Title:

Identifying spawning timing and locations of jack mackerel in a semi-closed bay using environmental DNA analysis

Running title: Identifying Jack Mackerel Spawning with eDNA

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Abstract

Understanding spawning ecology is vital for species/population management and conservation. However, conventional surveys are generally time- and labour-demanding and invasive. To overcome these challenges, environmental DNA (eDNA) analysis has emerged as a promising spawning survey method. This study aimed to identify the spawning season, times, and locations of Japanese jack mackerel (*Trachurus japonicus*) through eDNA analysis and to investigate the utility of eDNA analysis in spawning surveys. We expected that eDNA concentration and nuclear/mitochondrial DNA ratio should change overnight due to sperm-derived eDNA as this species is a nocturnal spawner. The eDNA concentrations at sunset and sunrise at three sites in Maizuru Bay were compared monthly for one year. Our results showed significant increases in eDNA concentrations and ratios at all sites in July, suggesting potential spawning occurrences. At the site with a particularly large increase in concentration, temporal monitoring showed a diurnal eDNA peak (9:00 PM–12:00 midnight), likely reflecting the jack mackerel spawning time window. Subsequently, our eDNA-based estimations were supported by the successful capture of their eggs using a plankton net. This is the first report providing evidence of saltwater fish spawning using eDNA analysis, underscoring the usefulness of this approach for spawning detection.

Keywords: spawning, eDNA, egg, sperm, saltwater fish, Maizuru Bay, qPCR

Introduction

Reproductive events are an important factor in the recruitment process of fish populations because the success and magnitude of spawning directly influence population reproduction and future growth (Mertz and Myers, 1994; Scott et al., 2006). Hence, comprehending the time of day and location of fish spawning has long been recognised as a critical challenge for effective species and/or population management and conservation (Danylchuk et al., 2011; Di Muri et al., 2022; Spear et al., 2015). Conventional methods employed by fishermen, researchers, and conservation managers to identify fish spawning involve collecting fish eggs, observing the gonads of parent fish or conducting acoustic telemetry surveys. However, these methods are generally time-consuming and labour-intensive as well as sensitive to monitoring biases and field conditions (ex. weather, time and turbidity) (Caswell et al., 2004; Ko et al., 2013; Vautier et al., 2023). Furthermore, additional mortality is inevitable with survey methods that require the collection of individuals and eggs (Engstedt et al., 2014; Lefort et al., 2015; Wei et al., 2009). Consequently, an efficient and non-invasive method that could solve these conventional challenges would be welcomed as a new approach in fish spawning surveys.

In recent years, environmental DNA (eDNA) analysis has emerged as a rapidly advancing biodiversity assessment method and has entered a new phase where it can estimate ecological events such as spawning as well as the presence of the target species (Ip et al., 2023; Sato et al., 2021; Tsuji et al., 2022b; Wu et al., 2023). Environmental DNA on macroorganisms refers to generic terms for DNA derived from epidermal tissue, faeces, mucus and other sources released from organisms into their habitat (Barnes and Turner, 2016; Merkes et al., 2014). In species that perform in-vitro fertilisation, the reproductive materials such as oocytes, ovarian fluid and sperm released during spawning are also an eDNA source. In eDNA-based spawning surveys, the occurrence of spawning can be identified by observing rapid and significant increases in eDNA concentration and/or changes in nuclear/mitochondrial DNA ratios caused by sperm-derived eDNA (Bylemans et al., 2017; Tsuji et al., 2022b). Since only environmental samples like water need to be collected in the field, there is no impact on the target species or their habitats, and monitoring bias resulting from the surveyor's experience and amount of knowledge

are minimised. These advantages would solve some of the challenges of conventional methods, leading to increasingly high expectations for eDNA analysis as a novel approach in spawning surveys.

Spawning surveys based on eDNA analysis are still in the developing stage, and their application for fish is currently limited to freshwater areas (ex. lake, Vautier et al., 2023; Wu et al., 2023; river, Bracken et al., 2019; Di Muri et al., 2022; Erickson et al., 2016; Saito et al., 2022; Tsuji and Shibata, 2023). Previous studies investigating fish which spawn in the sea, such as the Japanese jack mackerel (*Trachurus japonicus*) and the Japanese eel (*Anguilla japonica*), have reported significant increases in eDNA concentrations before and after spawning in both species when artificially spawned in laboratory conditions (Takeuchi et al., 2019; Tsuji et al., 2022b). These findings suggest that spawning surveys based on eDNA could be a useful method for investigating spawning in saltwater fish. The decline of marine biodiversity is a pressing concern, with fish populations inhabiting coral reefs and seagrass beds estimated to have decreased by 34–70% in the past 40 years until 2012 due to overfishing, ecosystem destruction and climate change (WWF 2015). Considering the importance of conserving marine biodiversity and managing fisheries resources sustainably, it would be of great value to investigate the applicability of spawning surveys based on eDNA analysis in saltwater areas, which are affected by more complex currents than freshwater areas.

