Identification of fish spawning based on size-selected sampling and diurnal concentration changes of environmental DNA resulting from sperm-release

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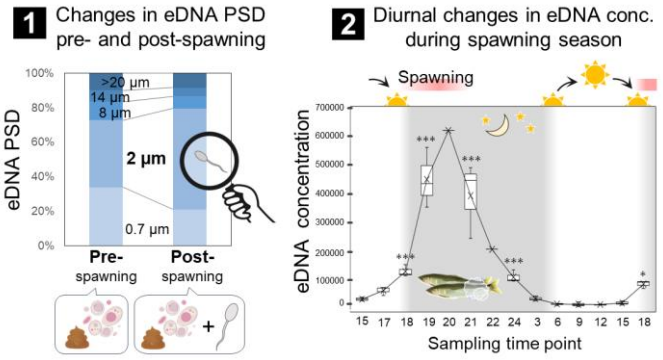
Abstract



Environmental DNA (eDNA) analysis is a useful method for detecting spawning behaviour by observing elevated eDNA concentrations and/or nuclear/mitochondrial DNA ratios. However, little is known about the characteristics and dynamics of sperm-derived eDNA, which is key to detection. This study focused on changes in eDNA particle size distribution (PSD) and concentrations pre-, post-, and during spawning of *Plecoglossus altivelis*, to investigate the potential for semi-selective recovery of sperm-derived eDNA and the importance of sampling time for improving detection sensitivity. Firstly, PSD changes between pre- and post-spawning were investigated by comparing concentrations and proportions of eDNA obtained from filters with different pore sizes. Secondly, the diurnal changes in eDNA concentration were monitored at the peak of the spawning season by collecting river water every 1-3 hours. Results showed that eDNA related to sperm-head size increased at post-spawning, and eDNA concentrations had significant diurnal changes with a peak during the spawning time window. These findings suggest that semi-selective recovery of sperm-derived eDNA based on particle size and/or sampling during the spawning time window with increased concentrations can improve the detection sensitivity of eDNA-based spawning surveys. This study provides essential fundamental information for advancing eDNA-based spawning surveys and contributes to their further development.

Keywords: spawning, sperm-derived eDNA, particle size distribution, diurnal change, Ayu

Introduction

The utilisation of environmental DNA (eDNA) analysis for the evaluation and administration of aquatic ecosystems is rapidly expanding and now becoming commonplace [1–6]. Environmental DNA analysis enables us to indirectly reveal the presence and/or biomass of macroorganisms based on the detection of DNA materials derived from their metabolic waste, sloughed skin cells and gametes released into the environments including soil, water, and air [7–11]. Moreover, recent studies have indicated that the eDNA concentration and the nuclear DNA (nuDNA)/mitochondrial DNA (mtDNA) ratio in environments significantly increases during the spawning season compared to the non-spawning season [12–18]. This phenomenon is attributable to the fact that gametes, primarily sperm, discharged during spawning behaviour, are detected as eDNA in species that perform external fertilisation [11,19].

By focusing on the eDNA concentration and/or ratio alterations that are distinct to the non-spawning season, eDNA analysis has the potential to enable us to estimate species spawning behaviour and its magnitude and to detect species with small biomass more sensitively. An understanding and knowledge of spawning are crucial for the conservation and management of species because it constitutes one of the most significant aspects of their life history [13,15]. Spawning surveys using eDNA analysis are potentially useful because they provide an opportunity to monitor and understand spawning with less effort, time, and invasiveness than conventional observation-based methods [20,21]. Additionally, it is probable that appropriate sampling during the spawning season, when eDNA concentrations are temporarily heightened, will enhance the probability of detecting species with small biomass compared to the non-spawning season[22,23]. The low eDNA concentrations are one of the primary reasons for false-negative results in surveys focused on detecting rare species and recently introduced non-native species [24,25]. A sampling strategy targeted at the spawning season could reduce the risk of false negatives.

Despite the key role of gamete-derived eDNA in eDNA studies during the spawning season, its characteristics and dynamics remain largely unexplored. As eDNA detectability and persistence are significantly influenced by its characteristics and dynamics, understanding these aspects is critical for designing effective sampling strategies and interpreting results accurately [26]. While substantial knowledge has been accrued on somatic-derived eDNA (such as from skin, feces, and mucus), it is pointed out that previous knowledge may not be applicable because the morphological features of gametes-derived eDNA differ significantly from them [27–29]. Teleost spermatozoa, for example, the range is between about 25 and 100 μm in length and mainly consists of a head tightly overlayed with the plasma membrane, a middle section containing a mitochondrial capsule with multiple disulfide bridges, and a long tail [29,30]. These sperm are released into the water in an almost intact state, and the size of each part is almost uniform within each species.

