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Molecular approaches to enhancing fish egg monitoring in the Pacific waters off Japan

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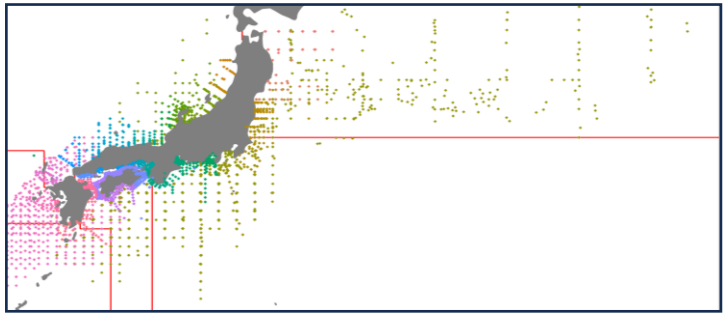
Egg and Larval Surveys

Purpose:

1. Monitoring the distribution of eggs and larvae.
2. Estimating the egg abundance and similar indicator values of egg and larvae distribution for use in resource assessment and basic research.

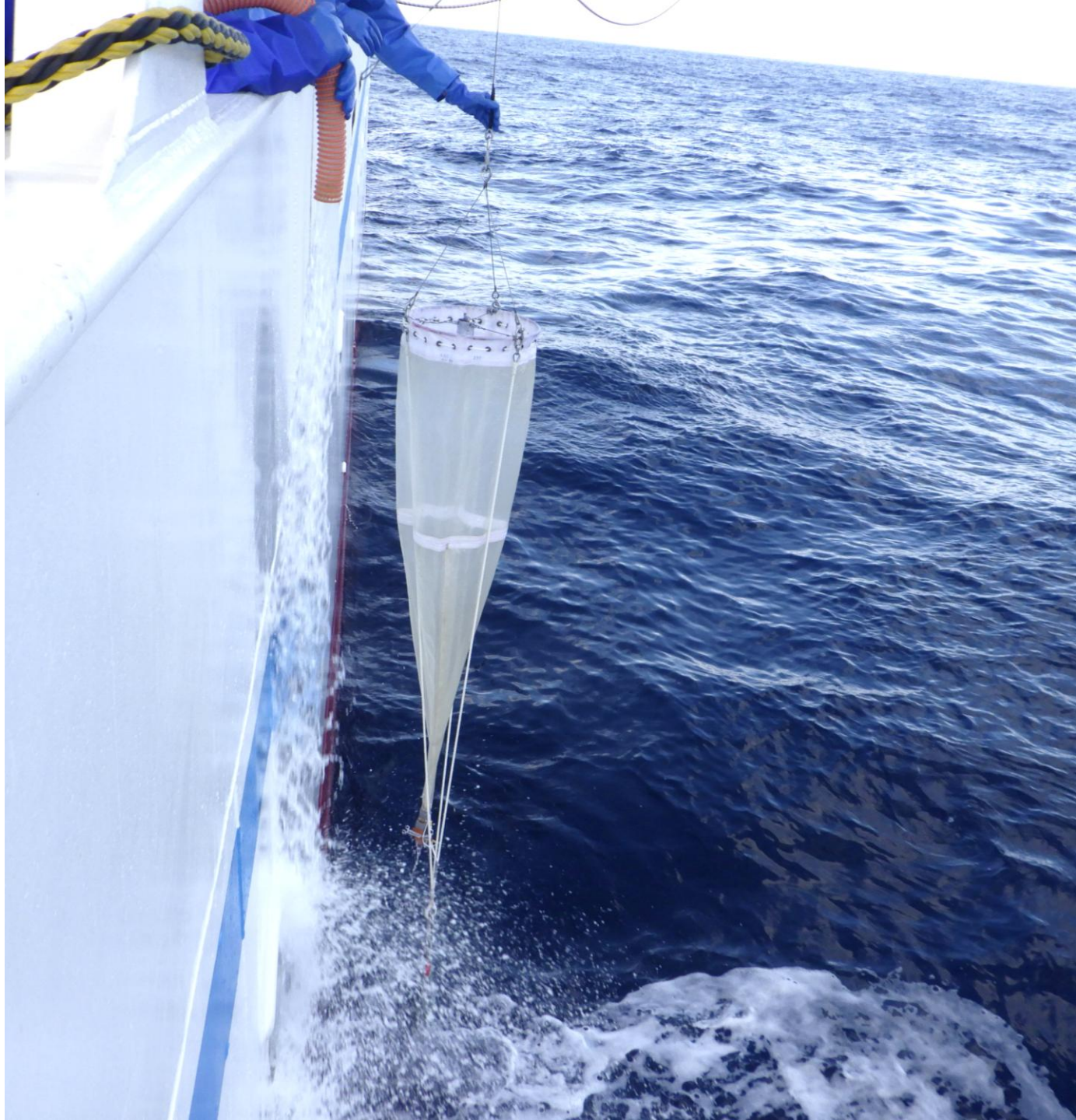
Analysis Targets: Eggs and larvae of multiple fish species, including sardine, anchovy, herring, and mackerel.

Survey Area:
（Example from Japan）



Fixation: 5% formalin

Identification: morphological characteristics



Traditional Identification Methods

- **Accurate identification** is crucial for:
  - Estimating species distribution
  - Understanding spawning patterns
- However, this method has **several limitations**:
  - **Time-consuming**
  - **Heavily dependent on expert knowledge**
  - **Lack of detailed information**

➥ These issues lead to:

- **Difficulty in accurate identification**
- **Limited understanding of marine ecosystems**

Identifiability of species after fixation	number of species	example
Identification is possible	26	***Sardinops melanosticta*** ***Engraulis japonicus*** ***Etrumeus micropus*** ***Konosirus punctatusniphonius*** ***Trachurus japonicus***
Identifiable to a large taxonomic group is possible	46	***Beryx splendens***
Identifiable to species level in the late developmental stage	1	***Lateolabrax japonicus***
Identifiable to a large taxonomic group is possible at a late stage of development	15	***Trichiurus japonicus***
Identification is not possible	100	***Laemonema longipes*** ***Physiculus japonicus*** ***Seriola quinqueradiata***
Information insufficient to make a determination	46	
No information on eggs	other species*	

*Japanese fish species: 4210 species (Nakabou *et al.* 2013)

The types of eggs listed in the Japanese Fish Egg Guide (e.g. Ikeda *et al.* 2014), classified by morphological characteristics.

➥ Only a small number of species can be identified morphologically.