The Japanese jack mackerel (*Trachurus japonicus*) is one of the most commercially important resources in East Asia and dominates in Maizuru Bay, Japan, from spring to autumn (Masuda, 2008; Masuda et al., 2008). They primarily spawn at the shelf break of the southern East China Sea (Sassa et al., 2006), although small-scale regional spawning also scatters along the Japanese coast (Nishida, 2006; Xie et al., 2005). Due to the complex ocean currents affecting the Japanese archipelago, Japanese jack mackerel spawn at different seasons and in different spawning grounds, contributing to the seasonal stock recruitment in fisheries. For example, they spawn from February to May in the main spawning ground of the East China Sea and from November to next August in the coastal waters of Japan (the eastern regions being later) (Nishida, 2006). While the spawning of Japanese jack mackerel in Maizuru Bay has not been demonstrated, the otolith microstructure of juveniles caught in Kunda Bay,

just west of Maizuru Bay, suggests the existence of a population that may have been spawned near Maizuru Bay and the southern Sea of Japan (Kanaji et al., 2009). Understanding the spawning season and grounds of Japanese jack mackerel in Maizuru Bay is essential for fishery resource managers to comprehend population dynamics and effectively manage resources.

In the present study, three experiments were conducted in Maizuru Bay with the aim of identifying the spawning season (seasonability), time window (diel spawning periodicity) and location of Japanese jack mackerel and examining the usefulness of eDNA analysis on spawning surveys. Since they spawn at night, it is assumed that eDNA concentrations are higher at sunrise than before sunset during the spawning season. Therefore, in Experiment 1 (Exp. 1), eDNA concentrations in Japanese jack mackerel were investigated once a month, both before sunset and at sunrise the following day at multiple sites to estimate the spawning season and locations. In Experiment 2 (Exp. 2), diurnal changes in eDNA concentrations were investigated during the estimated spawning season to identify the time window of spawning. Finally, in Experiment 3 (Exp. 3), fish eggs were collected using a plankton net to demonstrate the spawning of Japanese jack mackerel during the estimated spawning season. Based on the results, we discuss the potential of eDNA analysis as a novel approach for conducting spawning surveys in the sea.

Materials and Methods

The overview of sampling designs is presented in Fig.1. All experiments were conducted in Maizuru Bay, Japan, which has a surface area of ca. 11 km^2 (35.481°N, 135. 332°E).

Experiment 1: Estimation of the spawning season and spawning sites

The water sampling survey was conducted in three sites in Maizuru Bay once a month from January 2021 to January of the following year, on the day of the new moon (Fig. 1, Table S1). We chose a new moon day on the assumption that a darker environment would be less likely to be attacked by natural enemies and spawning would

be more likely to occur. As Japanese jack mackerel spawn at night; if spawning occurs, it is expected that higher DNA concentrations are observed at sunrise than before sunset. In each site, 1 L of surface water was collected in triplicate at two-time points, two hours before sunset and sunrise on the following day (Fig. 1b, Table S1). As Maizuru Bay is surrounded by mountains and some areas are dark before sunset, the water sampling time was set at two hours before sunset. The three duplicated samples were treated as independent samples. To preserve eDNA in water samples, benzalkonium chloride (BAC) was added and agitated well (final concentration, 0.01%; OSVAN S 10 w/v % benzalkonium chloride, Nihon Pharmaceutical). Collected water samples were promptly transported to the laboratory and vacuum-filtered using the Millipore Express PLUS disc PES (Polyethersulfone) philic filters (1 L per filter, pore size 0.45 µm, 47 mm; Merck Millipore) and filter holders (PP-47; ADVANTEC) within one hour of collection. To avoid contamination, filter holders for the number of samples were decontaminated by prior soaking in 10% hypochlorite for at least two hours. After filtration of the sunrise samples, 1 L of ultrapure water was filtered in the same way as the collected water samples and used as a filter negative control (Filt-NC). All filter samples were folded in half, wrapped in aluminium foil and immediately frozen at −20°C. DNA was extracted from the filter samples, and the nuclear internal transcribed spacer-1 region (ITS1) of the ribosomal RNA gene of Japanese jack mackerel was quantified using quantitative real-time PCR and species-specific primer-probe sets (detailed below). For July 2021 samples, as the spawning trend was indicated from the increase of ITS1 concentration (see results), the mitochondrial cytochrome b gene (cyt b) region was also quantified using a species-specific primer-probe set to examine the ratio of nuclear DNA (nu-DNA) to mitochondrial DNA (mt-DNA).

Experiment 2: Estimation of the spawning time window

Time-series water sampling was conducted between 29 and 30 June 2022. It was preferred to conduct Exp. 1 on the new moon of July when spawning was shown to be possible (see results), but in 2022 the new moon was on 28-29 July. Thus, Exp. 2 was conducted at the end of June, i.e. at a new moon closer to 10 July, the date of the new moon in July 2021 when Exp. 1 was conducted. At site 2, which was suggested as a possible main spawning location (see results), 1 L of surface water was collected in triplicate at nine-time points: 12:00 noon, 3:00, 6:00,

9:00 PM, 12:00 midnight, 3:00, 6:00, 9:00 AM, and 12:00 noon (Table S2). Each water sample was added with BAC (final concentration, 0.01%) and filtered on-site using the same techniques and equipment as in Experiment 1. After filtration of 12:00 samples on 30 June, 1 L of ultrapure water was filtered as a filter negative control (Filt-NC). All filter samples were folded in half, wrapped in aluminum foil and immediately frozen at −20°C. DNA was extracted from the filter samples, and ITS1 region of Japanese jack mackerel was quantified using the same way as in Experiment 1.