This study focused on examining the changes in particle size distribution (PSD) of eDNA pre- and post-spawning, as well as the diurnal changes in eDNA concentration during the spawning season. Firstly, the understanding of PSD is crucial for estimating the dispersion and settling rate of eDNA, and it may allow for selective recovery of gamete-derived eDNA based on particle size. Selective recovery

of sperm-derived eDNA could enhance the sensitivity of spawning activity detection, as well as aid in assessing genetic diversity within spawning groups. Secondly, since the increase in eDNA concentrations during the spawning season is mainly attributed to sperm release, eDNA concentrations are likely to show diurnal changes with peaks at the spawning time window [19]. The only previous study to investigate spatiotemporal changes in eDNA concentrations post-spawning of a single carp (*Cyprinus carpio*) pair by observing experimental ponds and data simulations found that concentrations peaked by spawning, distributed uniformly in the experimental pond, and returned to baseline within approximately 24 hours [31]. Nevertheless, in natural lotic habitats, eDNA concentrations may change more dynamically and over a shorter period due to multiple spawning individuals and continuous diffusion. Therefore, if this assumption is correct, it will be essential to pay attention to the timing of water sampling for conducting an accurate and quantitative survey based on eDNA analysis to monitor spawning.

This study aimed to explore pre- and post-spawning changes in eDNA particle size distribution (PSD) and diurnal eDNA concentration shifts during the fish spawning season in a river. Two experiments were conducted in a river using Ayu (*Plecoglossus altivelis*), which is known to spawn daily in the hours post-sunset during their spawning season, as a model species. In experiment 1 (Exp. 1), we collected water samples pre- and post-spawning at the initial and peak phases of the spawning season, examining eDNA PSD for two genetic regions. In experiment 2 (Exp. 2), diurnal changes in eDNA concentration were monitored during peak phases of the spawning season by time-series sampling over a 27-hour period with emphasis on the spawning window. Based on the results, we discussed the potential of selective recovery of sperm-derived eDNA, its advantages and the need for careful consideration of sampling timing in view of spawning ecology.

Materials and Methods

Overview of the experimental designs are presented in Fig.1. Both experiments were conducted in the lower reaches of Shiotu-o River, Japan (35°30'58.7"N 136°09'48.8"E, Fig. S1).

Experimentsl species

Ayu, an important species for fisheries and recreational fishing in Japan, holds a unique ecological position as an algivorous specialist, making it a key member of Japanese riverine ecosystems [32–34]. Ayu has a one-year life cycle, primarily migrating upstream in spring or autumn from the sea or lake to rivers, spawning and dying in the lower reaches of rivers in autumn [35,36]. During the peak of the spawning season (roughly late September to middle October), they form dense colonies and spawn daily around sunset to early night hours on gravel beds in riffles [34,37]. It means that eDNA derived from spawning, primarily sperm-derived eDNA, is only released within a limited time window. The sperm of Ayu is primarily composed of an oval capsule-shaped head (measuring 2.1 μm in length and 1.2 μm in width) and a lengthy flagellum (21.2 μm) (Fig. S2)[38-40]. The sperm head contains a nucleus and some mitochondria.

Experiment 1: Changes in PSD of eDNA pre- and post-spawning

Water sampling was carried out on September 13th (the earliest phase of the spawning season) and 22nd (the peak phase) in 2020. 300 mL of surface water was collected in triplicate at 15:00 (daytime, pre-spawning) and 19:00 (one hour after sunset, during spawning) using disposable bags (DP16-TN1000, Yanagi, Aichi, Japan). The collected water was fractionally filtered on-site via a series of filters with different pore sizes, 20, 14, 8, 2 μm (Track-Etched Membrane PCTE filter; GVS Japan, Tokyo, Japan), and 0.7 μm (GF/F glass-fiber filter; Cytiva, Tokyo, Japan). The on-site filtration is comprised of multiple filter holders connected (PP-47; ADVANTEC, Tokyo, Japan), for which the necessary number of folders were prepared by decontamination through immersion in 10% hypochlorite for at least 30 minutes, followed by adequate washing with water (same for Exp. 2). After filtering the 19:00 samples, 300 mL of ultrapure water was filtered using GF/F filters to serve as filtration-negative controls (Filt-NCs). All filter samples were immediately stored at −20 °C. DNA was extracted from the filter samples in the laboratory, and the mitochondrial cytochrome *b* (cyt*b*) gene and nuclear recombination activating gene 1 (RAG1) of Ayu were quantified using quantitative real-time PCR and species-specific primer-probe sets, respectively (detailed below).

