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Decline of unionid mussels enhances hybridisation of native and introduced bitterling fish species through competition for breeding substrate

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Abstract

1. Bitterling fishes (Subfamily: Acheilognathinae) spawn in the gills of living freshwater mussels and obligately depend on the mussels for reproduction. On the Matsuyama Plain, Japan, populations of unionid mussels—*Pronodularia japanensis*, *Nodularia douglasiae*, and *Sinanodonta lauta*—have decreased rapidly over the past 30 years. Simultaneously, the population of a native bitterling fish, *Tanakia lanceolata*, which depends on the three unionids as a breeding substrate, has decreased. Furthermore, a congeneric bitterling, *Tanakia limbata*, has been artificially introduced, and hybridisation and genetic introgression occur between them. Here, we hypothesised that decline of the unionids has enhanced this invasive hybridisation through competition for the breeding substrate.
2. Three study sites were set in three streams on the Matsuyama Plain. We collected adult bitterling fishes (native *T. lanceolata*, introduced *T. limbata*, and foreign *Rhodeus ocellatus ocellatus*) once a week from April to October 2013 to measure their densities in streams and to examine seasonal differences in female ovipositor length, which elongates in the breeding season. Simultaneously, we set quadrats and captured unionids and measured environmental conditions. Each unionid individual was kept separately in its own aquarium to collect ejected bitterling eggs/larvae. *Tanakia* eggs and larvae were genotyped using six microsatellite markers and the mitochondrial cytochrome *b* gene.
3. Introduced *T. limbata* was more abundant, had a longer breeding period, and produced more juveniles than native *T. lanceolata*. Hybrids between the two species occurred at all sites, and in total 101 of the 837 juveniles genotyped were hybrids. The density of *P. japanensis* was low, at most 0.42 individuals/m^2. *Nodularia douglasiae* and *S. lauta* have nearly or totally disappeared from these sites. Hybrid clutches of *Tanakia* species occurred more frequently where the local density of *P. japanensis* was low. Mussels were apparently overused and used simultaneously by three species of bitterlings.
4. Decline of freshwater unionid populations has enhanced hybridisation of native and invasive bitterling fishes through increasing competition for breeding substrate. We showed that rapid decline of host mussel species and introduction of

an invasive congener have interacted to cause a rapid decline of native bitterling fish.
5. Degradation of habitat and the introduction of invasive species interact to cause a cascade of extinctions in native species. In our study, obligate parasite species are threatened because the host species are disappearing, which means there is a serious threat of coextinction.

KEYWORDS
Acheilognathinae, endangered species, invasive species, stream habitat, Unionidae

1 | INTRODUCTION

Rivers and streams are one of the ecosystems most threatened by human impacts, such as habitat degradation, non-native species introduction, overexploitation, flow modification, and water pollution (Brookes, 1988; Dudgeon, 2006). Degradation of habitat and the introduction of invasive species interact to cause a cascade of extinctions of native species. In particular, obligate parasite species will go extinct when host species disappear; this is known as coextinction (Koh et al., 2004). Bitterling fishes (Acheilognathinae, Cyprinidae) are freshwater fishes with an unusual spawning symbiosis with freshwater mussels. Bitterling fishes spawn on the gills of living freshwater mussels of the families Unionidae and Margaritiferidae, and obligately depend on the mussels for reproduction (Smith et al., 2004; Wiepkema, 1961). However, freshwater mussel species are susceptible to habitat degradation and are threatened worldwide (Strayer, 2008; Williams et al., 1993; Zieritz et al., 2018). Decrease of breeding substrate directly endangers organisms that obligately depend on it. Furthermore, loss of breeding substrate may cause hybridisation between species that share the same breeding substrate (Hubbs, 1955).

Japan is a mountainous, wet, and forested country with a rich freshwater fauna and a high proportion of endemic species (Yoshimura et al., 2005). However, people are concentrated in densely populated urban areas along the coast and on alluvial plains, and recent urbanisation has destroyed and degraded natural freshwater habitats, and now most unionid species are endangered (Kondo, 2008; Negishi et al., 2008; Onikura et al., 2006, 2016). Due to coextinctions, 15 of the native 16 species/subspecies of bitterling fishes are also endangered and are listed on the Japanese Red List (Ministry of the Environment, Japan, 2013). On the Matsuyama Plain, Ehime Prefecture, Japan, populations of the unionids *Pronodularia japanensis*, *Nodularia douglasiae*, and *Sinanodonta lauta* have decreased rapidly over the past 30 years through habitat fragmentation by weirs (Kuwahara et al., 2017). Glochidium larvae of these unionids mostly parasitise an amphidromous goby, *Rhinogobius nagoyae*, on the Matsuyama Plain, but weirs prevent them from moving upstream and restrict unionid dispersion to downstream of the weirs. Simultaneously, the native population of one bitterling fish, *Tanakia lanceolata*, has decreased during these 30 years (Matsuba et al., 2014). Conversely, a congeneric species, *Tanakia limbata*, which was artificially introduced from the island of Kyushu, western Japan in the 1970s, has expanded its distribution (Hata et al., 2019; Matsuba et al., 2014; Uemura et al., 2018). Some *T. lanceolata* individuals are also suspected to have been introduced from Kyushu (Tominaga et al., 2020); our previous research determined that most individuals (in the study, 36 of 40, 90%) are native, but the rest (10%) are a Kyushu type via genotyping of the cytochrome *b* gene (cyt *b*; Uemura et al., 2018). These artificial disturbances of populations have caused hybridisation and genetic introgression between *T. lanceolata* and *T. limbata* in western Japan, including on the Matsuyama Plain, in recent decades (Hata et al., 2019; Uemura et al., 2018).

The rapid decrease of unionids may enhance invasive hybridisation through competition for breeding substrate at Matsuyama. Therefore, we aimed to clarify the process of bitterling hybridisation and estimate the effects of the decrease in unionid populations on the frequency of bitterling hybridisation. In this study, we caught bitterlings periodically and measured the ovipositor length of females to reveal their reproductive seasons. We captured unionid individuals that had bitterling eggs/larvae in their gills, kept them separately in aquaria, collected the bitterling eggs/larvae released from the unionids, and classified them as *T. lanceolata*, *T. limbata*, or hybrids using microsatellite markers and the mitochondrial cyt *b* region. These data have allowed us to test the prediction that hybridisation between congeneric bitterling species would increase in relation to decreasing unionid availability.

