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**Identifying a breeding habitat of a critically endangered fish, *Acheilognathus typus*,
in a natural river in Japan**

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Abstract

Freshwater biodiversity has been severely threatened in recent years, and to conserve endangered species, their distribution and breeding habitats need to be clarified. However, identifying breeding sites in a large area is generally difficult. Here, by combining the emerging environmental DNA (eDNA) analysis with subsequent traditional collection surveys, we successfully identified a breeding habitat for the critically endangered freshwater fish *Acheilognathus typus* in the mainstream of Omono River in Akita Prefecture, Japan, which is one of the original habitats of this species. Based on DNA cytochrome B sequences of *A. typus* and closely related species, we developed species-specific primers and a probe that were used in real-time PCR for detecting *A. typus* eDNA. After verifying the specificity and applicability of the primers and probe on water samples from known artificial habitats, eDNA analysis was applied to water samples collected at 99 sites along Omono River. Two of the samples were positive for *A. typus* eDNA, and thus, small fixed nets and bottle traps were set out to capture adult fish and verify egg deposition in bivalves (the preferred breeding substrate for *A. typus*) in the corresponding regions. Mature female and male individuals and bivalves containing laid eggs were collected at one of the eDNA-positive sites. This was the first record of adult *A. typus* in Omono River in 11 years. This study highlights the

value of eDNA analysis to guide conventional monitoring surveys and shows that combining both methods can provide important information on breeding sites that is essential for species' conservation.

Keywords: *Acheilognathus typus*; breeding site; environmental DNA (eDNA); Omono River; real-time PCR; species-specific detection

Introduction

In recent years, the loss of biodiversity caused by human activities has been one of the major concerns regarding the global ecosystem (Butchart et al. 2010). The loss of diversity in freshwater environments is a particularly critical situation, and numerous species are endangered (Dudgeon et al. 2006; WWF 2016). For example, approximately 40% of freshwater fish species are listed as endangered or threatened in the Red List of Japan (167 of the approximately 400 species registered in this region) (Japanese Ministry of Environment 2017). Factors reducing freshwater fish diversity include water pollution, habitat degradation and fragmentation, **biological invasions**, and large-scale capture for food and for aquariums (Allan and Flecker 1993; Vitousek et al. 1997).

Acheilognathinae species (bitterlings and related species) are distributed in

rivers, ponds, and waterways, and show a characteristic breeding system in which eggs are laid into bivalves. Hatched larvae stay within the bivalves for months and are released into the environment as juveniles. Fifteen out of the 16 Acheilognathinae species recorded in Japan are listed as either critically endangered, endangered, or threatened in the Red List of Japan, with habitat degradation being an important driver for the decline of these species (Japanese Ministry of the Environment 2015). The target species of this study, *Acheilognathus typus*, is one of these species. *Acheilognathus typus* is an annual fish, and adult fish are about 8 cm in length. In autumn, fish become mature, and females lay eggs inside small bivalves, such as *Sinanodonta lauta*, *Sinanodonta japonica*, and *Unio douglasiae*, while males spawn in the immediate vicinity of the bivalves. Short days in autumn are the main trigger initiating reproduction (Shimizu, 2010). The eggs hatch four to seven days after being laid, and juveniles leave bivalves in spring (Nakamura 1969). Although no information is available on the spawning migration of the species, adult bitterlings in general do not undertake large spawning migrations (Otokita et al. 2011). *Acheilognathus typus* used to inhabit large lakes, such as Kasumigaura, Izunuma, and Hachirougata, and the large rivers connected to these lakes, such as the Tone, Kitakami, and Omono rivers. Historically, *A. typus* was present in 13 Japanese Prefectures within the Kanto and Tohoku areas (Fig. 1; Nakamura 1969). This species has now been declared