Experiment 2: Diurnal changes in eDNA concentration during the spawning season

Time-series water sampling was performed between September 20th and 21st, 2021. 300 mL of surface water was collected in triplicate at 14-time points: 15:00, 17:00, 18:00, 19:00, 20:00, 21:00, 22:00 and 24:00 (day 1), 3:00, 6:00, 9:00, 12:00, 15:00 and 18:00 (day 2). Sampling time points were set to be hourly during the spawning time window from sunset to earlier in the night, and every three hours at other times. The collected water samples were filtered on-site using GF/F filter and filter holder. After filtering of all samples, 300 mL of ultrapure water was filtered using GF/F filters as Filt-NCs. All filter samples were immediately stored at −20 °C. DNA was extracted from the filter samples in the laboratory, and the Ayu cyt*b* gene was quantified using quantitative real-time PCR and species-specific primer-probe set (detailed below). However, for the 20:00 and 22:00 samples, we found accidental trapping Ayu larvae were accidentally captured in 2/3 of the filters, respectively. Thus, these samples were excluded from subsequent analyses. Water and ambient temperatures during the survey are shown in Table S1. The weather was fine for at least five days prior to the survey date until the end of the survey, and the flow velocity remained constant throughout the survey (Table S2).

DNA extraction from each filter sample

Firstly, a PCTE filter and a GF/F filter were respectively placed in the lower or upper part of the spin column (EconoSpin, EP-31201; GeneDesign, Inc., Osaka, Japan), with the silica gel membrane eliminated. Prior to DNA extraction, the GF/F filters contained excessive water due to their thickness, which was removed by pre-centrifugation at 6,000 g for 1 minute. Subsequently, 470 μL of a mixture consisting of 200 μL ultrapure water, 250 μL Buffer AL, and 20 μL proteinase K was placed on each filter, and the spin columns were incubated for 45 min at 56 °C. After the incubation, each PCTE filter was re-placed in the upper part of the spin column. The spin columns were then centrifuged at 6,000 g for 1 minute. After adding 500 μL ethanol to the collected liquid and mixing well by pipetting, the DNA

solution was transferred to a DNeasy mini spin column (Qiagen, Hilden, Germany). The solution was then purified following the manufacturer's protocol. Finally, the DNA was eluted in 100 µL of Buffer AE.

Species-specific primer/probe set development targeting for RAG1 of Ayu

The RAG1 sequences of Ayu and its closely related *Osmeriformes*, *Hypomesus nipponensis* and *Salangichthys microdon*, were downloaded from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The species-specific primers and probe were manually designed based on Ayu sequences, where the designed primers have the species-specific nucleotide at the 3' ends. For the forward primer, the 3' end is consistent with *S. microdon*; however, there are base mismatches at positions 4, 7 and 19. For the reverse primer has three consecutive mismatches from the 3' end, so it was judged not to anneal for *S. microdon* DNA (Fig. S3). The TaqMan probe was designed on the amplification range of primers, where it has four and five bases mismatches with *H. nipponensis* and *S. microdon*, respectively. To check the primer parameters and species specificity, *in silico* test was conducted using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with default settings. Additionally, to check the species specificity of primer/probe set, an *in vitro* test was performed using extracted genomic DNA from Ayu, *H. nipponensis* and *S. microdon* (three individuals for each species). As a DNA template, 100 pg of genomic DNA from each individual was used in each PCR reaction. The real-time PCR conditions were consistent with those used for the analysis of eDNA samples, as described below. Along with assessing the amplification curve by real-time PCR, PCR products were also checked for DNA bands by electrophoresis.

Quantitative real-time PCR (qPCR) assays

Quantitative real-time PCR was performed in triplicate utilizing either a Light Cycler 96 system (Roche, Basel, Switzerland) or StepOnePlus Real-Time PCR system (Life Technologies, CA, USA). The species-specific primer-probe set for each target region of Ayu was shown in Table 1. All reactions were performed in a 15-µL total volume comprising of 900 nM of each primer (Paa-cyt*b*-F/R or Paa-RAG1-F/R), 125 nM of TaqMan probe (Paa-cyt*b*-Pr or Paa-RAG1-Pr), 0.075 µL AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific, MA, USA), and 2.0 µL DNA template in 1× TaqMan Environmental Master Mix (Thermo Fisher Scientific). In all qPCR runs, plasmid DNA that contains the target regions at pre-set concentrations (dilution series of 3×10^1–10^4 copies per reaction for Exp. 1; 3×10^1–10^5 copies per reaction for Exp. 2) and PCR negative controls (PCR-NCs, ultrapure water) were also amplified with environmental samples. The qPCR thermal conditions were as follows: 50 °C for 2min, 95 °C for 10 min, followed by 55 cycles of 95°C for 15 s and 60°C for 60 s. For Exp. 1, the R^2 values for the standard lines of all qPCR for cyt*b* and RAG1 region ranged from 0.996 to 1.0 and 0.996 to 0.999, respectively. For Exp. 2, the R^2 values for the standard lines of all qPCR for cyt*b* were 1.0. No amplification was observed in any of the Filt-NCs and PCR-NCs.