DNA Barcoding Introduction

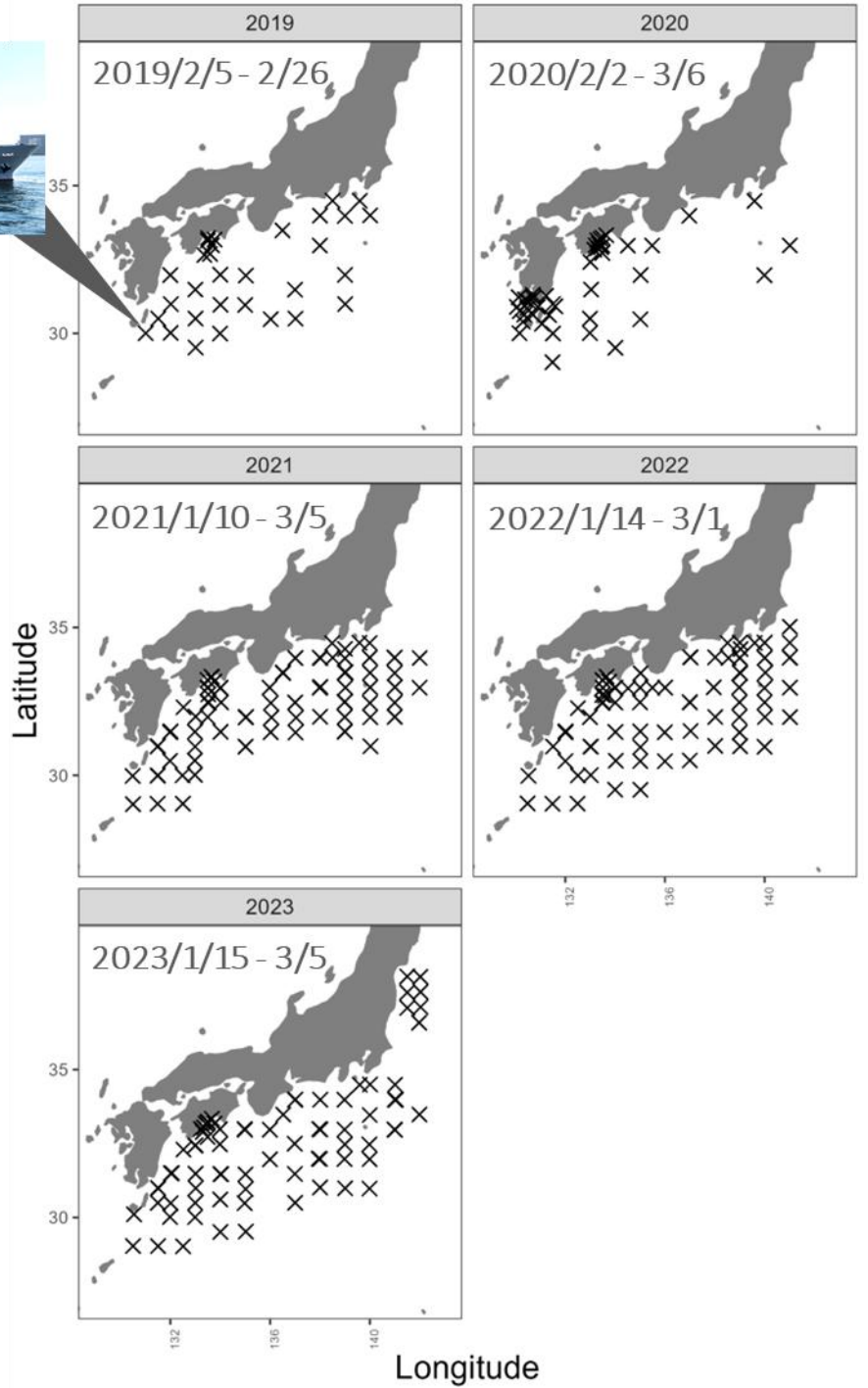
- **DNA barcoding** used to compare sequences with reference databases
  - Helps identify **spawning areas** and estimate **biomass** (Saitoh et al., 2009; Neira et al., 2015).
  - Traditional methods are **slow** and **contamination-prone**
- **Next-generation sequencing (Illumina MiSeq)** for **barcoding**
  - Fast and high-throughput
  - Effective for **mixed samples**
- **Universal primers** used to identify eggs to **genus or species level** (Miya et al., 2015)
  - Enables estimation of **relative abundance**

This method is faster, more reliable, and better suited for assessing fish egg diversity.

Materials and Methods

Egg Sampling and Preservation

- Covered inshore and offshore spawning grounds of small pelagic fish.
- January–March 2019–2023
- Specifications:
  - Diameter: 0.45 m
  - Mesh size: 0. 335 mm
- Vertical towing

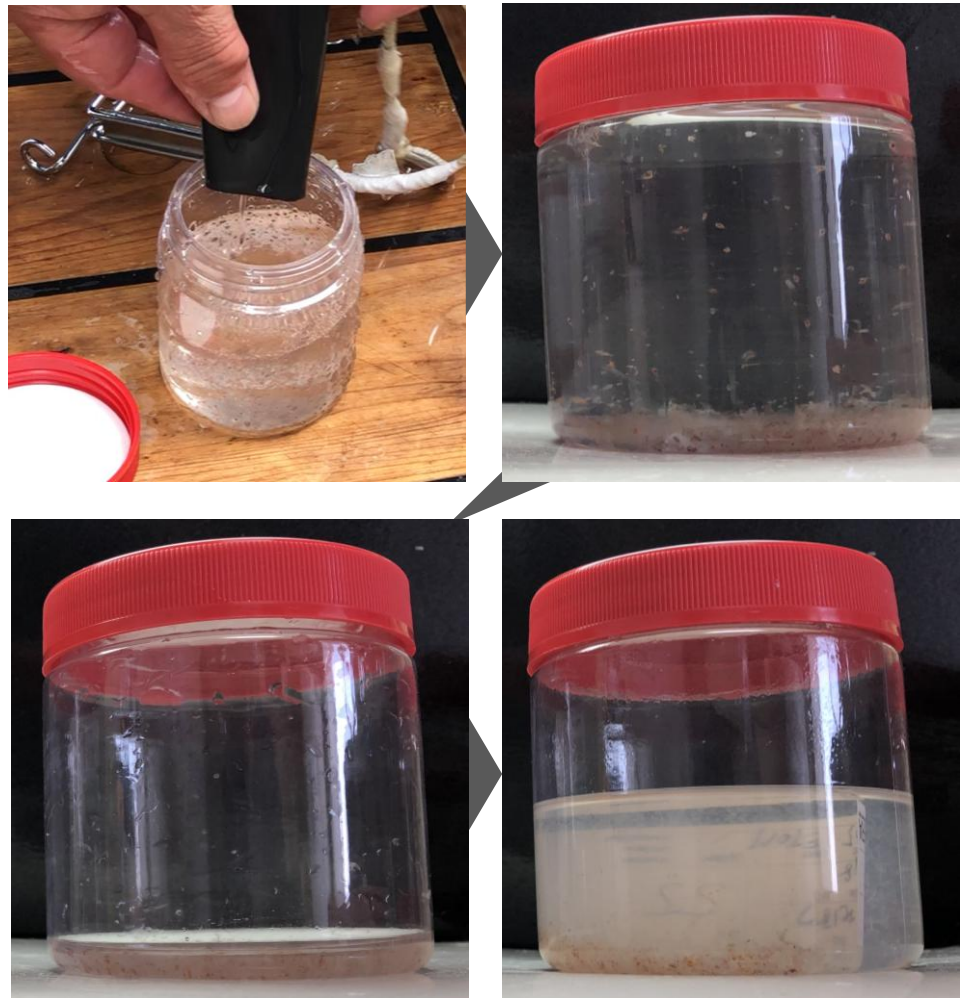


Materials and Methods

Specimen Processing Procedure for Plankton Samples

Method 1 (2019-2021)

1) **Sedimentation**: Allow the sample to settle for approximately 15 minutes.
2) **Removal of Excess Seawater**: Drain as much excess seawater as possible.
3) **Preservation**: Add 99% ethanol and a label to the sample. Store the sample in a freezer.