Experiment 3: Field collection of Japanese jack mackerel egg

Egg surveys were conducted on 29 (9:10–10:17 AM) and 30 (1:09–1:58 PM) June 2022 at three sites in Maizuru Bay (dashed lines, L1 to L3, in Fig. 1A). Oblique tawing of a plankton net (diameter 450 mm, mesh 0.3 mm) was performed three or four times for each site (average 3 min 10 sec trawl per trial, wire length 40 m, wire angle 63–78°). Tables S3 and S4 show the towing conditions for each trawling and water quality at each site. The collections were pooled for each site and immediately stored on ice. In the laboratory, approximately 0.6–1.2 mm sized spherical fish eggs were sorted under a stereomicroscope and then fixed with 70% ethanol. For each collected egg, DNA was extracted using the spin column (EconoSpin, EP-31201; GeneDesign Inc.) and the buffer reagents supplied in the DNeasy Blood and Tissue kit (Qiagen). Eggs were soaked in a mixture of 180 µL buffer ATL and 20 µL Proteinase K and incubated at 56°C for 3 hours to dissolve completely. 200 µL of ethanol was added and mixed well, after which the entire elute was transferred to a spin column. After centrifugation at 6,000 g for 1 min, DNA was purified according to the manufacturer's protocol of the DNeasy Blood and Tissue kit. Finally, the DNA was eluted in 50 µL of Buffer AE and stored at −20°C until use in the PCR assay. The species identification was conducted based on the sequencing of the mitochondrial 12S rRNA region with the MiFish fish-universal primer set (detailed below). Based on current laws and guidelines of Japan relating to animal experiments on fish, the collection of fish eggs and the use of DNA samples are allowed without any ethical approvals from any authorities.

DNA extraction from filter samples

A PES filter sample was folded in quarters and placed in the lower part of the spin column (EconoSpin) with the silica gel membrane preliminarily removed. A mixed extraction buffer comprising 200 μL ultrapure water, 200 μL Buffer AL (Qiagen), and 20 μL Proteinase K was added to each filter, and the spin columns were incubated for 30 minutes at 56°C. The filter was moved to the top of the spin column and then centrifuged at 6,000 g for 1 min. 600 μL of ethanol was added to the collected liquid and mixed well by pipetting. The DNA in the mixture was purified using a DNeasy Blood and Tissue kit following the manufacturer's protocol. Finally, the DNA was eluted in 100 μL of Buffer AE and stored at −20°C until use in the quantitative real-time PCR assay.

Quantitative real-time PCR (qPCR) assays

Quantitative real-time PCRs were performed in triplicate using the Light Cycler 96 system (Roche, Basel, Switzerland). The ITS and cyt b regions of Japanese jack mackerel were amplified with species-specific primer-probe sets, which were developed by previous studies (Jo et al., 2019; Yamamoto et al., 2016). The sequences of each primer-probe set were shown in Table 1. Each qPCR was conducted in a total volume of 15 μL, containing 900 nM of forward and reverse primers, 125 nM TaqMan probe, 0.075 μL of AmpErase uracil N-glycosylase (Thermo Fisher Scientific), 7.5 μL of 2 × TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), and 2 μL of DNA template. A four-step dilution series containing 3×10^1 to 3×10^4 copies of each target region was used for each qPCR run as quantification standards. As PCR-negative controls, three reactions were included in all qPCR runs in which 2.0 μL of ultrapure water was added instead of the DNA template. The qPCR thermal conditions for each target region were as follows: ITS1, 2 min at 50°C, 10 min at 95°C, then 55 cycles of 15 s at 95°C, and 75 s at 60°C; cyt b, 2 min at 50°C, 10 min at 95°C, then 55 cycles of 15 s at 95°C, and 60 s at 60°C. As the ITS1 primer-probe set had a lower amplification efficiency than the cyt b primer-probe set, the annealing and extension time was increased by 15 s compared to cyt b. To avoid contamination, reagent preparation and qPCR were carried out in separate rooms. The R^2 values for the standard curve of all qPCR for ITS1 and cyt b were shown in Table S5. No amplification was observed in any of the Filt-NC and PCR-negative controls.