Statistical analysis

All statistical analyses for this study were conducted using R version 4.1.1 software (R Core Team. R, 2021). Due to the limited amount of data in Experiment 1 (n = 3), it was difficult to determine the data distribution accurately. Thus, we conducted two statistical analyses, assuming either non-parametric or parametric data distribution in Experiment 1. The p-values obtained from the parametric analyses were shown in the figures and used for discussion, as statistical analyses assuming non-parametric data distribution can easily trigger Type II errors (Table S3). To compare the total copy number of cyt*b* or RAG1, which included all size fractions, we used the Kruskal–Wallis test followed by the Conover's test with Holm adjustment (PMCMRplus package; ver. 1.9.3) and one-way analysis of variance (ANOVA), followed by the parametric Tukey honest significant differences (HSD) test. Additionally, to examine changes in the PSD of eDNA pre- and post-spawning, the concentrations and proportions of eDNA obtained from filters with different pore sizes were compared between daytime and nighttime using the Mann-Whitney U test and t-test for each filter, respectively. To account for the multiplicity of tests, the Bonferroni correction was applied, and we set the minimum level of significance at $P < 0.01$. In Experiment 2, to examine diurnal changes in eDNA concentration, the eDNA concentration observed during the daytime (pre-spawning; day1-15, 17, day2-6, 9, 12, 15) was compared with those observed at each time point during the nighttime (post-spawning; day1-18, 19, 21, 24, 3, day2-18) using the Kruskal–Wallis test followed by the Conover's test with Holm adjustment. Day1-20 and Day1-22 were excluded from the analysis because larvae were accidentally trapped on one or two filters out of three replicate filters.

Results and Discussion

Changes in concentration and ratio of eDNA between pre- and post-spawning

In both DNA regions, a significant increase in total DNA copy number was observed only when comparing pre- to post-sunset on 22 September (Tukey HSD; $p < 0.01$, both DNA regions, Fig. 2a, b). Additionally, the RAG1 to cyt*b* ratio also showed a significant increase before and after the spawning time window on only 22nd September (Tukey HSD; $p < 0.01$, Fig. 2c). These findings suggested that simply comparing eDNA concentration and the ratio of ncDNA/mtDNA before and after the spawning time window is a useful approach for identifying spawning, but it might not be sensitive enough to identify slight spawning in the early spawning season. The total number of eDNA copies showed a tendency to increase in both DNA regions from the 13th of September towards the peak of spawning on the 22nd (Tukey HSD; Fig. 2a, b). As Ayu aggregates in the lower reaches of rivers for spawning [21,41], the concentration of eDNA originating from non-gametes sources (i.e. skin, feces, and mucus etc.) in the water is expected to increase from just before the spawning season to the peak. This baseline increase in eDNA concentration due to fish aggregation might have masked the increase in concentration and ratio due to sperm-derived eDNA released by slight spawning in the early spawning season. Hence, it was suggested that further development of both approach methods is needed to improve detection sensitivity and to identify the occurrence of slight spawning (Shiga Prefectural Fisheries Experiment Station).

Changes in eDNA PSD between pre- and post-spawning

On only the 22nd of September, the eDNA concentrations increased in both DNA regions of all particle sizes at nighttime (t-test; Table S3; Fig. 3a, b). This increase in eDNA concentration in both DNA regions was accompanied by alterations in the PSD, characterised by a decrease in 0.7-2 μm size and an increase in 2-8 μm size post-spawning (t-test; Table S4; Fig. 3a, b). These results would be explained by the size of the sperm. The spermatozoa of *P. altivelis* is approximately 23 μm in length and consist of a head part containing the nucleus (about 2 μm) and mitochondria (about 0.7 μm), along with a tail part (Fig. S2)[38-40]. Considering the overall size, it is likely that sperm-derived eDNA would be trapped by filters with a pore size of 20 μm. However, eDNA concentrations increased in all size fractions at post-spawning, suggesting that the long and thin tail did not significantly prevent the passage of sperm through the filter. Furthermore, the increase in eDNA trapped in the 2-8 μm size fraction suggests that sperm-derived eDNA is mainly ensnared in the filter depending on the size of the head part. The dependence of PSD of sperm-derived eDNA on head size is consistent with the only previous study using Japanese jack mackerel (*Trachurus japonicus*)[28]. The shapes and sizes of sperm vary widely between species and taxa [40,42]. Therefore, comprehending the relationship between more diverse sperm shapes and PSDs in future studies will contribute to a more profound understanding of the eDNA dynamics specific to the spawning period.

On 13th September (early spawning season), even though the obtained P-value did not reach the level of statistical significance, the RAG1 eDNA concentration and nuDNA/mtDNA ratio observed in the 2-8 μm size fraction showed a remarkably suggestive increase between pre- and post-spawning (Fig. 3bc). Considering that no change was observed when total eDNA concentrations and ratios were compared, this result suggests that the fractionation of eDNA according to sperm head size enables more sensitive detection of changes in eDNA concentration and ratio resulting from small amounts of released sperm in the early spawning season. Furthermore, the concentration of RAG1 within the 2-8 μm size fraction exhibited a significant increasing trend, indicating that nuDNA is a more sensitive indicator of spawning events than mtDNA. The superiority of nuDNA has also been reported in a previous study comparing detection sensitivity between mtDNA (cyt*b*) and nuDNA (RAG1) in the detection of Ayu spawning using the same primer-probe sets as in the present study [21]. Future studies should be worth investigating the detection sensitivity differences between regions of nuDNA, focusing on the number of copies per cell for spawning detection based on eDNA analysis. Due to the challenge of designing species-specific primers, this study and Saito et al. (2022) targeted RAG1 (a single-copy region). However, previous studies have shown that the use of multi-copy region nuDNA, such as the ribosomal internal transcribed spacer I (ITS1), significantly increases detection sensitivity [43,44].