Specimen Processing Procedure for Plankton Samples

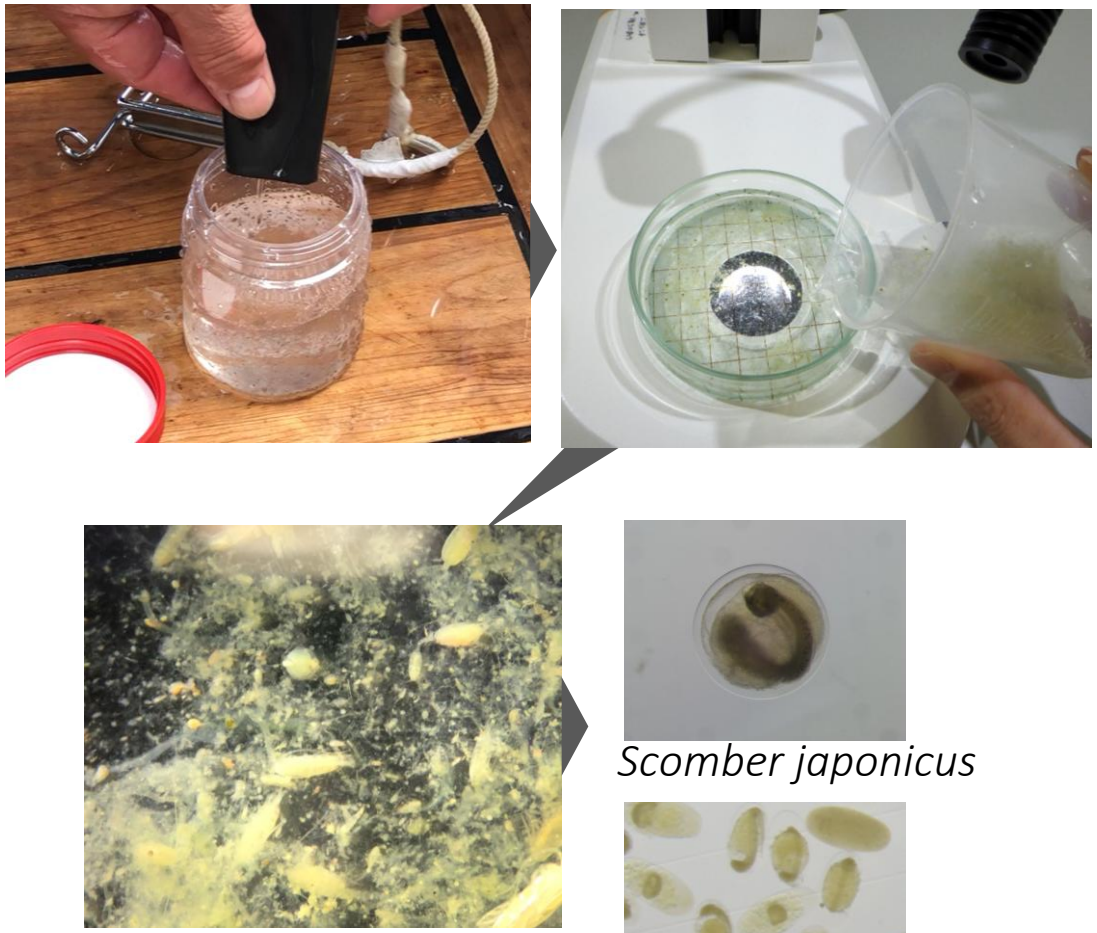
Method 2 (2022-2023)

1) **Filtration**: Pour the plankton net sample into a tea strainer.
2) **Preservation**: Submerge the tea strainer with the sample in 99% ethanol. Store the sample in a freezer.

Morphological Identification of Fish Eggs

- Samples preserved in **5% formaldehyde** for morphological identification
- Eggs examined under **light microscopy** based on key traits:
  - Shape
  - Yolk and oil globules
  - Chorion texture
- Species identified:

  Engraulis japonicus, Etrumeus micropus, Laemonema longipes, Sardinops melanosticta, Scomber japonicus, Scomber australasicus, Trachurus japonicus



Scomber japonicus

Engraulis japonicus

Materials and Methods

DNA Extraction and Analysis

DNA Extraction:

1. NaOH buffer or TE buffer
2. Crush egg, ultrasonicate at 55° C for 2 hours (2019 only).
3. Heat at 97° C for 20 minutes, neutralize with 1 M Tris–HCl (2019 only).

PCR and Sequencing:

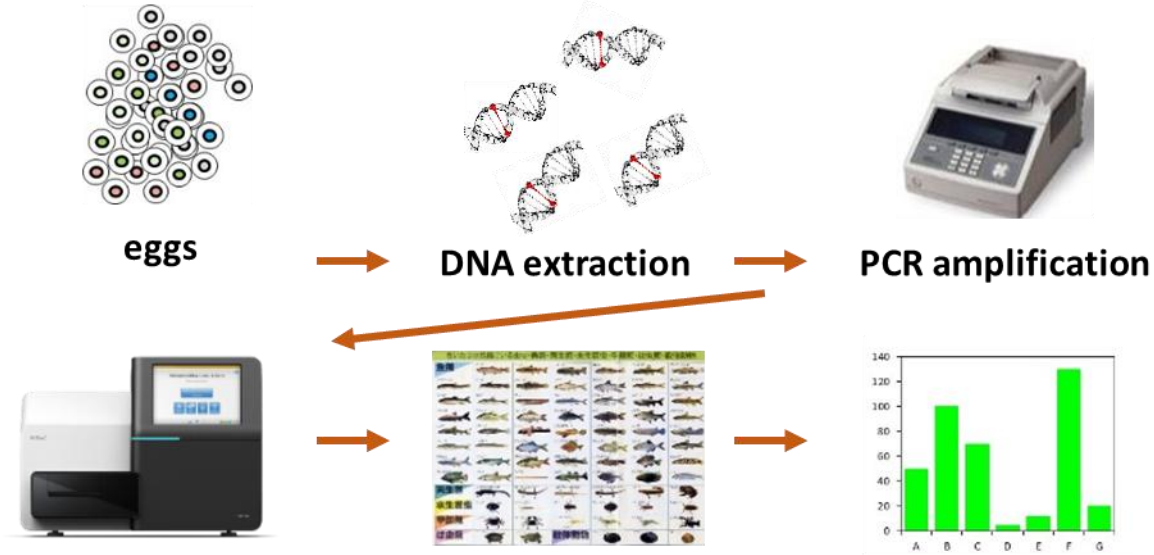
1. Amplify 12S rRNA genes with MiFish primers.
2. Two-step PCR:
   - First PCR: 1.0 µL DNA, 9 µL water, 12.5 µL GoTaq, 1.25 µL primers.
   - Conditions: 95° C for 3 min, 35 cycles of 95° C for 30 s, 60° C for 30 s, 72° C for 30 s.
   - Second PCR: Diluted first PCR products, dual-index primers. Conditions: 94° C for 3 min, 10-12 cycles of 94° C for 15 s, 59° C for 30 s, 68° C for 40 s.
1. Verify, purify, quantify, pool, and store at -30° C.

Bioinformatics:

1. Demultiplex, trim, filter, merge, align, and count operational taxonomic units (OTUs).
2. Deposit data in DDBJ.

Taxonomic Identification:

1. Download mtDNA, BLAST search.
2. Merge OTUs, detect and merge artificial OTUs.
3. Identify dominant OTUs (identity >0.980).