2 | METHODS

2.1 | Study sites and study species

The study was conducted in the Kunichi and Koyori streams (both in the Kunichi River system; main stem length 5.7 km) and the Nagaodani stream (length 6.9 km) on the Matsuyama Plain, the island of Shikoku, in southwestern Japan (coordinate range 33°46′N–33°48′N, 132°41′E–132°46′E; Figure 1). The region is in an alluvial fan formed by the Shigenobu River (watershed area 445 km^2; mainstem length 36 km). The Kunichi River system and the Nagaodani stream are irrigation canals with some weirs for water

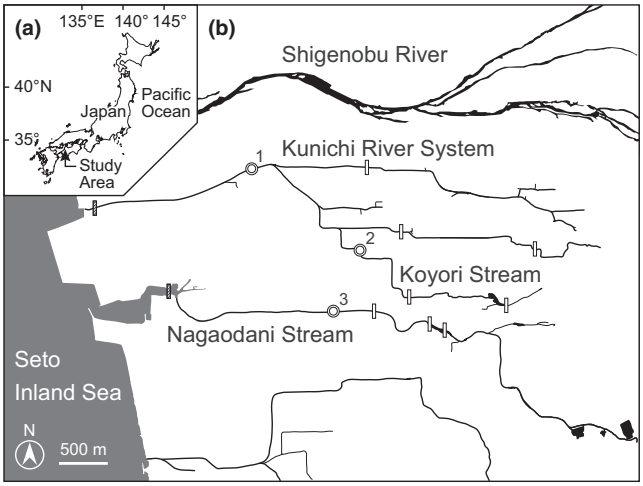

FIGURE 1 Map of study sites (b, double circles with numbers) in three streams on the Matsuyama Plain, Ehime, Japan (a, inset): 1, Kunichi stream; 2, Koyori stream; 3, Nagaodani stream. Open bars on the streams indicate weirs with > 1.0 m height. Bars with diagonal lines show floodgates. These maps are based on those provided under CC BY 4.0 by the Geospatial Information Authority of Japan (https://maps.gsi.go.jp)

intake into nearby paddy fields. The river banks are lined with concrete, and a floodgate (width 30 m) is installed at the mouth of each river or stream. We chose three study sites each inhabited by three bitterling species (native *T. lanceolata*, introduced congener *T. limbata*, and a species introduced from China, *Rhodeus ocellatus ocellatus*; Nakamura, 1955) and three unionid species (*P. japanensis*, *N. douglasiae*, *S. lauta*) (Hata et al., 2019; Kuwahara et al., 2017). The annual fecundities of *T. lanceolata*, *T. limbata*, and *R. o. ocellatus* are estimated as around 700, 400, and 700 eggs, respectively, which are spawned by about 6, 18, and 16 bouts during their breeding seasons (Solomon et al., 1984; Yokoyama, 2014). The number of eggs laid inside each unionid individual is estimated to be around 10–30, 5, and 14 in *T. lanceolata*, *T. limbata*, and *R. o. ocellatus*, respectively (Fukuhara et al., 1998; Kawamura et al., 2001; Kondo et al., 1984).

2.2 | Field survey

The field survey was conducted once a week from April to October 2013. We collected bitterling fishes using a minnow trap for 30 min with 100 g of silkworm pupa powder as a fish attractant. Adult bitterlings were assigned to species based on their external morphologies (Hosoya, 2013), and the number of individuals was counted. In the breeding season, female bitterlings develop a long ovipositor to place their eggs inside the gills of a mussel through the exhalant siphon (Kitamura, 2006). Therefore, regarding *T. lanceolata* and *T. limbata*, we determined sex, measured standard length (SL) and female ovipositor length (OPL), and calculated the ovipositor index (OPI) as follows:

$$\mathrm{OPI} = \mathrm{OPL(mm)}/\mathrm{SL(mm)}$$

After these measurements were taken, all individuals were released back to the sites where they were caught.

We set a line transect at a right angle to the flow direction every time sampling was carried out. Quadrats (1.0 m × 1.0 m) were set on the line transect at 1.0 m intervals and 50 cm away from the shoreline. To avoid using the same place twice, the line transect was moved upstream by 2.0 m for every sampling event. For each quadrat, stream width (cm), transparency (cm), water temperature (°C), water depth (cm), and flow velocity (cm/s) were measured as environmental factors. Water transparency was measured using a transparent tube, 100 cm long with a small Secchi disc at the bottom (01267, SANPLATEC CORP.). Water depth was measured at three spots randomly chosen within the quadrat and the mean of the three values was used. Flow velocity was measured at 1/10, 5/10, and 9/10 of the depth using a portable current meter (Model VR-301, KENEK) and the mean of these three values was used. In the quadrats, we collected unionids by hand, identified the species based on Kondo (2008) and Lopes-Lima et al. (2020), and recorded the number of individuals. If no unionid individuals were found we sampled additional quadrats until at least one unionid was collected and measured environmental factors. From June to October, we used a hand net for sampling in the Kunichi stream because of the high water level and low water transparency (Figure S1a). In the Nagaodani stream, unionid density was quite low, and therefore we searched for unionids from the whole area (7.0 m × 50 m) during sampling.

The unionids were tagged with ID codes on the shell surface using white marker, and shell length (mm) measured. Then, we opened the shells of unionids gently using a shell opener for pearl shells, observed their gills, and brought those individuals that had bitterling eggs/larvae to our laboratory. Unionid individuals without bitterling eggs/larvae were released back to the same place we caught them. We did not collect the same individual more than once.

2.3 | Collection of eggs and larvae of bitterlings from unionids

We kept each unionid individual in a net tank (H 130 mm × W 125 mm × L 80 mm; S-5330, Sudo & Company Inc.) at 23 ± 1°C water temperature until all bitterling eggs/larvae were ejected from the unionid shells. Unionids ejected bitterlings at all three stages—eggs, larvae, and fry. After ejection, these unionid individuals were released back to the place where they were collected. Eggs, larvae, and fry of the bitterling fishes were collected every day and identified to genus level, i.e. *Tanakia* or *Rhodeus*, following Nakamura (1969) and Suzuki and Hibiya (1985). Then, the developmental stages of *Tanakia* were determined as follows: A, immediately after hatching; B–C, primordial fin-fold at the caudal portion is well developed; D–E, notochord flexion is complete; F, melanophores appear on the body; G, the number of caudal fin-rays is complete; H, gas-bladder has grown larger but not divided into lobes; I, free-swimming stage; this was done following figures 2 and 4 of Suzuki and Hibiya (1985). Based on the developmental