Species identification of eggs

To identify fish species of each egg, approximately 170 base pair of 12S rRNA region was amplified by a two-step tailed PCR approach with two MiFish fish universal primer sets (Table S6, Miya et al., 2015). The first-round PCR (1stPCR) was performed in 12-µL reactions containing 6 µL of 2×KODone PCR Master Mix (TOYOBO), 1.4 µL of each primer mixture (MiFish-U: MiFish-U2 = 10: 3; 5µM) and 2 µl of template DNA. There was no PCR replication. The thermal conditions were as follows: 30 cycles of 10 sec at 98°C, 5 sec at 65°C and 5 sec at 68°C. Amplicons of the 1stPCR were purified using Sera-Mag SpeedBeads Carboxylate-Modified magnetic particles (Hydrophobic) (Cytiva) (PCR product : Sera-Mag beads = 1 : 0.8). The purified amplicons were adjusted to 0.1 ng/µL and used as the template in second-round PCR (2ndPCR). The 2ndPCR was performed in 12-µL reactions containing 6.0 µL of 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 2.0 µL of each primer with index (1.8 µM) and 2.0 µL of the purified 1stPCR amplicon. The sequences of the 2ndPCR primers were shown in Table S6. The thermal conditions were as follows: 3 min at 95°C, 12 cycles of 20 s at 98°C, 15 s at 72°C and 5 min at 72°C. The indexed 2nd PCR products were pooled in equal volumes, and the target band (approx. 370 bp) was excised using a 2% E-Gel SizeSelect Agarose Gels (Thermo Fisher Scientific). The 150 bp paired-end sequencing by NovaSeq was outsourced to Novogene. All raw sequences were deposited in the DDBJ Sequence Read Archive (accession number: DRA016903). The bioinformatics analysis was performed using the PMiFish pipeline (https://github.com/rogotoh/PMiFish). The top BLAST hit with a sequence identity ≥ 98% was identified as the species of the egg.

Statistical analysis

In experiment 1, we performed a Wilcoxon rank sum test to compare the eDNA concentration between sunset and sunrise samples for each DNA region in July 2021 by wilcox.exact function in exactRankTests package ver. 0.8.34. In experiment 2, to examine diurnal changes in eDNA concentration, the ITS1 concentration observed during the daytime (12:00 noon, 3:00 PM, 6:00PM of 29 June 2022, 6:00 AM, 9:00 AM, 12:00 noon of 30 June 2022) was compared with those observed at each time point during the nighttime (9:00 PM, 12:00 midnight of 29 of June 2022, 3:00 AM of 30 June 2022) using the Kruskal–Wallis test followed by the Conover's test with Holm

adjustment by kwAllPairsConoverTest function in PMCMRplus package ver.1.9.3. All statistical analyses and graphic illustrations for this study were conducted using R version 4.2.2 software (R Core Team, 2022). Statistical values were evaluated at a significance level of $\alpha = 0.05$.

Results

Japanese jack mackerel eDNA was detected and successfully quantified at all sites throughout the year. A comparison of eDNA concentrations at two hours before sunset (Hereafter "sunset") and sunrise in each month showed significantly higher ITS1 concentrations at sunrise than at two hours before sunset at all sites in July (Fig. 2 and Fig. 3a). Additionally, in March and October, higher DNA concentrations were found at sunrise than at sunset in 2/3 of the sites (Fig. 2). In July, both ITS1 and cyt b region showed a significant increase from sunset to sunrise ($P < 0.001$ for both region, Wilcoxon rank sum test; Fig. 3a,b). The nu-DNA/mt-DNA ratio, known to increase with the presence of sperm-derived eDNA, , although not significant when all sites were considered, read an increasing trend from sunset to sunrise at site 2 (Fig. 3c).

Diurnal changes in eDNA concentrations were observed in the time-series water sampling at site 2 (Fig. 4). Environmental DNA concentrations were significantly higher at nighttime than during the daytime ($P < 0.01$ for 9:00 PM and 12:00 midnight, $P < 0.05$ for 3:00 AM, Conover's test), peaking between 21:00 and 24:00.

In the egg collection survey, out of a total of 260 fish eggs successfully identified to species (or taxon) by sequencing, two eggs were determined to be Japanese jack mackerel eggs. The two Japanese jack mackerel eggs were recovered from line L3 in Fig. 1a. All remaining eggs were also determined to belong to species inhabiting Maizuru Bay (Fig. S1A). Ultimately, 25 species were identified, which corresponds to approximately 90% of the expected species diversity based on the accumulation curve of the number of analyzed eggs and species (Fig. S1b and c, Table S7). Eggs of species with relatively low occurrence frequencies, including Japanese jack mackerel, were detected from only one of the two days of surveys (30 June 2022).

Discussion

This study successfully identified the spawning season, time window and location of Japanese jack mackerel in Maizuru Bay through eDNA analysis. The eDNA-based findings were supported by the capture of Japanese jack mackerel eggs using trawling with a plankton net. This is the first report to provide evidence of saltwater fish spawning using eDNA analysis, indicating the high potential of this method for spawning detection.