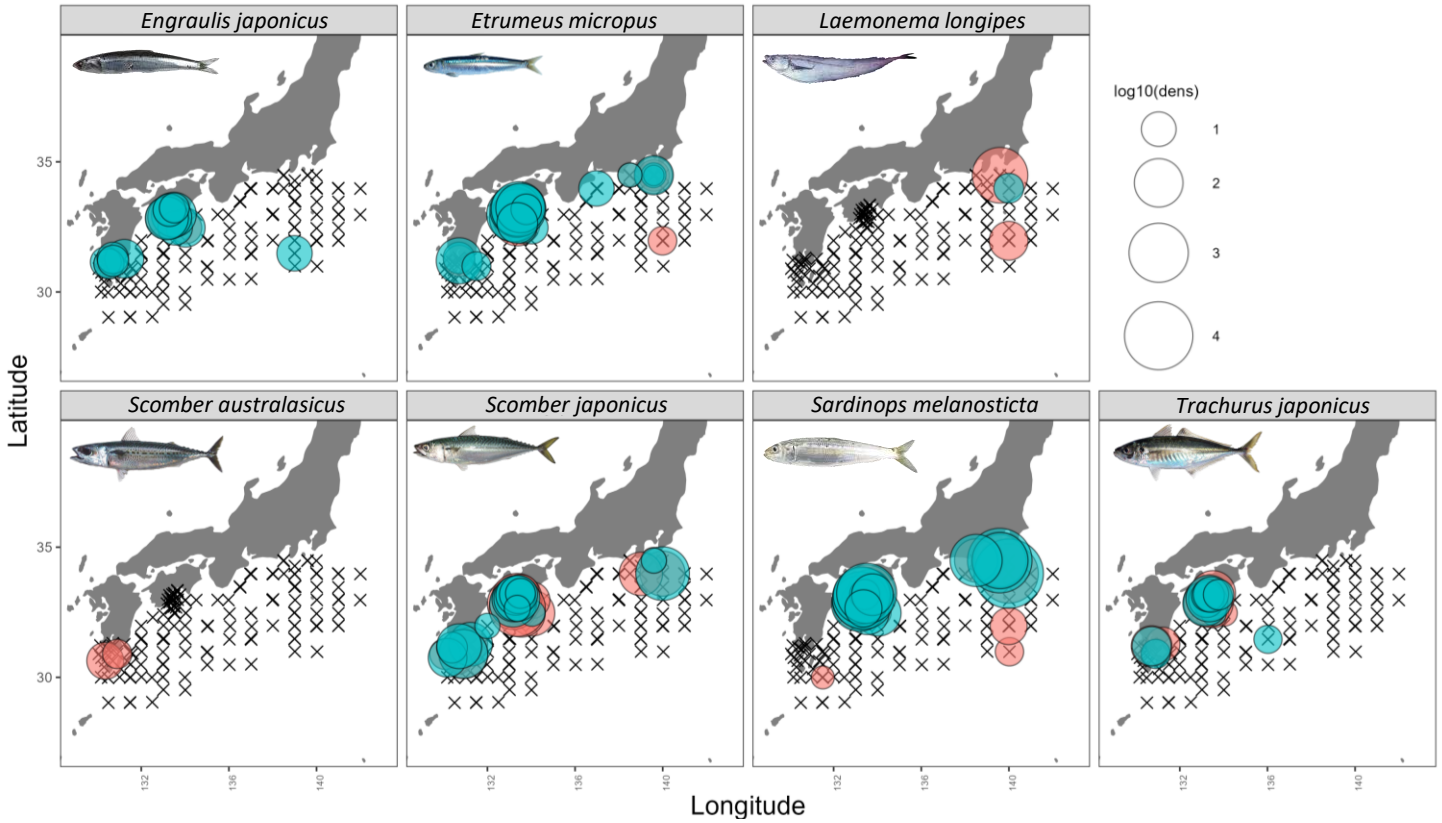
Results

Evaluating Ethanol Fixation Techniques: DNA vs. Morphological ID

Method 1
(2019-2021)

Formalin-fixed
for morphological ID

Supernatant discarded,
ethanol-fixed for DNA ID



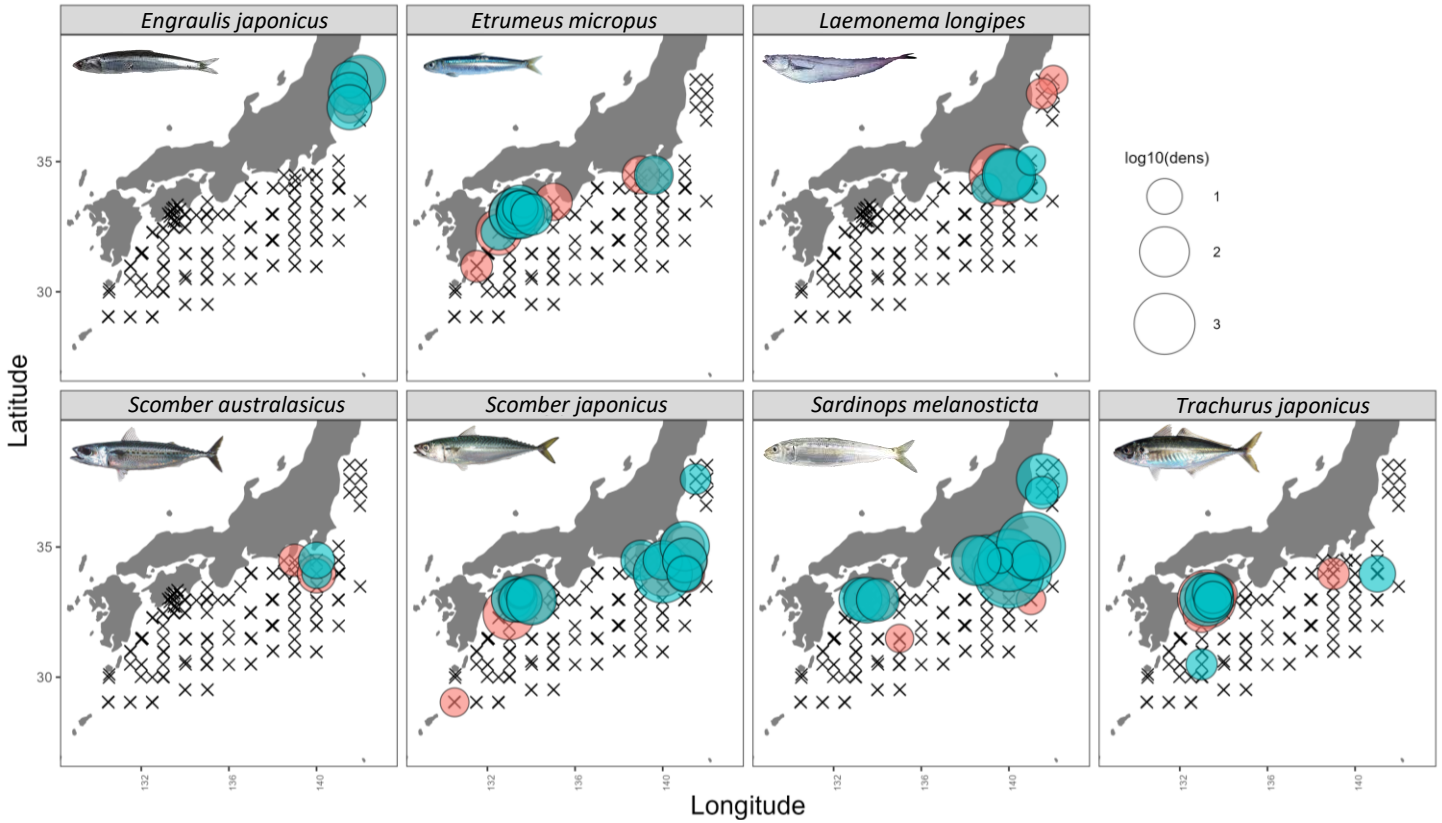
Results

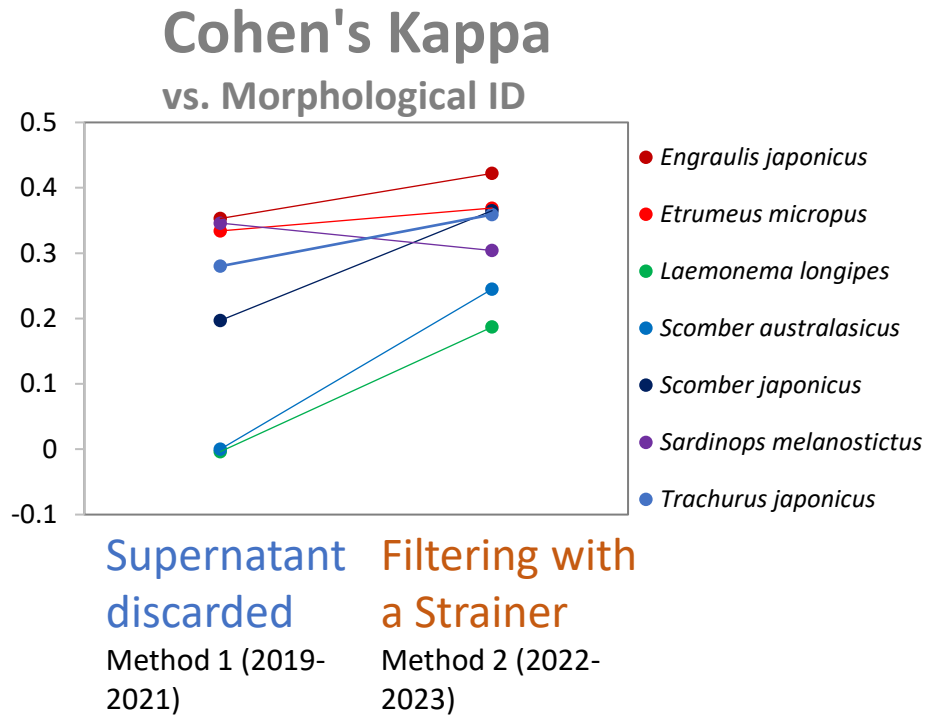
Evaluating Ethanol Fixation Techniques: DNA vs. Morphological ID

Method 2
(2022-2023)