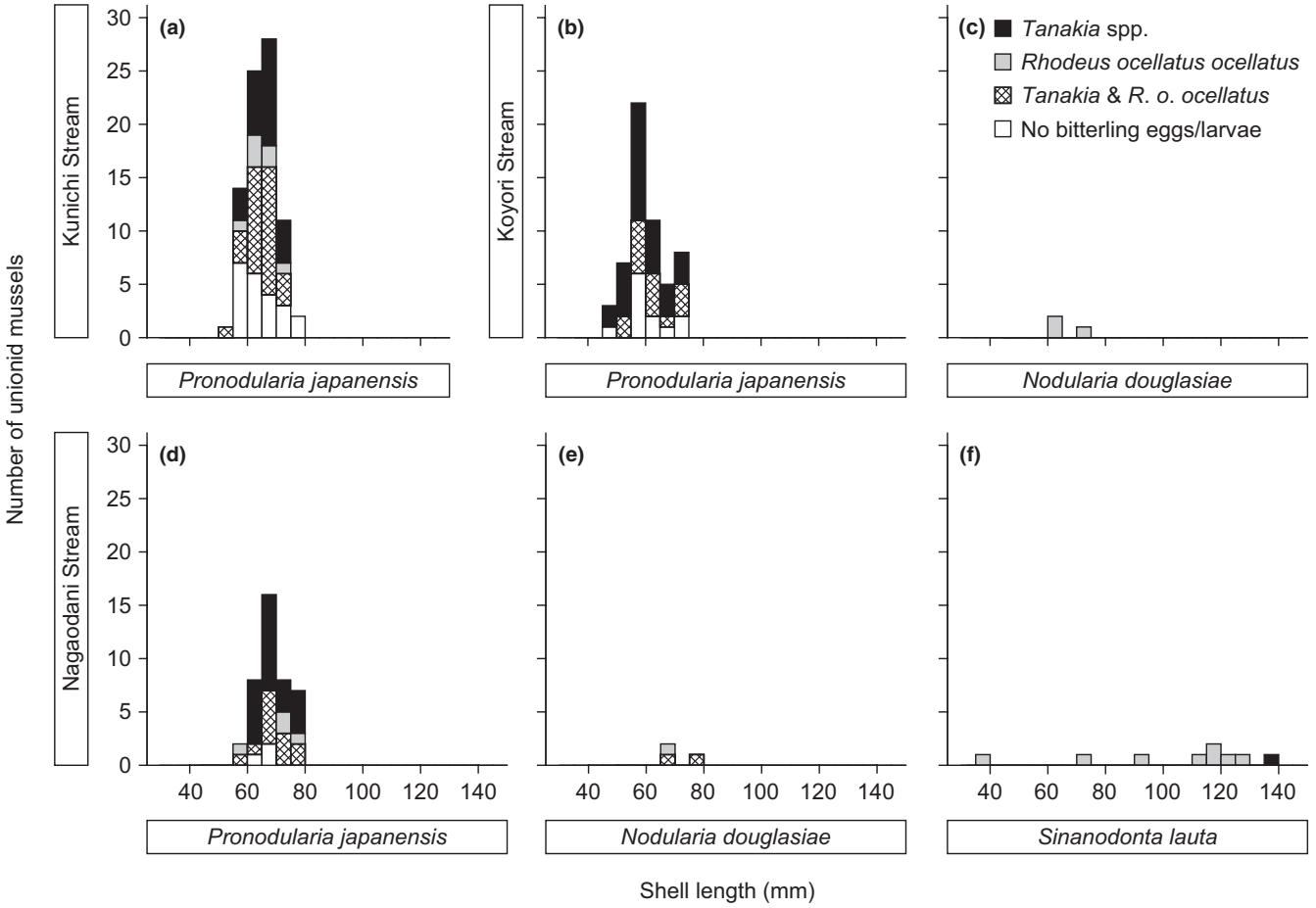

FIGURE 2 Size-frequency distribution of unionid mussels at our study sites; *Pronodularia japanensis* at the Kunichi stream (a), *P. japanensis* and *Nodularia douglasiae* at the Koyori stream (b, c), and *P. japanensis*, *N. douglasiae*, and *Sinanodonta lauta* at the Nagaodani stream (d–f). Closed bars, grey bars, and crosshatched bars represent the use of the mussels by *Tanakia* spp., *Rhodeus ocellatus ocellatus*, and both bitterlings, respectively, as their spawning substrate. Open bars indicate no such use

stage, we estimated the fertilisation date of each individual by deducting days for their development as follows: eggs, 1 day; stage A, 3 days; stage B, 5 days; stage C, 6 days; stage D, 8 days; stage E, 11 days; stage F, 17 days; stage G, 19 days; stage H, 23 days; stage I, 28 days. Collected specimens were fixed in 100% ethanol.

2.4 | DNA sequencing and cytochrome *b* genotyping

The ethanol-fixed bitterling eggs and larvae were wholly and partially used for DNA analysis, respectively. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit following the manufacturer's protocol (Promega). We amplified the cyt *b* region of mitochondrial DNA (mtDNA) by polymerase chain reaction (PCR) with the forward primer NEW-FOR, 5′-AGCCTACGAAAAACACACCC- 3′ (Gilles et al., 2001), and the reverse primer cytb-Rev, 5′-GATCTTCGGATTACAAGACC-3′ (Hashiguchi et al., 2006). The reaction mixture contained 6.05 μl of sterile distilled water, 1.0 μl of 10 × ImmoBuffer (Bioline), 1.0 μl of dNTP mix (10 mM), 0.3 μl of $MgCl_2$ (50 mM), 0.3 μl of each primer (10 μM), 0.05 μl of BIOTAQ HS DNA polymerase (5 U/μl, Bioline), and 1.0 μl of DNA template. The reaction protocol consisted of an initial denaturation at 95°C for 10 min; this was followed by 30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 7 min. The samples ($n = 837$) were genotyped by PCR-restriction fragment length polymorphism using the restriction enzyme *Hha*I (TaKaRa Bio). This method is well established and has been used in previous studies. *Hha*I produced fragments of 91, 218, 252, and 369 bps for *T. lanceolata*, and fragments of 18, 109, 252, and 821 bp for *T. limbata* (Hata et al., 2019; Uemura et al., 2018). We incubated 5.0 μl of the PCR products with 0.2 μl of *Hha*I (10 U/μl), 3.8 μl of sterile distilled water, and 1.0 μl of 10 × M buffer at 37°C overnight. We conducted gel electrophoresis with the reaction products for 40 min at 100 V on 3% agarose gel and genotyped individual fish according to the resulting fragment patterns.