extinct in eight of these 13 Prefectures and has not been recorded in one of the Prefectures in the last 16 years. Currently, *A. typus* is distributed in only about 10 sites within four Prefectures in the Tohoku area (Akita, Iwate, Miyagi, and Fukushima Prefectures; Figure 1). The main reasons for the decline in *A. typus* are predation by piscivore alien species, competitive exclusion by alien bitterling species, and the decrease in their spawning substrate, bivalves (Suguro 1995; Takahashi 2002; Kitajima 2005; Kawagishi et al. 2007). Its known current habitats are isolated small ponds or small irrigation streams, which are quite different from the original habitats (Sugiyama 2015). For effective conservation, it is important to evaluate whether *A. typus* populations still persist in the original habitat (i.e., large lakes and rivers). However, monitoring these systems for *A. typus* is challenging as conventional survey methods have a low detection rate and require considerable resources.

In recent years, environmental DNA (eDNA) analysis methods have been developed and applied to various ecosystems to examine the distribution of macro-organisms (Ficetola et al. 2008; Minamoto et al. 2012; Thomsen et al. 2012a,b). The presence or absence of organisms can be inferred from the presence or absence of their DNA in the environment, which is considered to be released via egestion, excretion, secretion, exfoliation, reproduction, or decomposition (Burnes and Turner, 2016). These

new methods are reportedly more sensitive than traditional survey methods, although their cost efficiency is debatable (Jerde et al. 2011; Dejean et al, 2012; Takahara et al. 2012; Davy et al 2015; Sigsgaard et al. 2015; Smart et al. 2016; Evans et al. 2017). Compared to disruptive survey methods, such as gill netting or the usage of toxins, eDNA methods are less disruptive for the target species and ecosystems and can be used to survey the distribution of rare species (Jerde et al. 2011; Lefort et al. 2015). For example, eDNA surveys have been conducted for Alabama Sturgeon (*Scaphirhynchus suttkusi*; Pfleger et al. 2016), European Weather Loach (*Misgurnus fossilis*; Sigsgaard et al. 2015), and Japanese Giant Salamander (*Andrias japonicus*; Fukumoto et al. 2015). Recent studies have shown that eDNA analysis can be applied for specifying the breeding season or spawning activity by monitoring the change in eDNA concentration or ratio of nuclear to mitochondrial eDNA markers (Spear et al. 2014; Erickson et al. 2016; Buxton et al. 2017; Bylemans et al. 2017). Although eDNA can be used to monitor breeding activity and locate breeding sites, subsequent conventional surveys are essential to confirm eDNA results.

Here, we present a case study in which we combine both eDNA analyses and conventional monitoring to evaluate whether *A. typus* populations are still present within Omono River (one of this species original habitats) and to assess whether they

are still reproductively active. After the development and validation of a species-specific real-time PCR assay for *A. typus* eDNA detection, we screened 99 water samples collected from Omono River for the presence of *A. typus*. Those sites testing positive for *A. typus* were subsequently monitored using conventional methods to confirm that this species is still reproductively active (i.e., collection methods for mature fish and egg).

Materials and Methods

Development and validation of the real-time PCR assay

Mitochondrial DNA cytochrome B sequences of *A. typus* and closely related species, namely *Acheilognathus tabira tohokuensis*, *Tanakia lanceolata*, *Tanakia limbata*, and *Rhodeus ocellatus ocellatus*, which all inhabit Omono River, were obtained from the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov>). Based on these sequences, we designed species-specific primers satisfying two conditions: melting temperature around 60 °C and at least two specific bases within the five bases at the 3' end of both forward and reverse primers. Additionally, we designed a TaqMan probe in Primer Express 3.0 (Life Technologies, Carlsbad, CA, USA) using the “TaqMan Quantification” setting. The specificity of the assay was evaluated by real-time PCR using target and non-target DNA templates.