Formalin-fixed for morphological ID

Filtering with a Strainer, ethanol-fixed for DNA ID

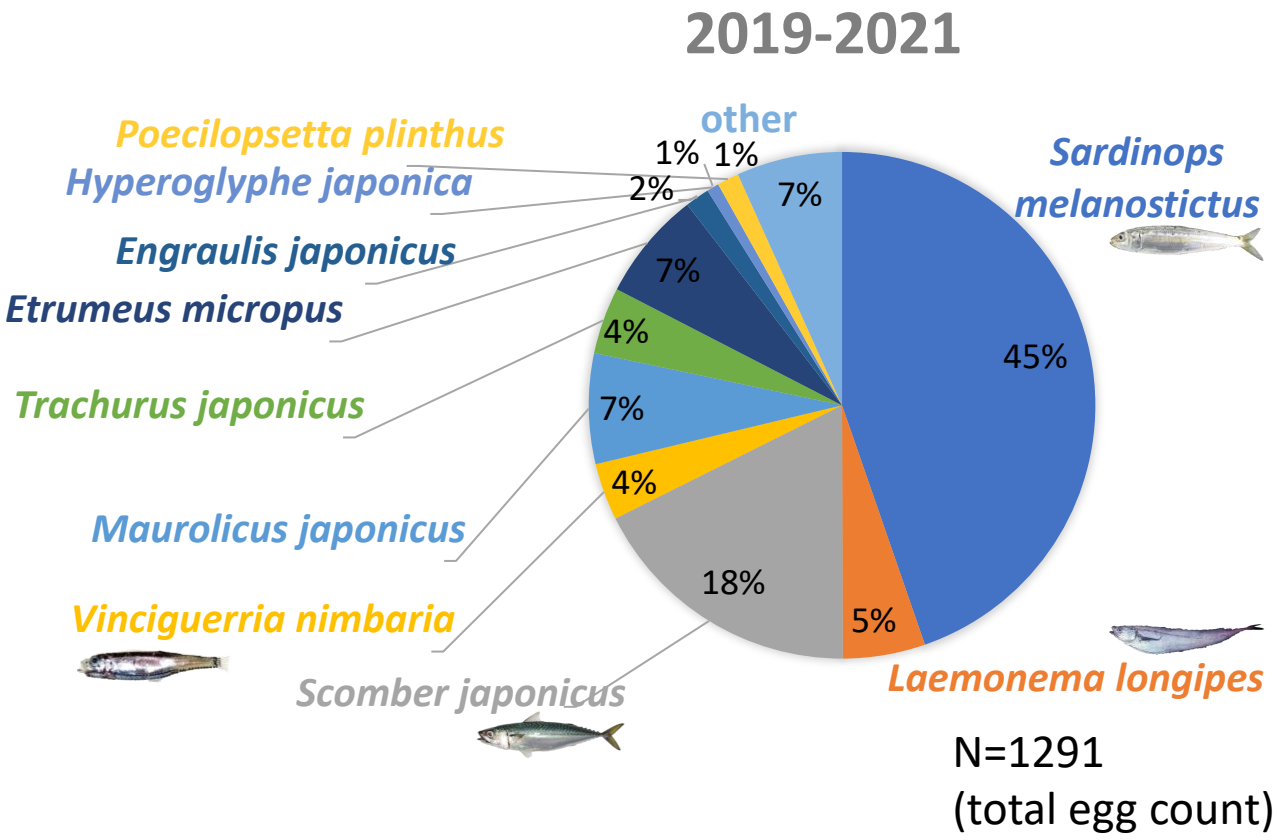


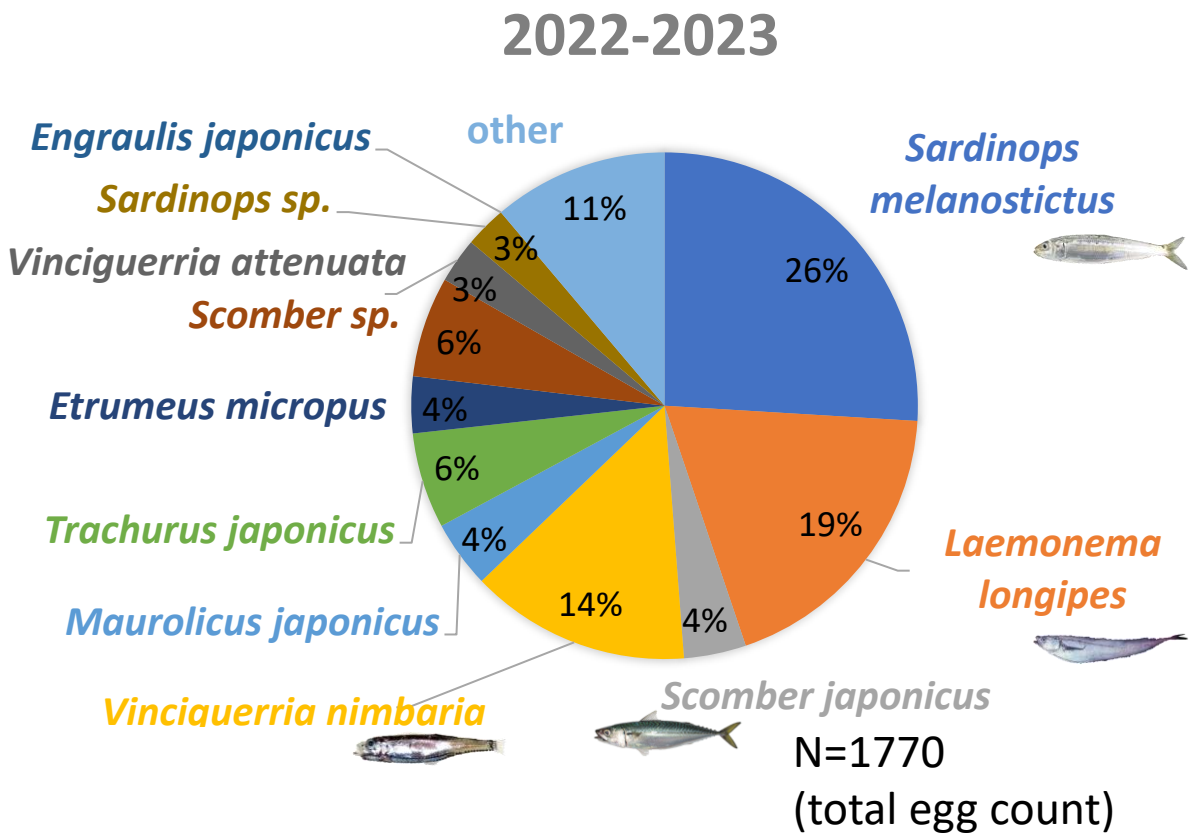


➥ The method using a strainer showed occurrences more closely aligned with morphological identification.

Results

Yearly Comparison (2019–2023)





➥ *Sardinops melanosticta*, *Laemonema longipes*, *Scomber japonicus*, and *Vinciguerria nimbaria* account for 60% of occurrences. The proportion of species varies by year.