Substantial diurnal change in eDNA concentration during the peak of spawning season

During the peak spawning season, a significant fluctuation in cyt*b* eDNA concentration was observed, with the highest concentrations occurring within the spawning time window (day1-20:00, 623,250 copies/2μL), approximately 20.5 times greater than the levels observed during the daytime (day1-15, 17, day2-6, 9, 12, 15; average, 30,332 copies/2μL; median, 24,040; SD 19,305 copies/2μL; Fig. 4). The

eDNA concentration increased immediately after sunset when Ayu began spawning, and reached a maximum at day1-20:00, after which they decreased to the same level as the diurnal concentration seven hours later on day2-3:00 (P=1.0). Additionally, on day2-18:00, eDNA concentrations tended to increase again, surpassing the levels observed during the day (P<0.05). In the only previous study about diurnal changes in eDNA concentration, a slight increase in eDNA levels associated with nocturnal activity in nocturnal fish, Japanese eels (*Anguilla japonica*), was reported by aquarium experiments[45]. In contrast, previous studies on nocturnal amphibians found no differences in eDNA concentrations between daytime and nighttime (*Cryptobranchus a. alleganiensis*[16], *Ascaphus montanus* and *Dicamptodon aterrimus*[46]). Ayu, the target species in this study, is a diurnal fish, and their activity level at nighttime is significantly lower than during the daytime in the non-spawning season [34,47]. Additionally, the fact that no differences in eDNA concentrations were observed before and after sunset during the early spawning season (13th September) makes it reasonable to assume that Ayu eDNA does not usually show significant diurnal changes with peaks at night. Therefore, the substantial diurnal changes in eDNA concentrations observed in this study were caused by spawning behaviour and are expected to be specific to the spawning season. This study is believed to be the first to monitor and report the diurnal changes in eDNA concentration during the spawning season.

The presence of diurnal changes in eDNA concentration has implications for the planning of sampling design in future eDNA surveys. Firstly, in spawning surveys based on eDNA, the timing of sampling should be determined by whether the researcher wants to estimate the occurrence and amounts of spawning or fish aggregation amounts. In previous spawning surveys based on eDNA analysis, the spawning time has not been considered in determining the timing of eDNA sampling. Almost all previous results from eDNA-based spawning surveys conducted in natural habitats showed a reasonable relationship with the abundance of aggregated fish observed using conventional methods, but either contradicted or showed very weak relationships with the abundance of eggs and larval recovered [11,17,18,20,48–50]. This could be due to the fact that fish remain in the vicinity of the spawning area, but sperm-derived eDNA is released and then diffuses downstream, with concentrations becoming comparable to the non-spawning time window within a few hours. So, in estimating the occurrence and/or amounts of spawning, it would be detected more sensitively and accurately by sampling before and after the spawning time window and comparing concentrations and ratios. On the other hand, in estimating the fish aggregation amounts, sampling at a fixed time, at least 8 hours after the spawning time window, would reduce the effect of increased concentration due to sperm-derived eDNA. However, it is assumed that diurnal changes in eDNA concentrations will not be observed or will be weakened in species that spawn regardless of time and in lentic environments. Thus, it would be desirable to investigate the relationship between spawning and eDNA in species and habitats with diverse spawning ecology in the future and to continue to seek further sampling strategies to appropriate research objectives. Secondly, eDNA sampling during the spawning time window would increase the probability of detecting species with small biomass, such as invasive species at the initial invasion stage and/or rare species. The low eDNA concentration due to the small biomass is one of the main causes of false-negative results in eDNA studies [24,25]. Thus, some previous studies have suggested that eDNA sampling

during the spawning season, when eDNA concentrations are likely to increase for species with small biomass, may be effective in avoiding false negative results [19,22,23]. Additionally, this study proposes that sampling during the spawning season, while taking into account the spawning time window, may further enhance detectability. Increased detection sensitivity could contribute to expanding the applicability of eDNA analysis and leading to reliable results.