Total DNA was extracted from the tissues of two *A. typus* individuals and from one individual of *A. tabira tohokuensis*, *T. lanceolata*, *T. limbata*, and *R. ocellatus* using the DNeasy Blood & Tissue Kit (QIAGEN Science, Hilden, Germany), following the manufacturer's instructions. The concentration of DNA was measured on a fluorescence quantification apparatus (Qubit 2.0 Fluorometer; Life Technologies). Real-time PCRs were carried out in triplicate using extracted DNA of each species as template. Each reaction (20 µl final volume) contained 900 nM primers and 125 nM TaqMan probe in 1× Environmental Master Mix 2.0 (Life Technologies) and 20 pg DNA. The real-time PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 55 2-step cycles of 15 s at 95 °C and 60 s at 60 °C. To detect false positives due to contamination during the real-time PCR procedures, ultrapure water was used instead of DNA in three reaction mixtures (non-template negative controls).

We further validated the real-time PCR assay using eDNA obtained from two ponds (7.7 and 638 m²) with a known presence of *A. typus*. To avoid contamination between samples or from previous samplings, all equipment for water collection and filtration was bleached in 0.1% sodium hypochlorite before use. One 1-l sample was collected from the surface of each pond. One milliliter of 10% benzalkonium chloride was added to the samples to suppress eDNA degradation, and samples were filtered within

48 h using through two glass-fiber filters (GF/F; GE Healthcare Life Science) per sample (Yamanaka et al. 2017). To control for false-positives due to eDNA contamination during filtration and subsequent processes, one negative filtration control was included. For each sample, the two filters were combined for eDNA extraction using the Salivette (Sarstedt, Nümbrecht, Germany) and DNeasy Blood & Tissue Kit methods and stored at -25°C (Uchii et al. 2016). All PCRs were set-up in a dedicated pre-PCR room using the conditions described above (Fukumoto et al. 2015). Triplicate reactions were run for all samples, negative filtration controls, non-template negative controls (for each plate), and positive controls (consisting of DNA derived from *A. typus* tissue). The amplification products were sequenced and checked by a commercial Sanger sequencing service.

Environmental DNA field survey

A field survey was conducted in Omono River, which flows through the Akita Prefecture in Tohoku area, Japan (Fig. 1). Historically, *A. typus* was present in this river, however, it has not been recorded for the last 11 years. The eDNA survey was conducted in August 2016 before the actual spawning season for *A. typus* (i.e., October). From the 8th to the 10th of August, 99 sites over a 112-km stretch from the river mouth to upstream Omono were sampled for eDNA-based monitoring. Sampling and eDNA extractions were

performed as described above: a single 1-l water sample was collected at each site. One negative filtration control was adopted for every 15 samples (seven in total) to monitor potential contamination during the filtration and eDNA extraction process. Because of clogging in 15 out of 99 samples, 500 ml instead of 1 l were filtrated using two filters. The same precautions to prevent contamination described above were adopted.

All DNA samples were examined for the presence of *A. typus* eDNA using the real-time PCR assay developed in this study. Real-time PCR conditions were as described previously with triplicate reactions performed for all samples, negative filtration controls, non-template controls and positive controls. While we initially used 2 µl of template DNA in the PCRs, we performed a second round of PCRs using 5 µl of template DNA as the initial results were all negative (see results). Samples that showed amplification in at least one of the replicates were considered positive, and were then sequenced to confirm the presence of *A. typus* eDNA.

Conventional field surveys

Fish were captured using two small fixed nets and three to five bottle traps installed near the water sampling points (~0.2 ha each). The capturing surveys were conducted at two eDNA-positive sites: from 7th to 12th of October, 2016 at Site A, and from 13th to 17th

of October, 2016 at Site B. Two or three individuals of bivalves were placed in both fixed nets and bottle traps because matured bitterlings are known to be attracted by amino acids released by bivalves (Kano 2014). The fishing gears were checked five (Site A) or four (Site B) days after their installation to check for the presence of *A. typus* adults. Five trays were set up for a breeding survey on 7th of October 2016 at Site A. Trays were placed in the water, and five bivalves comprising high-preference breeding substrates for *A. typus* were added to each tray. On 22nd of November 2016, the trays were collected and checked for laid eggs.