Genotyped individuals that had been ejected from the same unionid individual, had similar fertilisation days (within 5 days), and also shared the same mtDNA type, were treated as a clutch. Using this criterion, all individuals were successfully classified into a clutch. We used clutches including five or more individuals for analysis to

avoid both overestimation and underestimation of the hybrid and purebred clutches, respectively.

2.5 | Genotyping using microsatellite markers

We used six microsatellite loci as markers following previous studies (Uemura et al., 2018): *RC363* and *RC317A* (Shirai et al., 2009), and *Rser02*, *Rser03*, *Rser07*, and *Rser10* (Dawson et al., 2003). The target sequences were amplified by PCR following the method of Schuelke (2000), and each microsatellite primer was labelled with one of four fluorescent dyes: *RC363* and *Rser07* were labelled with PET; *RC317A* and *Rser03* with FAM; *Rser02* with NED; and *Rser10* with VIC. The reaction mixture contained 3.15 μl of sterile distilled water, 0.5 μl of 10 × ImmoBuffer (Bioline), 0.5 μl of dNTP mix (10 mM), 0.15 μl of $MgCl_2$ (50 mM), 0.02 μl of forward primer (10 μM), 0.08 μl of reverse primer (10 μM), 0.08 μl of fluorescent dye label (10 μM), 0.025 μl of BIOTAQ HS DNA polymerase (5 U/μl, Bioline), and 0.5 μl of DNA template. The reaction consisted of an initial denaturation at 95°C for 10 min; this was followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s; 10 cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. We determined the lengths of fragments using an ABI 310 Genetic Analyzer (Thermo Fisher Scientific) and Peak Scanner software version 2.0 (Thermo Fisher Scientific). The number of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using Genepop on the Web (Raymond & Rousset, 1995; Rousset, 2008; Table S1). The population genetic structure was analysed using Structure version 2.3.4 (Pritchard et al., 2000) with the following parameters: 100,000 burn-in; 1,000,000 Markov chain Monte Carlo steps; admixture model with independent allele frequencies; and five replicates of each simulation from $K = 1$ to 11 genetic clusters. The optimum K value was two (Figure S2) calculated by Structure Harvester version 0.6.94 (Earl & vonHoldt, 2012). We used a threshold q-value of 0.20 and defined hybrids as those with more than 20% of their genetic contents matching both purebred types (Vähä & Primmer, 2006). Using NewHybrids (Anderson & Thompson, 2002) we further assigned each individual to one of six genotypic classes based on the posterior probabilities: the two parental species (P_0, P_1), first-generation hybrid (F_1), second-generation hybrid (F_2), backcross of F_1 with P_0, and backcross of F_1 with P_1. We used a threshold value for the posterior probability of $q = 0.5$ with assignment to the aforementioned categories following van Dongen et al. (2012). Parameters used in NewHybrids were set as follows: without individual or allele frequency prior information; Jeffreys-like prior for both mixing proportions and allele frequencies; 25,000 sweeps of burn-in; and 100,000 iterations of the Markov chain Monte Carlo. To assess the efficiency and accuracy of genotype assignment by NewHybrids, we conducted a simulation method using HYBRIDLAB version 1.0 (Nielsen et al., 2006). A total of 100 simulated hybrids of each hybrid class were generated based on the allelic frequencies of *T. lanceolata* and *T. limbata* that were identified as purebreds in the previous Structure analysis for the ancestry coefficient. This dataset was analysed by NewHybrids to assign each individual to a genotypic class with $q = 0.5$.

2.6 | Statistical analyses

First, we conducted a generalised linear model (response variable, shell length of *P. japanensis*; experimental factor, streams; family, gaussian) and post hoc Tukey's multiple comparison test using the R package 'multcomp' version 1.4–11. Then, we conducted a generalised linear mixed model: response variable, hybrid clutch or purebred clutch; fixed factor, each environmental factor (water transparency [cm], water temperature [°C], water depth [cm], flow velocity [cm/s], number of bitterling eggs/larvae from the unionid individual, unionid density in the quadrat [N/m^2], shell length of unionid [mm]) and river system; a random factor, unionid identity; family, binomial. Generalised linear mixed model analysis was conducted using the R package *glmmML* version 1.1.0. In this analysis, we used river system because we wanted to increase the number of clutches being analysed and because the two sites in Kunichi river system are only 1.5 km apart from each other. All analyses were conducted using R version 3.6.2 (R Core Team, 2019).

3 | RESULTS

3.1 | Environmental conditions of the study sites

At the three study sites, water transparency was quite limited (around 25 cm), especially in June. In the Nagaodani stream, water temperature was higher than the other two sites by 1–11°C, and it exceeded 30°C from June to August (Figure S1). At the Kunichi stream only, water depth rapidly increased from June to September because of the control of the floodgate at the river mouth (Figure S1c).

3.2 | Relative abundance of three bitterling fish species

At the three sites, a native bitterling—*T. lanceolat*a, an introduced congener—*T. limbata*, and an exotic bittering—*R. o. ocellatus* were collected simultaneously. The composition of the populations of these three bitterlings was significantly different among the three streams (χ^2 test, $p < 0.05$), but *T. limbata* was the dominant species at all the sites (Figure S3). Note that adult bitterlings are not genotyped in this study, and hybrid individuals can be included in numbers of *T. lanceolata* and *T. limbata*. The rates of hybrids are 11.3% (22 individuals) of all collected *Tanakia* individuals (in total 193) in 2011 (Hata et al., 2019), and 10.7% (18 individuals) of all *Tanakia* individuals (in total 168) in 2014 (Uemura et al., 2018).

3.3 | Density of unionid species, their size distributions, and their exploitation by bitterling fishes as spawning substrate

The density of *P. japanensis* was low in the Kunichi and Koyori streams—at most 0.42 individuals/m^2. *Nodularia douglasiae* and *S. lauta* have nearly or totally disappeared from these sites (Table 1). The shell length of *P. japanensis* was largest in Nagaodani (mean ± *SD*, 68.6 ± 5.6 mm, $n = 41$), smallest in Koyori (60.3 ± 6.3 mm, $n = 56$), and intermediate in Kunichi (64.9 ± 5.1 mm, $n = 81$; Tukey's multiple comparisons, $p < 0.01$ for all pairs; Figure 2). The smallest individual of *P. japanensis* was 49.1 mm in shell length, and no juvenile of any unionid species was found.