During monthly eDNA monitoring throughout the year, the increase in nighttime eDNA concentrations observed at all three sites in July was considered to reflect the occurrence of Japanese jack mackerel spawning. At site 2, both ITS1 and cyt b exhibited the most significant increases in eDNA concentrations between sunset and sunrise, and the ITS1/cyt b ratio also showed an increasing trend, suggesting the presence of sperm-derived eDNA due to spawning (Bylemans et al., 2017). In Wakasa Bay, the outer bay of Maizuru Bay, two groups of Japanese jack mackerel with distinct morphologies and ecological characteristics coexist: the offshore, seasonally migrating group (black-type) and the coastal, non-migratory group (yellow-type). Observations of body length composition and otoliths of their juveniles caught in Wakasa Bay suggested that black-type and yellow-type are derived from eggs spawned between January and March and between June to July, respectively (Azeta and Ochiai, 1962). The presence of cohorts presumably hatched between mid-June and early August has also been observed in Kunda Bay, located to the west of Maizuru Bay (Kanaji et al., 2009). Taken together, the July spawning estimated by eDNA analysis is likely attributed to the coastal yellow-type. Furthermore, while the water depth range in the main spawning grounds has been estimated between 100 to 200 m (Nishida 2006), Maizuru Bay has a maximum depth of approximately 30 meters near the bay's mouth and north of Toshima Island, and the depth is generally shallower towards the back of the bay (cf. Minami et al., 2018). Thus, the depths of Maizuru Bay are not quite comparable to those of the main spawning grounds, but if Japanese jack mackerel spawn in the bay, they are likely to prefer deeper sites. However, the tidal currents are fast from the mouth of the bay to Toshima. So,

assuming that they spawned around Toshima where the currents begin to slow down, the most noticeable increase in eDNA concentration and ratio observed at site 2 seems reasonable. (Miwa and Ikeno, 2008).

Increases in eDNA concentrations between sunset and sunrise were also observed at 2 out of 3 sites in March and October, but this could be migration-related noise given Japanese jack mackerel ecology. In March, the quantified eDNA concentrations were relatively low throughout the locations, both at sunset and sunrise, making it unlikely that spawning behaviour with the release of large amounts of sperm had occurred. On the other hand, October is the time when juveniles that entered from the outer bay during summer are growing and actively swimming in the bay (Masuda et al., 2008). Therefore, relatively high eDNA concentrations were observed at sunset at all sites. Although there was an increase in concentrations at sites 2 and 3, the increase was only 1.4 to 1.9-fold, which is hardly a remarkable increase. The amount of faeces, one of the sources of eDNA, should increase as fish actively feed in the early morning. Therefore, it is reasonable to assume that the changes in eDNA concentrations observed in October were most likely caused by fish migration and increased activity.

The significant diurnal changes in eDNA concentrations observed in the time-series sampling suggested that Japanese jack mackerel mainly spawn between 9:00 PM and 12:00 midnight. This study is the first to identify their spawning time window in the sea. This result is consistent with the peak spawning window observed in a previous study when Japanese jack mackerel were artificially spawned in tanks (Nyuji et al., 2013). It is also consistent with information on other *Trachurus* species, *T. symmetricus* and *T. trachurus*, which spawn at night (Karlou-Riga and Economidis, 1997; Macewicz and Hunter, 1993). The spawning time window of Japanese jack mackerel under natural conditions has been predicted to be before dawn, but this has not been clearly reported and remained unknown (Nishida, 2006; Yoda et al., 2006). It is hoped that intensive egg recovery surveys during the estimated spawning time window will be carried out in future studies, providing direct observations of eggs immediately after spawning. Quantifying mt-DNA (cyt b) concentrations in the same samples and observing time-series changes in nu-DNA to mt-DNA ratios would provide further insight. However, as the observations in Exp. 1 showed (Fig. 3b), the concentration of cyt b was about 1/10th of ITS1, and daytime samples were

considered difficult to quantify. In addition, due to a change in the author's affiliation (S.T.), it was difficult to perform the analysis in the same experimental environment and the same qPCR machine as in Exp. 3. Therefore, these studies remain as future work.

The occurrence of Japanese jack mackerel spawning in Maizuru Bay during July (end of June), as estimated based on eDNA analysis, was confirmed by the successful direct recovery of their eggs. The fish egg recovery surveys conducted in this study were not of an experimental design that provided quantitative data. However, considering that the Japanese jack mackerel is one of the dominant species in Maizuru Bay, the fact that only two out of the analysed 260 eggs were recovered likely indicates a low spawning quantity. Based on the water temperature during the survey (20–25°C, Table SX), the recovered Japanese jack mackerel eggs may have been floating for a maximum of one day (Ochiai et al., 1982). So, the possibility that the eggs were introduced from outside the bay cannot be completely ruled out, depending on the tidal current conditions. However, as it would have taken more than a day for the eggs to flow from the outer bay to Maizuru Bay, we concluded that the eggs recovered in this study were likely spawned within the bay or at least inshore near the bay mouth.

In conclusion, this study demonstrated the utility of eDNA analysis in saltwater fish spawning surveys through the identification of the spawning of Japanese jack mackerel in Maizuru Bay. While only Japanese jack mackerel was focused on in this study, future research could potentially estimate the spawning ecology of multiple species within the target taxonomic group simultaneously by employing quantitative eDNA metabarcoding methods (Tsuji et al., 2022a; Ushio et al., 2018). Although eDNA analysis currently does not provide some of the information available through conventional methods (e.g., size, age, sex, and hybridization of fish) (Minamoto, 2022), its advantage lies in the ability to efficiently and non-invasively narrow down spawning seasons, time of day, and locations. These characteristics could significantly alleviate the challenges associated with spawning surveys in marine areas and foster advancements in the research field.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Authors' contributions

S.T.: Conceptualization, Funding acquisition, Methodology, Fieldwork (all experiments), Molecular analysis, Visualization, Writing−original draft. H.M.: Methodology, Fieldwork (experiment 1), Writing−review & editing. R.M.: Conceptualization, Funding acquisition, Methodology, Fieldwork (experiment 3), Writing−review & editing, Supervision.