Results

Overview of Identified Fish eggs

family	species
Clupeidae	*Sardinops melanosticta*
	Sardinops sp.
Dussumieriidae	*Etrumeus micropus*
Engraulidae	*Engraulis japonicus*
Argentinidae	*Glossanodon semifasciatus*
Microstomatidae	*Lipolagus ochotensis*
Gonostomatidae	*Sigmops gracilis*
Sternoptychidae	*Maurolicus japonicus*
	Polyipnus matsubarai
	Sternoptyx diaphana
Phosichthyidae	*Vinciguerria attenuata*
	Vinciguerria nimbaria
Chauliodontidae	*Chauliodus macouni*
	Chauliodus sloani
	Chauliodus sp.
Melanostomiidae	*Opostomias mitsuii*
Stomiidae	*Stomias atriventer*
	Stomias sp.
Synodontidae	*Synodus cf. oculeus*
	Synodus sp.
Myctophidae	*Benthosema pterotum*
	Dasyscopelus asper
	Nannobrachium sp.
	Notoscopelus japonicus
	Symbolophorus californiensis
Lophotidae	*Lophotus capellei*

family	species
Moridae	*Gadella jordani*
	Laemonema longipes
	Physiculus japonicus
Macrouridae	*Coelorinchus hubbsi*
	Coelorinchus macrochir
	Nezumia convergens
Melamphaidae	*Scopeloberyx sp.*
Berycidae	*Beryx sp.*
Trachichthyidae	*Aulotrachichthys sp.*
Aulostomidae	*Aulostomus chinensis*
Macroramphosidae	*Macroramphosus sp.*
Mugilidae	*Mugil cephalus*
Atherinidae	*Atherion elymus*
Triglidae	*Chelidonichthys sp.*
	Lepidotrigla guentheri
	Lepidotrigla sp.
Hoplichthyidae	*Hoplichthys fasciatus*
	Hoplichthys sp.
Howellidae	*Howella sp.*
Serranidae	*Caprodon schlegelii*
	Selenanthias analis
Callanthiidae	*Callanthias japonicus*
Branchiostegidae	*Branchiostegus sp.*
Scombropidae	*Scombrops boops*
Carangidae	*Kaiwarinus equula*
	Trachurus japonicus

family	species
Bramidae	*Brama japonica*
	Brama sp.
Caristiidae	*Caristius macropus*
Sparidae	*Rhabdosargus sarba*
S	
M	
G	
C	
P	
N	
L	
P	
C	
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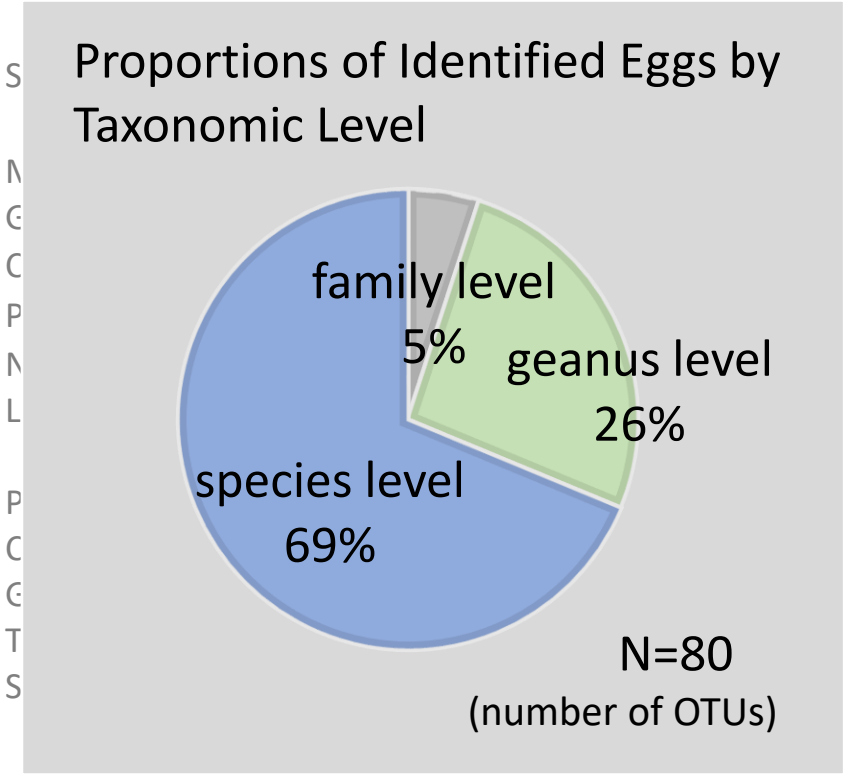
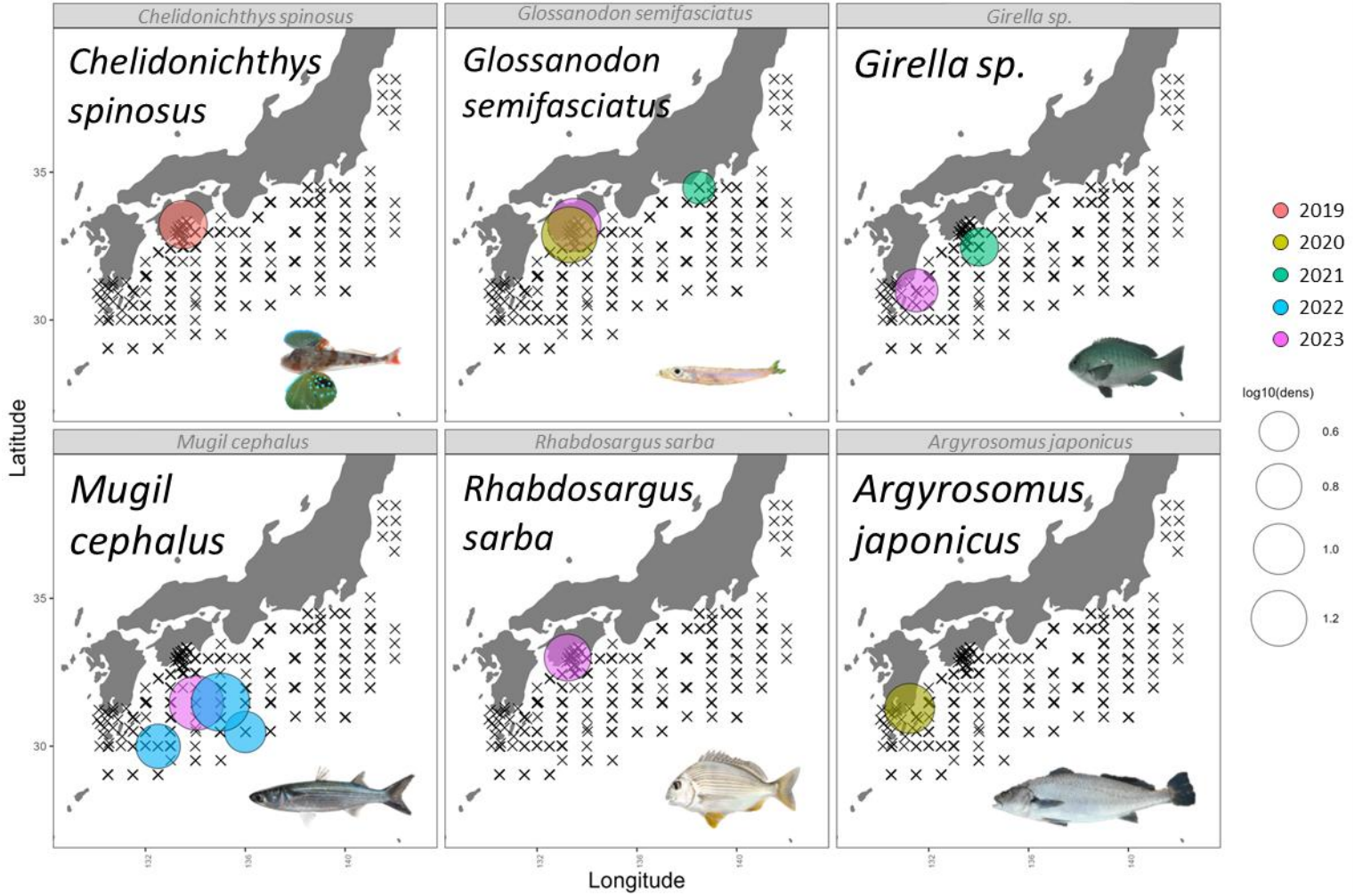
S	
	Scomber japonicus
	Scomber sp.
Pleuronectidae	*Glyptocephalus zachirus*
Poecilopsettidae	*Poecilopsetta plinthus*
Cynoglossidae	Cynoglossidae sp.