In the Kunichi stream, 28% of *P. japanensis* individuals were used solely by *Tanakia* spp., 9% by *R. o. ocellatus*, 36% by both *Tanakia* spp. and *R. o. ocellatus* simultaneously, and the remaining 27% were empty (Figure 2a). In the Koyori stream, 52% of *P. japanensis* individuals were used by *Tanakia* spp., 27% by both *Tanakia* spp. and *R. o. ocellatus* simultaneously, and the remaining 21% were empty (Figure 2b). In the Nagaodani stream, 54% of *P. japanensis* individuals were used solely by *Tanakia* spp., 10% by *R. o. ocellatus*, 29% by both *Tanakia* spp. and *R. o. ocellatus* simultaneously, and the remaining 7% were empty (Figure 2d). There was no difference in preference on mussel size among two *Tanakia* species and hybrids (Figure S4). *Nodularia douglasiae* and *S. lauta* were more frequently used by *R. o. ocellatus*.

The highest numbers of bitterling eggs found in the gills of a single *P. japanensis* individual were 87, 99, and 222, respectively, for the Kunichi, Koyori, and Nagaodani streams (Figure 3).

3.4 | Genotyping of bitterling eggs and larvae and direction of hybridisation

As a result of analysis with Structure and NewHybrids based on the six microsatellite loci, hybrids between *T. lanceolata* and *T. limbata* were found to be 4.5, 11.8 and 18.4% of all *Tanakia* eggs/larvae from the Kunichi, Koyori, and Nagaodani streams, respectively (Figure 4; Table 2). Most hybrid individuals turned out to be F_2 according to NewHybrids. However, efficiency to distinguish genetic classes among hybrids was limited (Table S2), and thus we do not distinguish them and instead use a category, hybrid. Introduced *T. limbata* was more prevalent than native *T. lanceolata* among *Tanakia* eggs/juveniles at all sites, as was also the case in adult populations. With regard to the mtDNA types of the hybrid individuals, the ratios were significantly different among streams; in Kunichi and Koyori the *T. limbata* type was dominant (8/10, 35/39, respectively), but in Nagaodani both *T. limbata* and *T. lanceolata* types were equivalent (19/50 and 31/50, respectively; Fisher's exact test, $p < 0.001$; Table 2).

The *Tanakia* clutches were classified into four types: purebred *T. lanceolata*, purebred *T. limbata*, hybrid with *T. lanceolata* mtDNA (that is, female was *T. lanceolata* or hybrid), hybrids with *T. limbata* mtDNA (female was *T. limbata* or hybrid; Table 2). Hybrid clutches occurred frequently, especially in Koyori (13 of 30 clutches, 43%) and Nagaodani (15/26, 58%).

3.5 | Environmental and biological factors affecting the frequency of hybridisation

The probability of hybrid clutch occurrence was significantly higher where the local density of *P. japanensis* was low (Table 3; Figure 5). No relationship was detected between the hybrid probability and the other environmental/biological factors such as water transparency, water temperature, depth, or flow velocity (generalised linear mixed model, all $p > 0.05$).

3.6 | Seasonal differences in breeding of bitterlings and occurrence of hybrids

Following the OPI, the spawning season of *T. lanceolata* was shorter than that of *T. limbata*, and started in May, peaked in June, and almost finished by the end of June (Figure 6). In contrast, the breeding season of *T. limbata* was longer, from April to July, covering the whole spawning season of *T. lanceolata*. The number of *Tanakia* eggs laid in *P. japanensis* reached its peak from May to June, and then the number declined gradually (Figure 3). Spawning of *R. o. ocellatus* started in early May, and the season overlapped with the latter half of *Tanakia* breeding in the Kunichi and Koyori streams, whereas

TABLE 1 Density of unionid mussels—*Pronodularia japanensis*, *Nodularia douglasiae*, and *Sinanodonta lauta*—in the Kunichi, Koyori, and Nagaodani streams on the Matsuyama Plain, Ehime, Japan

	Kunichi stream			Koyori stream			Nagaodani stream		
Mussel species	Individuals/m^2	*SD*	*N*	Individuals/m^2	*SD*	*N*	Individuals/m^2	*SD*	*N*
P. japanensis	0.42	0.30	62	0.21	0.34	41	0 (0.12)	0	54
N. douglasiae	0	0	62	0.05	0.15	41	0 (0.03)	0	54
S. lauta	0	0	62	0	0	41	0 (0.01)	0	54
Total	0.42	0.30	62	0.26	0.34	41	0 (0.15)	0	54

Note: Density in parentheses for the Nagaodani stream is calculated by dividing the total number of collected individuals by the study site area (7 m × 50 m).

Abbreviation: *N*, number of quadrats (1.0 m × 1.0 m).

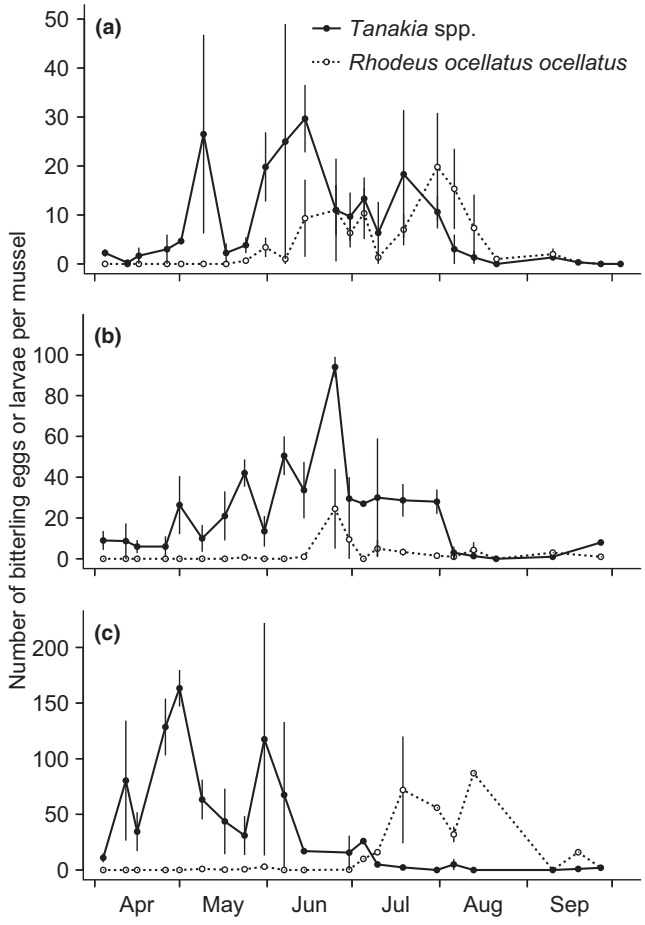

FIGURE 3 Number of bitterling eggs or larvae laid in the gills of unionid mussels at the Kunichi (a), Koyori (b), and Nagaodani (c) streams. Solid line with closed circles and dotted line with open circles represent *Tanakia* spp. and *Rhodeus ocellatus ocellatus*, respectively, and error bars indicate standard deviation

in the Nagaodani stream, spawning of *R. o. ocellatus* started in July and finished in September. Both hybrid individuals with *T. lanceolata* mtDNA and those with *T. limbata* mtDNA occurred from May to June, when the breeding seasons of the two species overlapped. By contrast, in April, and from July to August, most hybrids had *T. limbata* mtDNA (Figure 7).