Implications and Perspectives

In this study, we observed changes in the PSD resulting from spawning and demonstrated the possibility of semi-selectively recovering sperm-derived eDNA by using a filter with pore size corresponding to the size of the sperm head. Semi-selective recovery of sperm-derived eDNA could contribute to improving the detection accuracy of spawning surveys based on observations of changes in the concentration and ratio of eDNA. Furthermore, the existence of significant diurnal changes in eDNA concentrations peaking during the spawning time window was revealed for the first time, emphasising the necessity of carefully considering the sampling times. Our findings provide essential fundamental information for improving the detection sensitivity and accuracy of spawning surveys based on eDNA analysis, advancing our comprehension of the characteristics and dynamics of eDNA during the spawning season. There is no doubt that eDNA analysis can be one of the most efficient and non-invasive strategies for spawning surveys, although attempts to apply it are still in their infancy. To maximise the applicability and usefulness of eDNA analysis for spawning surveys, it is crucial to advance the understanding and accumulation of basic information on the characteristics and dynamics of sperm-derived eDNA in various taxa and habitats in future research.

Authors' contributions

S.T.: Conceptualization, Methodology, Fieldwork (Exp. 2), Molecular analysis, Visualization, Writing − original draft, Funding acquisition. N.S.: Fieldwork (Exp. 1 and 2), Molecular analysis, Writing − review & editing.

Data availability

Full details of the qPCR results for each experiment of the present study are available in the supporting information (Table S5 and S6).

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Table

Table 1 Species-specific primer-probe set for each target region of Ayu. Each primer-probe set was developed by Yamanaka & Minamoto (2016) (cyt*b*) and this study (RAG1), respectively.

Target region	Primer/probe	Sequence (5' -> 3')	Length	Tm °C	Product length
mtDNA, cyt*b*	Paa-cyt*b*-F	CCTAGTCTCCCTGGCTTTATTCTCT	25	61.17	131
	Paa-cyt*b*-R	GTAGAATGGCGTAGGCGAAAA	21	58.73	
	Paa-cyt*b*-Pr	[FAM]–ACTTCACGGCAGCCAACCCCC–[BHQ]	21	67.59	
nuDNA, RAG1	Paa-RAG1-F	GGACCACGAGACCCTGACA	19	60.91	132
	Paa-RAG1-R	TAGCCTGTTCCCCTGAAACAG	21	59.65	
	Paa-RAG1-Pr	[FAM]–ACTCATCCTCTCCATGGGTGGCCTCC–[BHQ]	26	69.16	

Figure

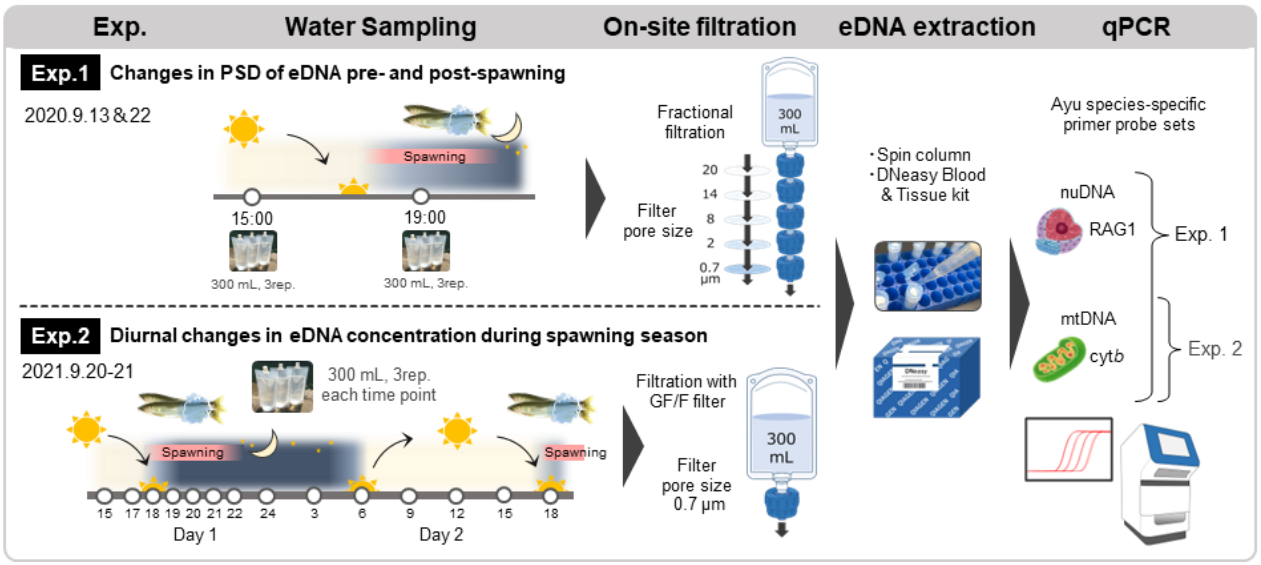

Figure 1. Overview of the two experimental designs.