Results

Development and validation of the real-time PCR assay

This image is not open for conservation of rare species

This specificity was confirmed by the amplification of *A. typus* samples only, i.e., closely related species and negative controls were not amplified. In addition, we checked potential cross-reactivity of the assay with DNA of a closely related fish, *Acheilognathus rhombeus*, which is distributed in the past habitats of *A. typus*, by *in silico* analysis. In

total, seven and three mismatches were observed between the *A. rhombeus* sequence and the forward and reverse primers in our assay, respectively. Within the five bases at the 3' end of each of the forward and reverse primers, we found two mismatches (Fig. 2). Consequently, the amplification of *A. rhombeus* DNA with the proposed primers is unlikely. Further, amplification was confirmed in water samples collected from the two ponds where *A. typus* was present and sequencing of the amplified products confirmed the presence of *A. typus* DNA.

Environmental DNA field survey

Real-time PCR using 2 µl of template DNA per reaction was unsuccessful for all field samples from the 99 locations. We thus increased the amount of template DNA per PCR reaction to 5 µl which resulted in a positive amplification in two of the 99 samples. Sequencing of the amplicons confirmed the presence of *A. typus* eDNA at both sites (Table 1). In all PCR amplifications, positive controls were amplified normally, and negative controls, including filtration and PCR controls, did not show positive amplification.

Conventional field survey

In the capturing surveys, we collected 754 individual fish consisting of 25 different species from seven different families. Two adult *A. typus* specimens were captured at one of the sites (Site A) where *A. typus* eDNA was detected (Fig. 3a): a male was captured in the bottle trap and a female was captured in the small fixed net (Table 1). The male showed a clear nuptial coloration and the female showed markedly elongated oviposition tubes (Fig. 3a). In the **tray** set for breeding confirmation at Site A, we recovered 24 of the 25 bivalves, eight of which contained *A. typus* eggs (Fig. 3b; Table 1).

Discussion

In this study, we show through a combination of eDNA-based and conventional monitoring techniques that *A. typus* still occupies its original habitat within Omono River and that these populations are still reproductively active. This was the first report of adult *A. typus* in the mainstream Omono River in 11 years. In 2010 and 2015, large-scale inventories, using casting nets, gill nets, hand nets, and set nets, were performed by the Ministry of Land, Infrastructure, Transport and Tourism at eight sites different from the eDNA-positive sites in this study. In that survey, *A. typus* was not detected using any of these methods. The successful detections in the current study highlight the combined strength of using eDNA analyses for habitat identification and the use of bivalves as an

egg laying substrate. While recent studies have shown that eDNA analysis can be used to determine the breeding season or detect spawning activity, accurately locating breeding habitats by eDNA analysis alone remains difficult (Spear et al. 2014; Schmidt et al. 2013; Erickson et al. 2017; Buxton et al. 2017; Bylemans et al. 2017).

The eDNA surveys are suitable for large-scale surveys as the time for sampling collection is lower than that in conventional methods. In the present study, water samples from 99 sites were collected within three days by two persons, showing the simplicity of the eDNA approach and its applicability to a whole-river survey (Trèguier et al. 2014; Fukumoto et al. 2015; Eva et al. 2016). When performing a large-scale survey in a short period and with limited budget, this simplicity is essential. For example, based on our experience, we estimate that conventional surveys at these 99 sites would take more than 30 days and involve three persons. In addition, by using eDNA analysis, we can perform uniform surveys, because collecting water samples needs no specialized technique or knowledge. Environmental DNA analysis sometimes yields false-positive and false-negative results (Ficetola et al. 2016; Guillera-Aroita et al. 2017). Regarding the false positives, the amplicons obtained in this study were confirmed as belonging to the target species, and contamination between samples is highly unlikely because the samples from the two eDNA-positive sites were treated separately. On the other hand, there could be