4 | DISCUSSION

4.1 | Invasive hybridisation between two *Tanakia* species that share endangered unionids

Artificial introduction of bitterling fishes causes invasive hybridisation between *Tanakia* species on the Matsuyama Plain (Hata et al., 2019; Uemura et al., 2018). Previously, only *T. lanceolata* inhabited the Matsuyama Plain exclusively, but *T. limbata* was first discovered in this area in 1979 (Ehime Prefecture, Japan, 1979), introduced from western Kyushu (Matsuba et al., 2014; Uemura et al., 2018). Although some bitterling fish species prefer different mussel species

TABLE 2 Genotype frequencies of individual eggs/larvae and clutches of *Tanakia lanceolata* (*Tlan*), *Tanakia limbata* (*Tlim*), and their hybrid (Hyb) in the Kunichi, Koyori, and Nagaodani streams

Genotype	mtDNA	Kunichi	Koyori	Nagaodani
Individual				
Tlan	*Tlan*	36	48	75
Tlim	*Tlim*	177	244	156
Hyb	*Tlan*	2	4	31
Hyb	*Tlim*	8	35	19
Hyb	(Total)	10	39	52[a]
Total		223	331	283
Clutch				
Tlan	*Tlan*	5	4	2
Tlim	*Tlim*	7	13	9
Hyb	*Tlan*	0	2	9
Hyb	*Tlim*	1	11	6
Hyb	(Total)	1	13	15
Total		13	30	26

[a] mtDNA genotype of two individuals are missing.

at their coexisting sites (Fukuhara et al., 1998; Kitamura, 2007), on the Matsuyama Plain *P. japanensis* is the only species really available as the breeding substrate, and both *T. lanceolata* and *T. limbata* use *P. japanensis* intensively. These two fish species and the other foreign species, *R. o. ocellatus*, frequently spawned eggs simultaneously in the same mussel individual. Unionids are apparently overused and in our study, at most, 222 bitterling eggs were found in a single unionid individual. The limited availability of suitable breeding substrate due to the rapid decline of mussel populations, which remain in a small midstream region, enhances encounters and competition for breeding substrate between the native *T. lanceolata* and the introduced *T. limbata*. Bitterling males are known to use alternative reproductive tactics depending on the availability of females and the breeding substrate; these are: (1) pair spawning in which male defends a mussel within its territory; (2) sneaking towards a pair; or (3) group spawning (Kanoh, 1996, 2000; Mills & Reynolds, 2003; Smith et al., 2002). When bitterling density is much higher than that of unionids, territoriality collapses, and sneaking and group spawning become common (Kanoh, 2000; Nagata, 1985; Reichard et al., 2004). In both of these strategies, different bitterling species can spawn simultaneously and incidentally hybridise with each other when they are closely related species (Hubbs, 1955; Taborsky, 2004). Males of *T. lanceolata*, *T. limbata*, and *R. o. ocellatus* are known to defend breeding territories around unionids against not only conspecific males but also other bitterling males (Kanoh, 2000; Smith, 2011; Yokoyama, 2014). However, this study shows that *T. lanceolata* and *T. limbata* breed simultaneously on the same *P. japanensis* individuals at our study site, and hybrid clutches appear frequently. Hybridisation occurs in both directions, but in our study was skewed towards female *T. limbata*–male *T. lanceolata*. This bias seems to be caused by the rareness of introduced species comparing with native