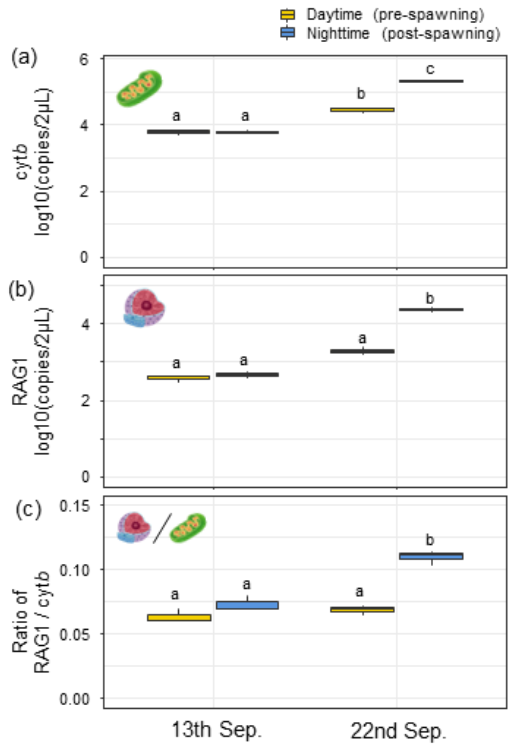

Figure 2. Comparisons of total eDNA concentrations and nuDNA/mtDNA ratio between daytime (pre-spawning) and nighttime (post-spawning) at two seasons. The orange and blue box plots indicate daytime and nighttime, respectively. The sum of the copy numbers detected on the five filters with different pore sizes was used as the total eDNA concentration. Significant differences are indicated by different letters (Conover's test, $P < 0.01$).

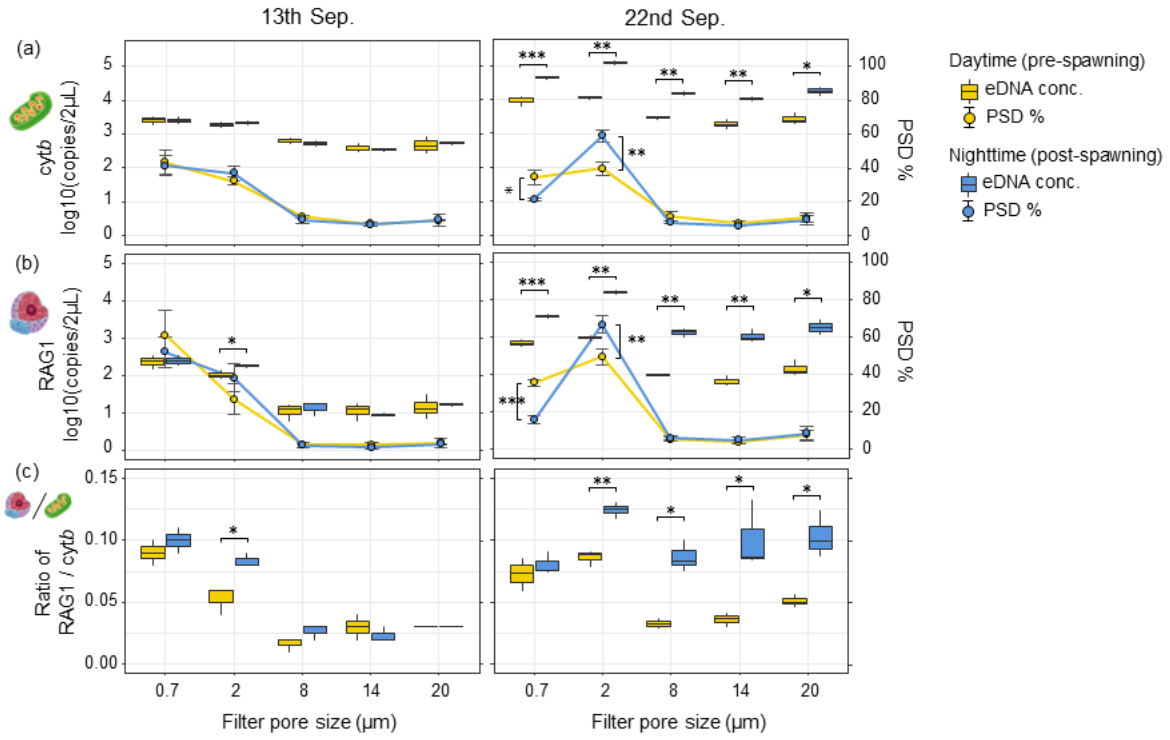

Figure 3. Comparisons of eDNA PSDs and its nuDNA/mtDNA ratio between daytime (pre-spawning) and nighttime (post-spawning) at early (13th Sep.) and peak (22nd Sep.) of spawning seasons. The orange and blue indicate daytime and nighttime, and the box plot and line graph indicate the eDNA concentration and percentage of distribution, respectively. Significant differences are indicated by asterisks (t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; the minimum level of significance was $P < 0.01$).