false negatives. Multiple factors affect the detectability of eDNA, such as sampling location, sampling depth, sample number, sample volume, and number of PCR replicates (Ficetola et al. 2015; Schultz & Lance 2015; Adrian-Kalchhauser & Burkhardt-Holm 2016; Furlan et al. 2016). We took one water sample per site, and therefore, there is a possibility of missing *A. typus* eDNA. Future surveys would benefit from testing more samples per site and more PCR replicates. We could detect eDNA of *A. typus* despite the many related species inhabiting the surveyed area. This shows the advantage of the species-specific detection of eDNA when looking for a single rare species. eDNA metabarcoding surveys, recently developed as an extension of species-specific detections, are likely to be more costly as high levels of replication and high sequencing depth is needed for the detection of rare species (Ficetola et al. 2015; Simmons et al. 2015; Alberdi et al. 2017; Stoeckle et al. 2017). On the other hand, metabarcoding has the potential to provide important additional information, such as the presence of bivalves as spawning substrate, and thus, both methods have complementary benefits.

We detected eDNA of *A. typus* at two of the 99 surveyed sites, but the presence of adult fish and laid eggs in bivalves was confirmed at only one of the sites (Site A). The number of individuals at Site B, where we detected eDNA but failed to collect fish, might be low. Because numerous individuals of related Acheilognathinae species, such as *T.*

271 *lanceolata* and *A. tabira tohokuensis* (95 and 14 individuals were collected, respectively),
272 inhabit Site B, this is a suitable habitat for Acheilognathinae. Therefore, the small number
273 of *A. typus* at this site seems to be the most plausible explanation for not capturing *A.*
274 *typus*, despite the positive eDNA result. Alternatively, inconsistent results between the
275 eDNA survey and conventional survey might be a consequence of the two-month lag
276 between both surveys. Not much is known about the migration behavior of *A. typus*.
277 However, in general, adult bitterlings do not show large-scale migration (Otokita et al.
278 2011), and *A. typus* is a relatively small fish among the bitterlings, suggesting they have
279 limited migratory ability. Therefore, it is possible that the fish stay in the same area from
280 August to October. If one needs to identify the breeding habitat of rare species that show
281 migration over long distance, it should be considered that the time-lag between eDNA
282 and conventional surveys may affect the result. The distance traveled by eDNA in rivers
283 is still controversial (Deiner and Altermatt 2014; Jane et al. 2015; Shorgen et al. 2017).
284 Possibly, the eDNA detected in Site B was transported from outside the survey site by
285 water flow or other media. For example, detected eDNA might have been excreted by
286 birds or other predators that consumed the fish in another location. At Site A, mature male
287 and female *A. typus* (one of each) were captured and eggs were laid within bivalves,
288 showing that this species is currently reproducing in the mainstream of Omono River, its

original habitat. For future conservation, the specific conditions of breeding habitats of *A. typus* need to be clarified. Because we identified only a single breeding site in this study, it is difficult to draw definitive conclusions about such requirements. Therefore, more breeding sites within Omono River have to be evaluated, and to this purpose, finer-scale samplings and/or the use of the novel method proposed by Bylemans et al. (2017), in which the breeding season and habitats can be identified by the combined use of mitochondrial and nuclear DNA, would be helpful.

In summary, the present study identified a breeding site for *A. typus*, a critically endangered fish species with a characteristic life history, in a large river that is its original habitat, by combining eDNA analysis with subsequent conventional survey. Our findings provide proof-of-concept that this approach can be applied in future studies on *A. typus* as well as other endangered species. Future studies combining eDNA and conventional surveys are expected to improve breeding site detection of *A. typus* in Omono River and other rivers and lakes. We believe that eDNA surveys combined with conventional method can provide a powerful tool for mapping out breeding sites and determining site characteristics essential for the conservation of *A. typus* and other endangered species.