Data availability

Full details of the qPCR results for each experiment of the present study are available in the supporting information (Table S8 and S9). All raw sequences were deposited in the DDBJ Sequence Read Archive (accession number: DRA016903).

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Table

Table 1 Species-specific primer-probe set for each target region of Japanese jack mackerel. Each primer-probe set was developed by Yamamoto et al. (2016) (cyt b) and Jo et al. (2019) (ITS1), respectively.

Primers/probe	Target region	Sequences (5'-> 3')	Tm (°C)	Amplicon length (bp)
Tja_Cyt b_F	cyt b	CAG ATA TCG CAA CCG CCT TT	58.7	127
Tja_Cyt b_R	(mt-DNA)	CCG ATG TGA AGG TAA ATG CAA A	57.6	
Tja_Cyt b_Pr		[FAM]-TAT GCA CGC CAA CGG CGC CT-[BHQ1]	67.9	
Tja_ITS1_F	ITS1	GCG GGT ACC CAA CTC TCT TC	60.1	164
Tja_ITS1_R	(nu-DNA)	CCT GAG CGG CAC ATG AGA G	60.5	
Tja_ITS1_Pr		[FAM]-CTC TCG CTT CTC CGA CCC CGG TCG-[BHQ1]	70.8	

Figure legends

Figure 1. Overview of the sampling designs. (a) Study sites in Maizuru Bay, Japan, (b)-(d) Sampling design for each experiment. The water sampling in Exp.1 and the egg surveys in Exp.3 were performed at st. 1 to 3 and L1 to 3, respectively.

Figure 2. Changes in eDNA concentrations of Japanese jack mackerel two hours before sunset and at sunrise on the following day observed in each month. Points indicate mean values and error bars indicate standard deviations. The grey background colour indicates months when eDNA concentrations were higher at sunrise than at sunset at multiple sites.

Figure 3. Comparisons of Japanese jack mackerel (a) ITS1 concentration, (b) cyt b concentration and (c) nu-DNA/mt-DNA ratio between two hours before sunset and at sunrise on the following day in July 2021. Yellow, pink, and blue plots indicate a different site, respectively. *P* values are indicated by asterisks (Wilcoxon rank sum test; *** $P < 0.001$).

Figure 4. Diurnal changes in eDNA concentration of Japanese jack mackerel ITS1 at site 2 on 29–30 June 2022. Orange and blue box-plot colour indicates the daytime (12:00 noon, 3:00 PM, 6:00 PM, 6:00 AM, 9:00 AM and day2 12:00 noon) and nighttime (9:00 PM, 12:00 midnight and 3:00 AM), respectively. The ITS1 concentration observed during the daytime was compared with those observed at each time point during the nighttime. *P* values are indicated by asterisks (Conover's test; * $P < 0.05$, ** $P < 0.01$). Sunset and sunrise times are as follows: 7:18 PM and 4:46 AM.

Figure S1. (a) species and their frequency of the collected eggs by egg surveys, (b) the number of detected species per survey date; (c) the number of analysed eggs and species diversity.

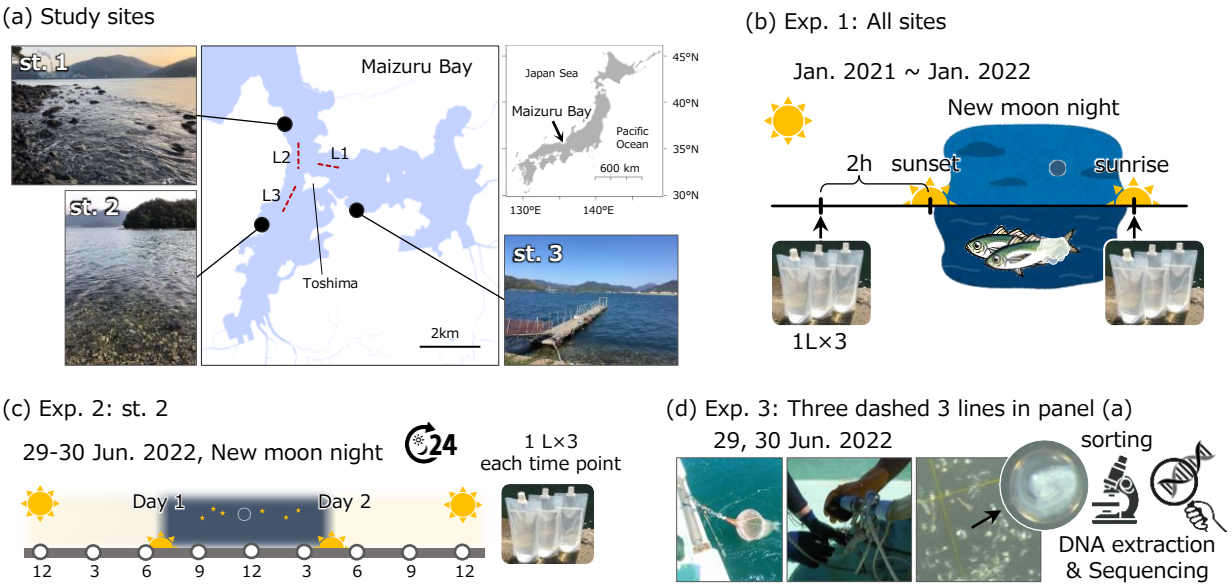
(a) Study sites
st. 1
st. 2
st. 3
Maizuru Bay
L1
L2
L3
Toshima
2km
Japan Sea
Maizuru Bay
Pacific Ocean
600 km
45°N
40°N
35°N
30°N
130°E
140°E
(b) Exp. 1: All sites
Jan. 2021 ~ Jan. 2022
New moon night
2h
sunset
sunrise
1L×3
(c) Exp. 2: st. 2
29-30 Jun. 2022, New moon night
24
1 L×3
each time point
Day 1
Day 2
12
3
6
9
12
3
6
9
12
(d) Exp. 3: Three dashed 3 lines in panel (a)
29, 30 Jun. 2022
sorting
DNA extraction
& Sequencing