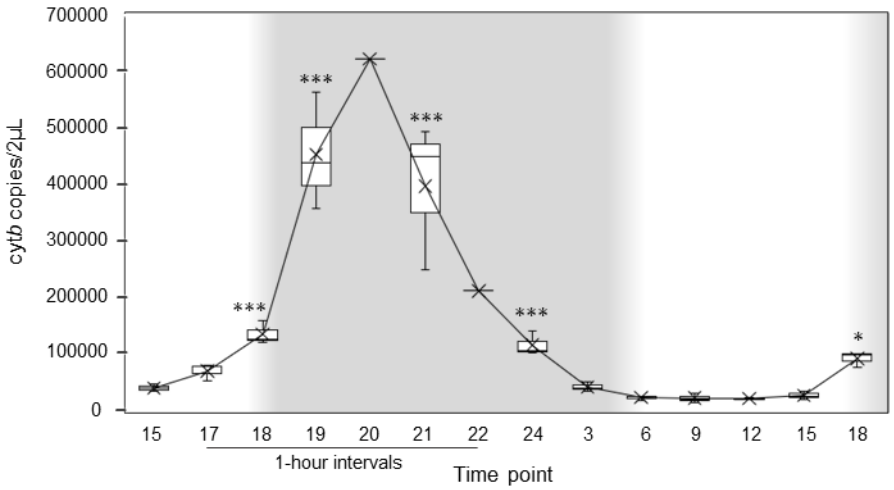

Figure 4. Diurnal changes in eDNA concentration of Ayu cytb during the peak of spawning season. To clearly show the diurnal changes in concentration, the mean concentration observed at each time point was indicated by x-mark and connected by a line. Samples at 20:00 and 22:00 were excluded from the analysis due to the accidental capture of Ayu larvae in 2/3 of the filters, respectively. the eDNA concentration observed during the daytime (day 1-15, 17, day 2-6, 9, 12, 15) was compared with those observed at each time point during the nighttime by Conover's test. Significant differences are indicated by asterisks (t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Sunset and sunrise times are as follows: 17:56 and 5:43.

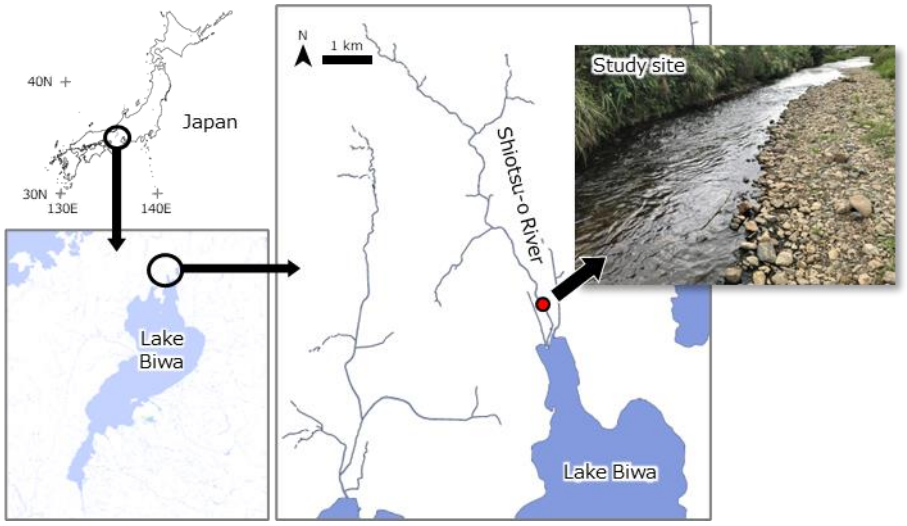

Figure S1. Study site.

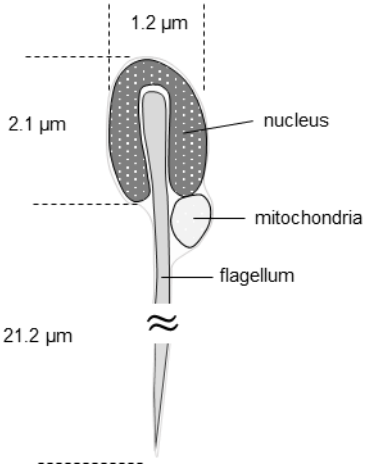

Figure S2. Simple schematic representation of the ultrastructure of *P. altivelis* sperm.

Species	Paa-RAG1-F	Paa-RAG1-Pr	Paa-RAG1-R (reverse compliment)
Plecoglossus altivelis	GGACCACGAGACCCTGACA ...	ACTCATCCTCTCCATGGGTGGCCTCC ...	CTGTTTCAGGGGAACAGGCTA
Hypomesus nipponensis	GGACCACGAGACCCTGACG ...	CCTCATCCTGGCCATGGGCGGCCTCC ...	CCACTTCAGGGGCACAGGCTA
Salangichthys microdon	AGACCACGAGACTCTCACA ...	ACTCATCCTGAGCATGGGCGGCCTCT ...	TCATTTCAGGGGCACGGGCTA

Figure S3. Sequences of the target region of the designed primer-probe set for nuclear recombination activating gene 1 (RAG1) of P. altivelis and its closely related species in Japan. The amplicon length was 132 bp.