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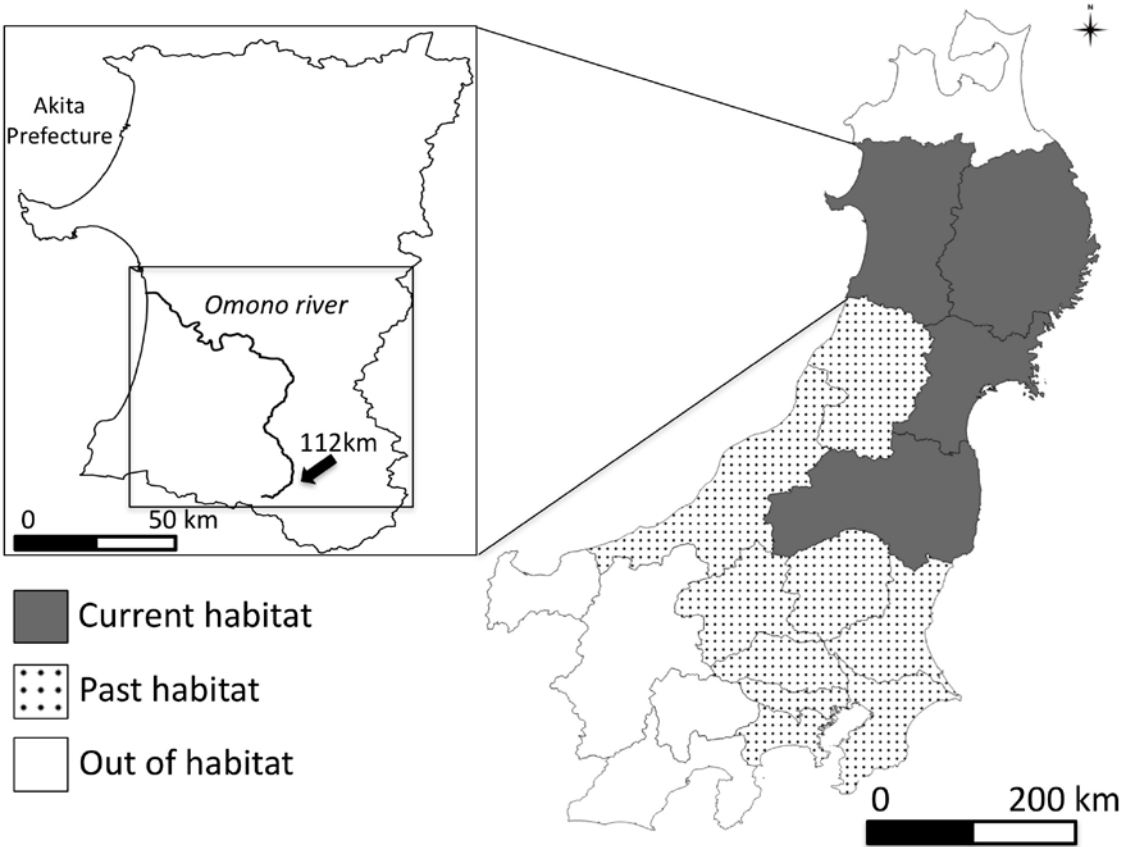
Figure legends

Figure 1. (a) Past and current distributions of *Acheilognathus typus* in Japan. This species used to be distributed in a wide area across the Kanto and Tohoku regions in eastern Honshu, mainland Japan. Currently, its distribution is limited to four Prefectures in the Tohoku area. (b) Enlarged view of the Omono River. Environmental DNA surveys were conducted at 99 sites (shown in red circles) along the Omono River.