Figure 1

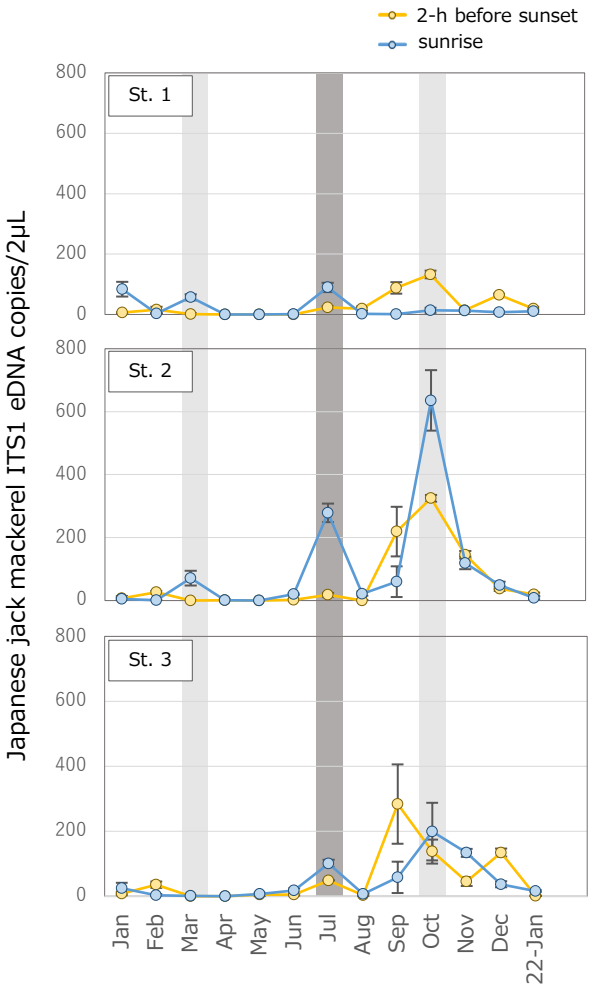
2-h before sunset
sunrise
St. 1
St. 2
St. 3
Japanese jack mackerel ITS1 eDNA copies/2μL
800
600
400
200
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
22-Jan

Figure 2

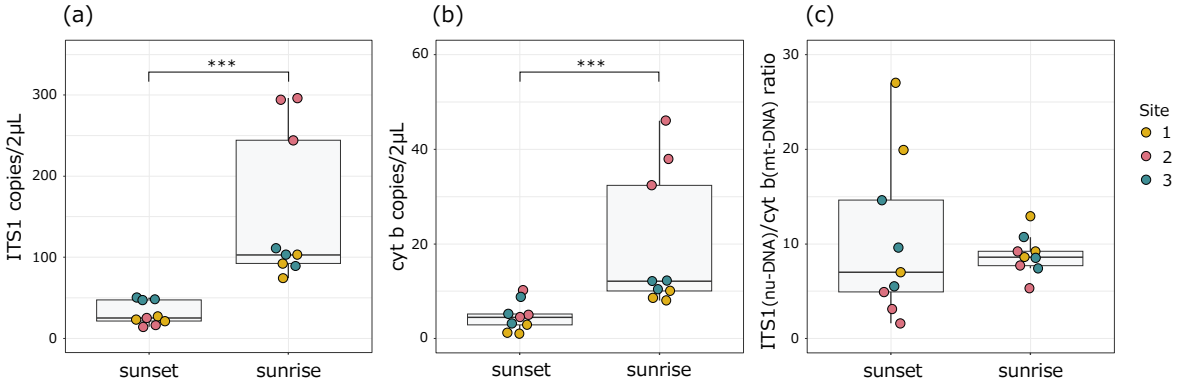
(a)
(b)
(c)
***
***
ITS1 copies/2µL
cyt b copies/2µL
ITS1(nu-DNA)/cyt b(mt-DNA) ratio
0
100
200
300
0
20
40
60
0
10
20
30
sunset
sunset
sunset
sunrise
sunrise
sunrise
Site
1
2
3