Results

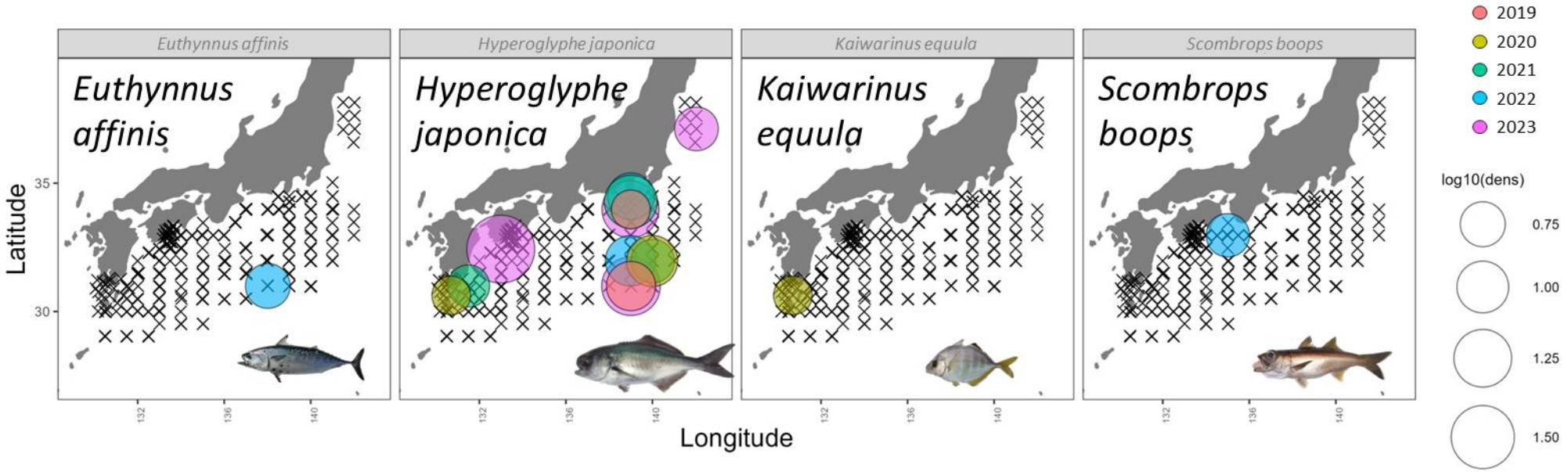
Newly Recorded Key Fish eggs



➥ They are an important commercial fish, but their eggs had never been recorded in the region before.

写真：WEB魚図鑑　（https://zukan.com/fish/）

Species with No Prior Morphological Descriptions



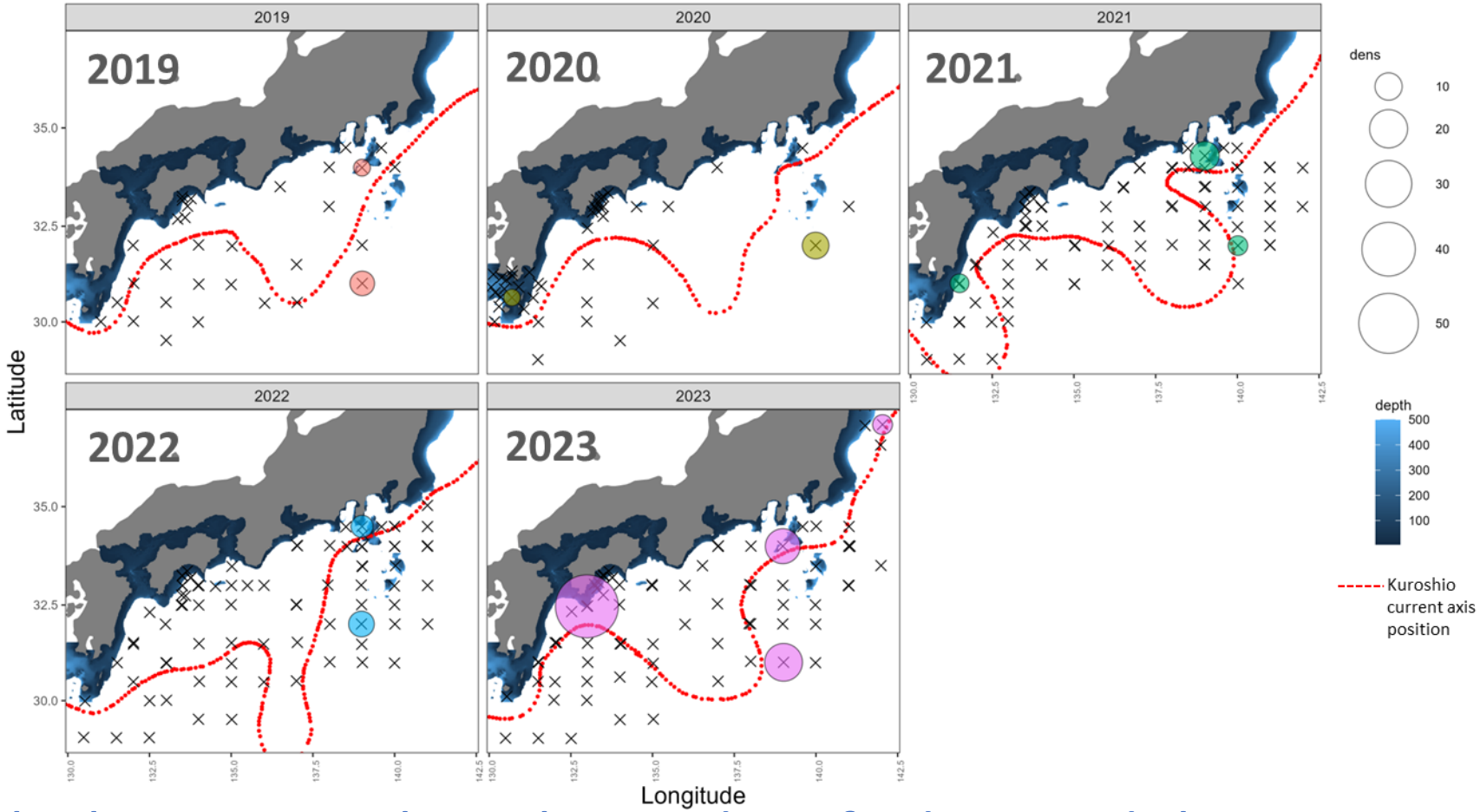
➥ These species are also important commercial fish, but there is no information on the morphology of their eggs. This is the first insight into their distribution.