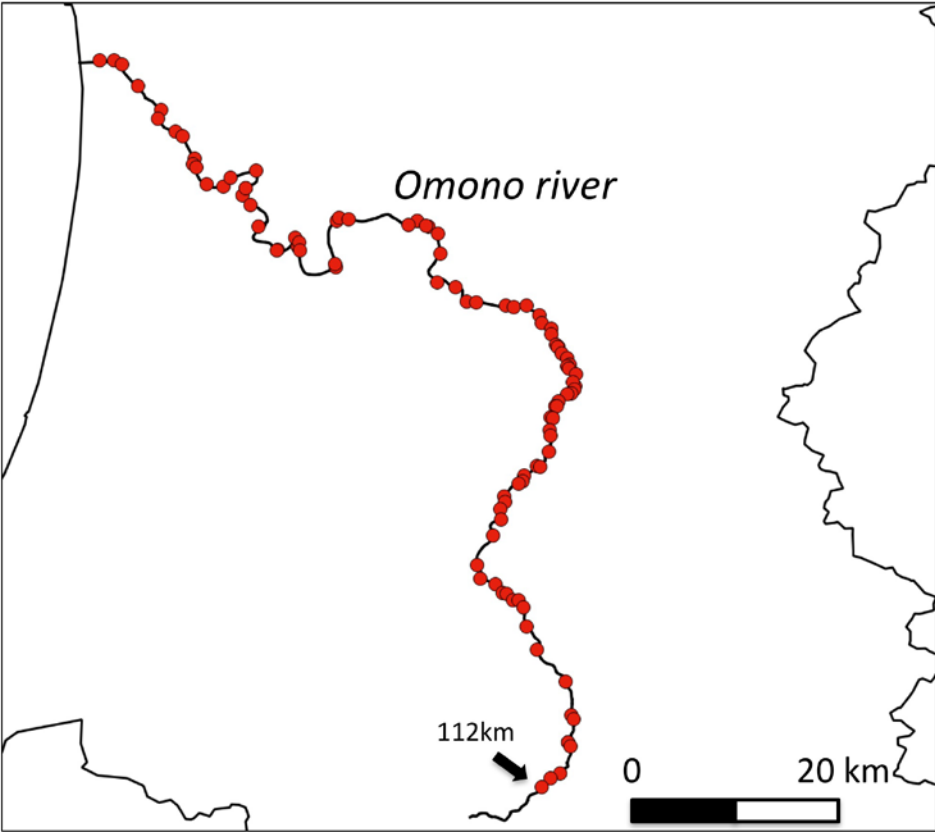
Figure 2. Alignment of the amplified region of the cytochrome *b* gene of *Acheilognathus typus* and closely related, potentially sympatrically distributed species with *A. typus*. Asterisks indicate *A. typus*-specific nucleotides.

Figure 3. Mature *Acheilognathus typus* adults (a) and their eggs laid within bivalves (b). Mature female (upper) and male (lower) individuals were collected at one of the environmental DNA-positive sites. The egg duct of the female is extended and the male shows remarkable nuptial colors.