Figure 3

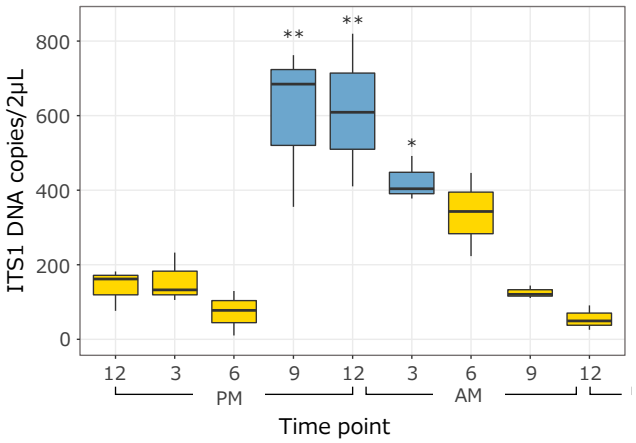
ITS1 DNA copies/2µL
800
600
400
200
0
**
**
*
12
3
6
9
12
3
6
9
12
PM
AM
Time point

Figure 4

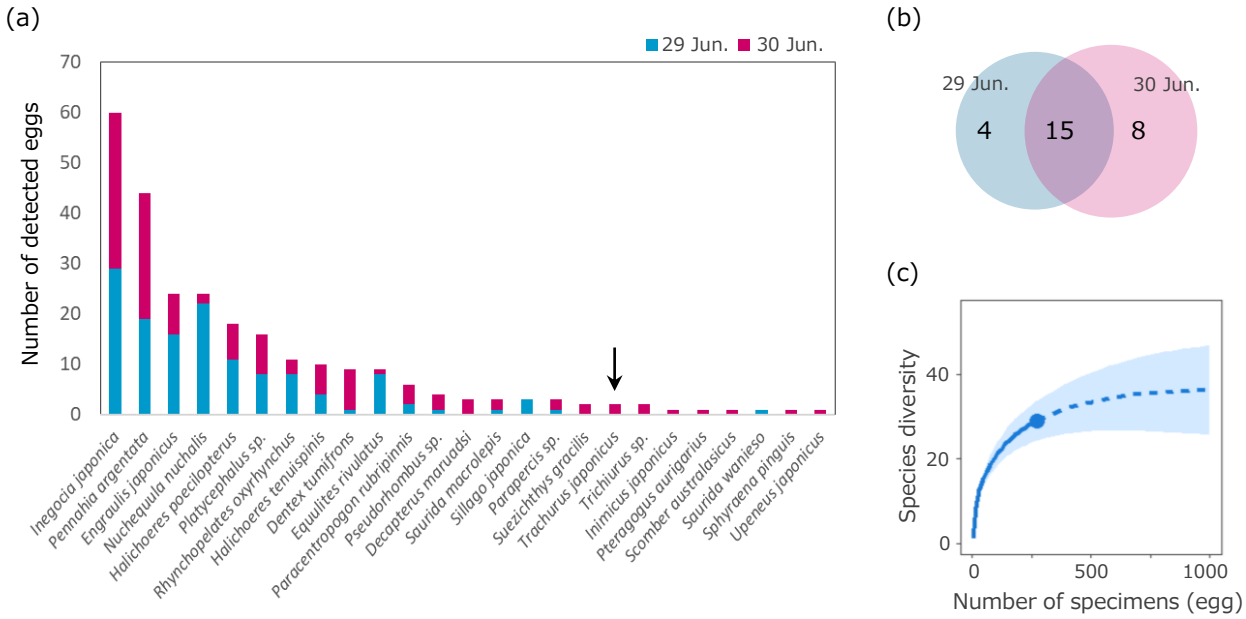
(a)
29 Jun.
30 Jun.
Number of detected eggs
70
60
50
40
30
20
10
0
Inegocia japonica
Pennahia argentata
Engraulis japonicus
Nuchequula nuchalis
Halichoeres poecilopterus
Platycephalus sp.
Rhynchopelates oxyrhynchus
Halichoeres tenuispinis
Dentex tumifrons
Equulites rivulatus
Paracentropogon rubripinnis
Pseudorhombus sp.
Decapterus maruadsi
Saurida macrolepis
Sillago japonica
Parapercis sp.
Suezichthys gracilis
Trachurus japonicus
Trichiurus sp.
Inimicus japonicus
Pteragogus aurigarius
Scomber australasicus
Saurida waniesо
Sphyraena pinguis
Upeneus japonicus
(b)
29 Jun.
30 Jun.
4
15
8
(c)
Species diversity
40
20
0
0
500
1000
Number of specimens (egg)

Figure S1