写真：WEB魚図鑑　（https://zukan.com/fish/）

Species with No Prior Morphological Descriptions

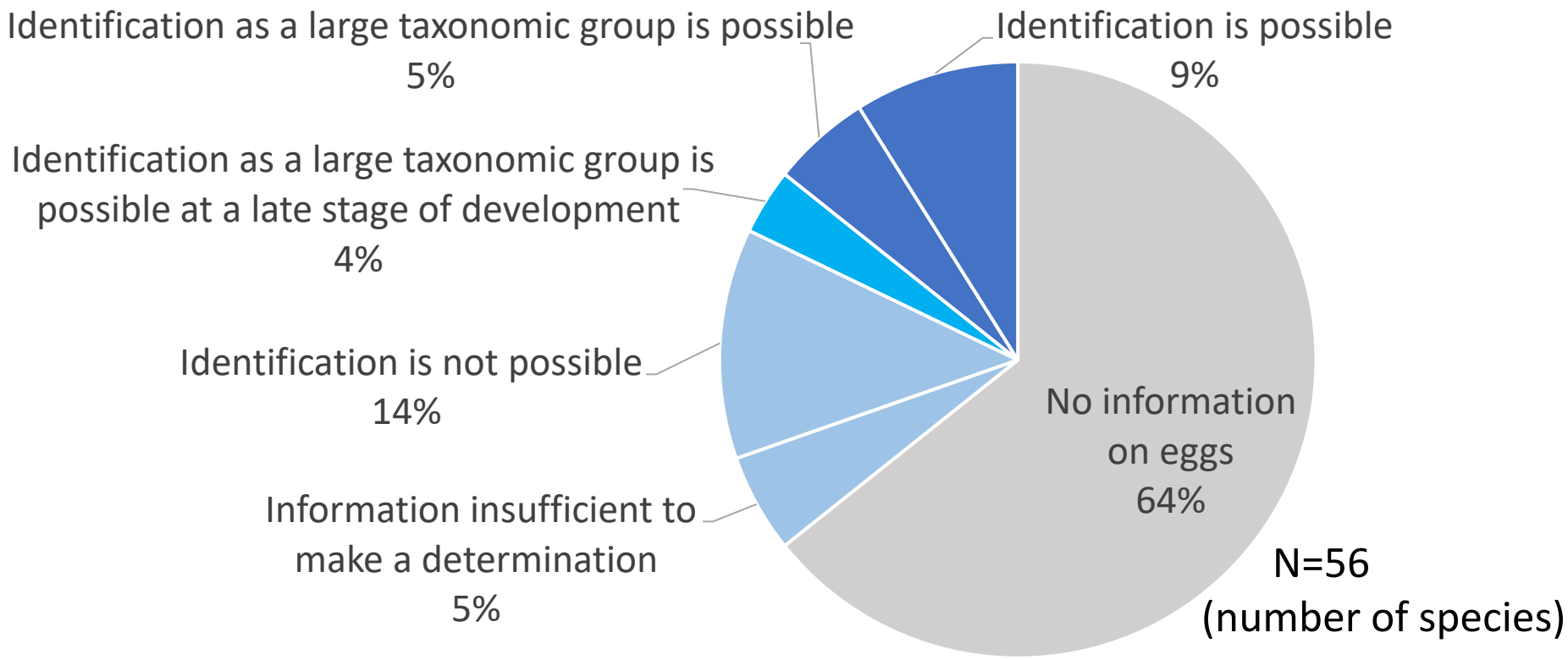
Hyperoglyphe japonica

- Mid-water zones near Japan
- Deep-sea handline fishing
  — around the Izu Islands and off the Kii Peninsula



➥ The eggs of *Hyperoglyphe japonica* have been identified around the Satsunan and Izu islands, providing valuable information for estimating spawning grounds.

Morphological Identification Possibilities

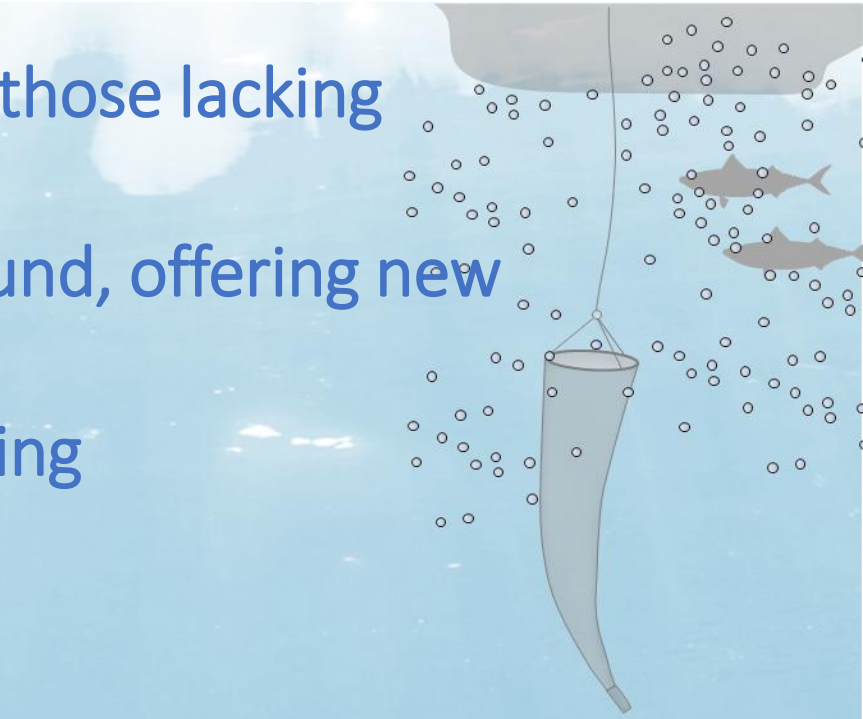


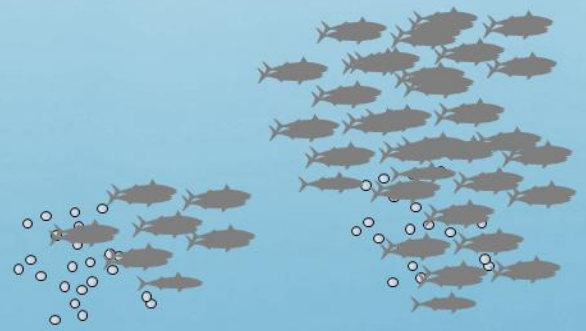
The types of eggs listed in the Japanese Fish Egg Guide (e.g. Ikeda *et al.* 2014), classified by morphological characteristics.

➥ 64% of which lack morphological trait information. Only 9% of the remaining species can be identified at the species level.

Significance of the Study

- A wide range of species was detected, including those lacking prior morphological descriptions
- Eggs of commercially important species were found, offering new occurrence records
- The method is applicable to many species, allowing comprehensive assessment of egg diversity





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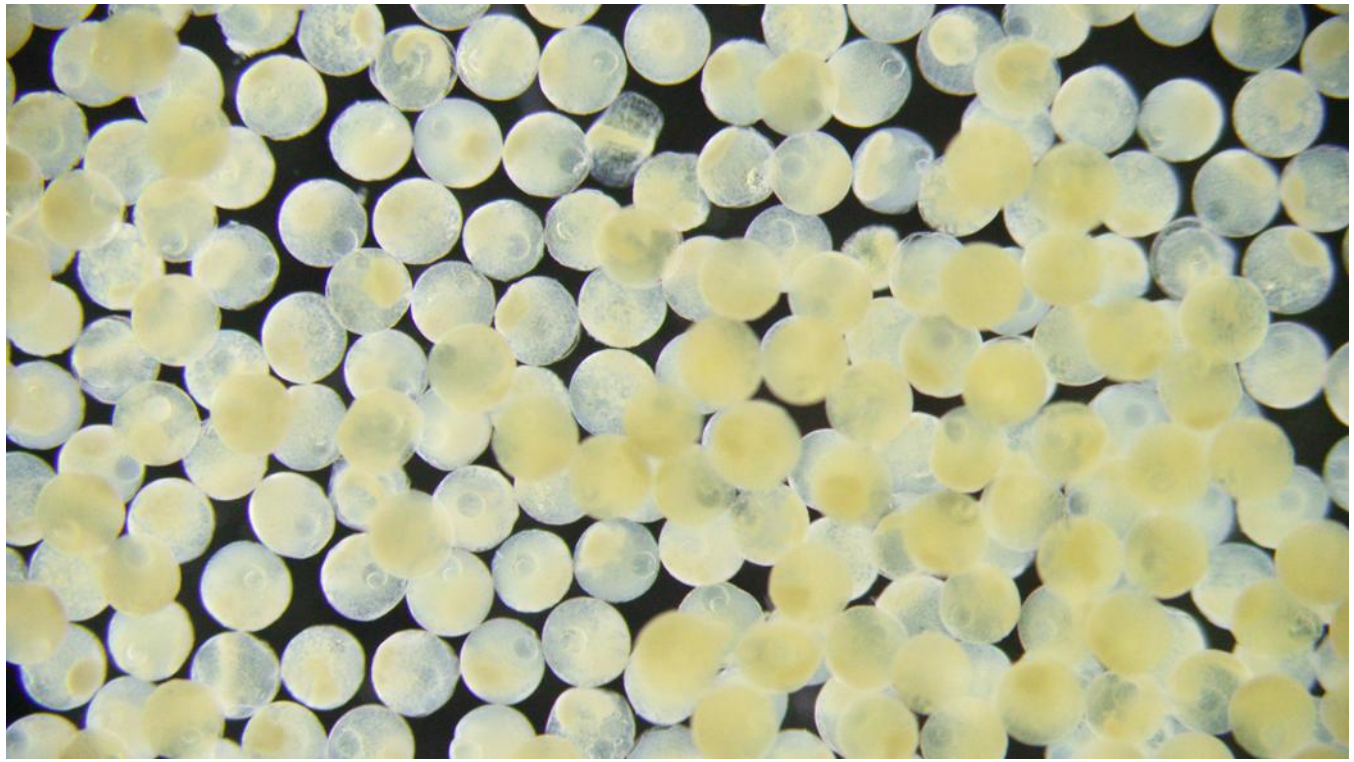

Diaphus theta