Fig. 1a

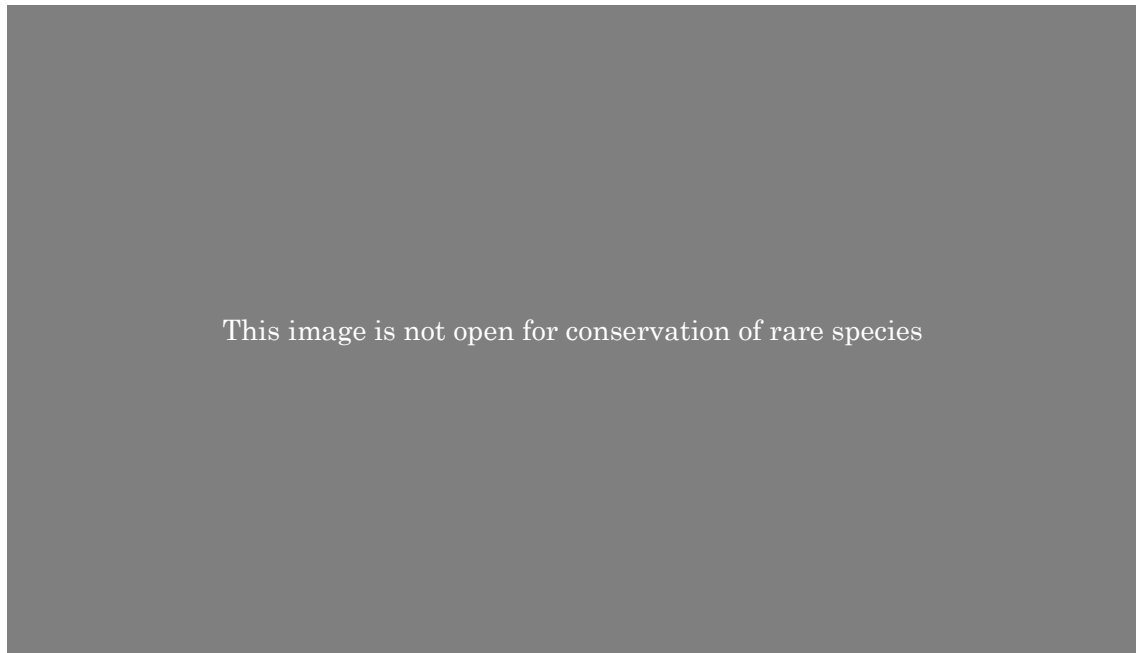


494 Fig. 1b



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497 Fig. 2



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Fig. 3

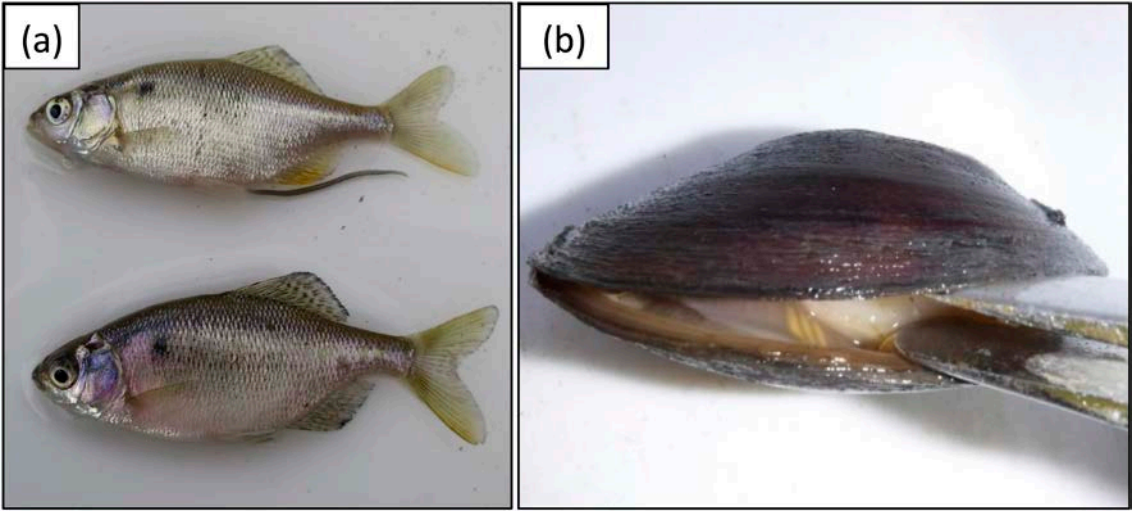


Table 1. The results of eDNA and conventinal surveys (only for eDNA positive sites).

eDNA positive site	eDNA survey			Conventional survey		
	Template 2µl	Template 5µl		fixed net	bottle trap	breeding survey
A	---*	+--**		+ (a matured female)	+ (a matured male)	+
B	---*	+--**		-	-	n.a.

* all three PCR reactions were negative

** one of three PCR reactions was positive