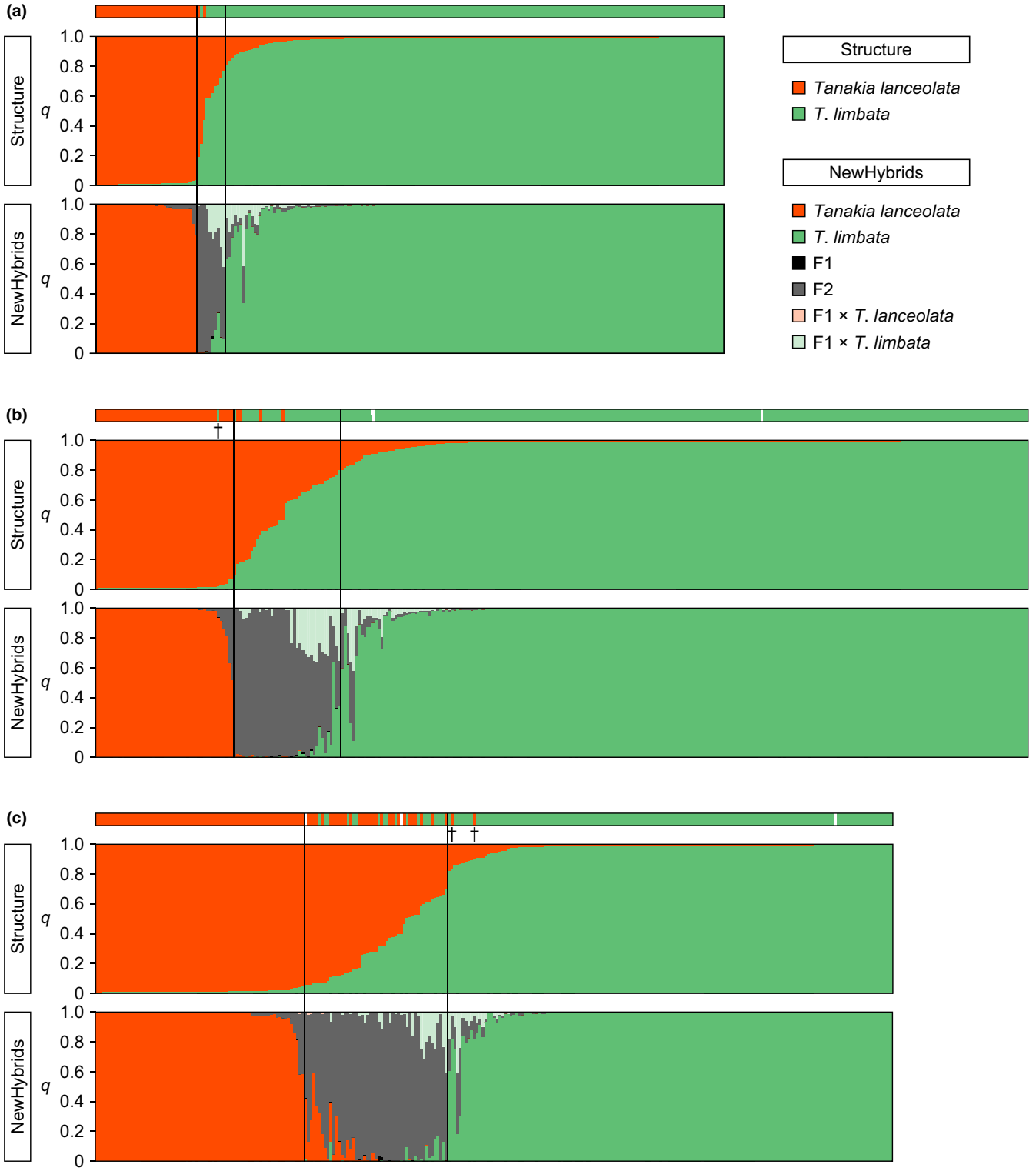

FIGURE 4 Population genetic structure of eggs/larvae of *Tanakia lanceolata*, *Tanakia limbata*, and their hybrids at the Kunichi (a), Koyori (b), and Nagaodani (c) streams in Ehime Prefecture, Japan. Squares above columns indicate mitochondrial cytochrome *b* genotypes. Upper columns are outcomes from Structure, and lower columns from NewHybrids analyses based on six microsatellite loci. Vertical lines on the boxes indicate thresholds between purebreds and hybrids. Dagger marks (†) indicate mismatch between mtDNA types and genotypes based on the microsatellite markers

species during colonising (Wirtz, 1999), and therefore colonising females may relax their mate choice to avoid gamete losses under the scarcity of conspecific males (Hórreo et al., 2011; Kaneshiro, 1983). By contrast, no hybridisation between *Tanakia* species—*T. lanceolata* and *T. limbata*—and *R. o. ocellatus* was observed. Further, their artificial F_1 hybrids are sterile because of failure in meiosis II (Kawamura

TABLE 3 Occurrence frequency of purebred clutches or hybrid clutches affected by unionid density and river systems

Variables	Coefficient	Standard error	*Z*-value	*p*-value
(Intercept)	−1.165	1.288	−0.905	0.365
Unionid density per quadrat	2.278	1.118	2.038	0.042
Nagaodani River system	−2.179	1.159	−1.881	0.060

Note: The result of generalised linear mixed model analysis with purebred/hybrid as a response variable, unionid density and river system as fixed factors, unionid identity as a random factor.

& Hosoya, 2000). This study shows that introduced *R. ocellatus ocellatus* negatively affects native *T. lanceolata* only through competition for breeding substrates.

4.2 | Other possible environmental factors affecting the hybridisation

This study suggests that the decline of unionid populations causes an increase in invasive hybridisation between two bitterling fish species. Our study sites are irrigation canals and turbid agricultural runoff flowed in from May to July, coinciding with the breeding period of bitterlings. In the Kunichi stream, the visibility was quite limited (Figure S1), and this may reduce interspecific recognition resulting in hybridisation, as observed in swordtail fishes in Mexican rivers (Fisher et al., 2006) and in cichlid fishes in the African Great Lakes, where turbidity is increased by human impact (Maan et al., 2010; Seehausen et al., 1997).

4.3 | Conservation implications

Invasive hybridisation can result in the extinction of native species through genetic introgression (Todesco et al., 2016). Hybrid individuals can also endanger native species through competition for habitat, food, and breeding opportunities (e.g. Ryan et al., 2009; Wolf et al., 2001). The present study found that lower unionid density was associated with more frequent invasive hybridisation of bitterlings. The sharp decline of unionids on the Matsuyama Plain is mostly caused by weirs. The weirs prevent mussel larvae dispersal being carried out by their host amphidromous goby, *Rhinogobius nagoyae*, to the upstream side of these barriers (Kuwahara et al., 2017). Weirs that are 1 or 2 m high are sufficient to hinder the recruitment of unionids to upstream areas, as observed in North America (Watters, 1996) and Australia (Brainwood et al., 2008). In our study sites, streams have multiple weirs, at most 2 m high, and without a fish ladder. This study also indicates that there is a recruitment failure of unionids because juvenile *P. japanensis* and *N. douglasiae* (<30 mm in shell length) (Brainwood et al., 2006) were never found. On the Matsuyama Plain, glochidium larvae of *P. japanensis* are frequently observed attached to the goby, *R. nagoyae*, during the mussel's breeding season (Kuwahara et al., 2017). Therefore, failure of settlement and/or survival of early juveniles cause this recruitment failure. Unionid recruitment can be restricted by artificial disturbances such as the input of agricultural runoff and negative effects of its chemical contents (Brainwood et al., 2006; Strayer & Malcom, 2012), and degradation of stream substrates (Geist & Auerswald, 2007). Without recruitment, unionid populations would become extinct within a few decades. Local extinction of a unionid population due to recruitment failures has been observed within as little as 8 years (Brainwood et al., 2006).

There remains only a short time for us to conserve endangered unionid species and to prevent coextinction of a bitterling fish on the Matsuyama Plain. *Tanakia lanceolata* and two unionid species—*P. japanensis* and *N. douglasiae*—are now listed as critically endangered species by the local government (Ehime Prefecture, Japan, 2019) based on previous studies (Hata et al., 2019; Kuwahara et al., 2017; Matsuba et al., 2014; Uemura et al., 2018). Firstly, a conservation zone needs to be established within the remaining habitat of these

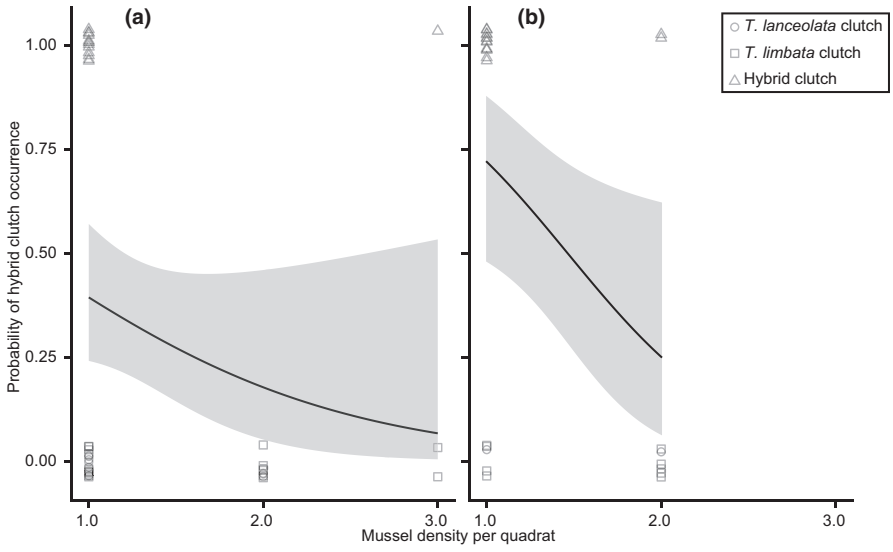

FIGURE 5 Relationship between the probability of hybrid clutch occurrence and the mussel density per quadrat in the Kunichi (a) and Nagaodani (b) river systems, fitted by a generalised linear model curve including a 95% confidence band (grey). *Tanakia lanceolata*, *Tanakia limbata* and hybrid clutches are illustrated by open circles, open squares, and open triangles, respectively

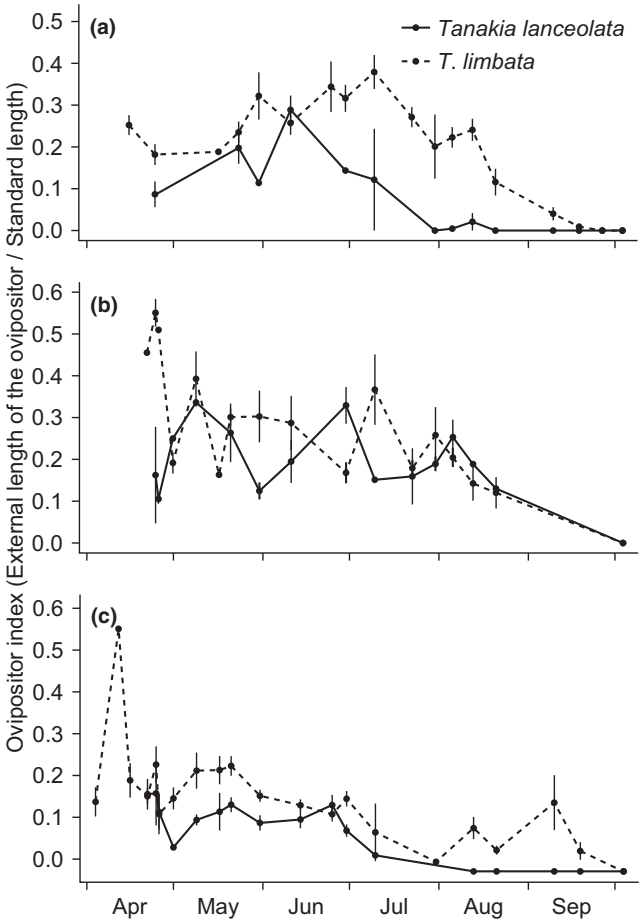

FIGURE 6 Seasonal changes in the ovipositor length index (ovipositor length/standard length) of female *Tanakia lanceolata* and *Tanakia limbata* in the Kunichi (a), Koyori (b), and Nagaodani (c) streams. The solid line and dashed line represent *T. lanceolata* and *T. limbata*, respectively, and error bars indicate standard deviation

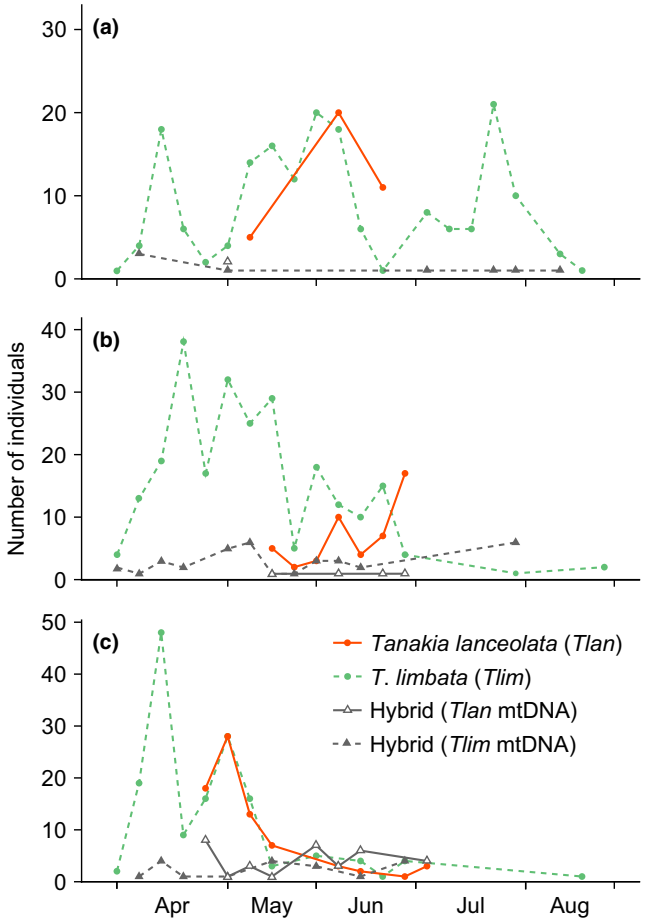

FIGURE 7 Seasonal differences in the numbers of *Tanakia lanceolata*, *Tanakia limbata*, and their hybrid eggs laid in mussels at the Kunichi (a), Koyori (b), and Nagaodani (c) streams based on genetic analyses and observations of the developmental stages (Suzuki & Hibiya, 1985). The solid line with closed circles (red), dashed line with closed circles (green), solid lines with open triangles (grey), and dashed lines with closed triangles (grey) represent *T. lanceolata* (*Tlan*), *T. limbata* (*Tlim*), their hybrids with *Tlan* mtDNA, and hybrids with *Tlim* mtDNA, respectively

species, and from there, introduced *R. o. ocellatus* and *T. limbata*, and hybrid individuals should be eliminated. Eliminating hybrids is problematic because some of them are only identifiable by genotyping. Therefore, morphologically distinct hybrids should be removed, and the genetic composition of the remaining population monitored using microsatellite markers. Secondly, the construction of fishways would provide accessibility to upstream regions for the host fish and lead to a recovery of habitat for unionids (Benson et al., 2018). Subsequently, larger-scale habitat restorations need to be implemented to improve stream substrates for the survival of unionid juveniles.

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DATA AVAILABILITY STATEMENT

All relevant data are available from the authors upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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