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DNA Barcodes for Identifying Fish Egg Species Diversity in Summer and Autumn in the Southwest Daya Bay, China

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Abstract: Identifying fish eggs and understanding fish reproductive periods are necessary for informed fishery management. However, accurate the identification of fish eggs is difficult because eggs have few distinct characters, and their morphology varies ontogenetically. Using cytochrome *c* oxidase subunit I, we identified fish eggs from ichthyoplankton samples collected in the summer and autumn of 2021 from southwestern Daya Bay, China. Of 567 fish eggs, 498 high-quality cytochrome *c* oxidase subunit I sequences were obtained, of which 116 eggs (23.3%) could be identified to species; 364 (73.1%) to genus, family and/or order; and 18 (3.6%) could not be assigned. Of 51 apparent taxa, 46 were identified to 6 orders, 19 families, and 30 genera; 20 to the species and 25 to the genus and/or family, and 1 to the order. Among these 51 taxa, 35 occurred in summer, 29 occurred in autumn, and 13 occurred in both seasons; 22 occurred only in summer and 16 only in autumn, indicating species-specific spawning periods. High-resolution photographs of eggs are provided to facilitate subsequent identification based on morphology. These results will facilitate the identification of spawning grounds and their protection, to more holistically manage fishery resources in Daya Bay, China.

Keywords: Southwest Daya Bay; fish egg diversity; molecular species identification; spawning time; spawning ground conservation

Key Contribution: In the following study, planktonic fish eggs collected in monthly surveys over the summer (June–August) and autumn (September–November) of 2021 in Southwestern Daya Bay (SDB) were identified using DNA barcoding. Key findings include the successful identification of 51 taxa of fish eggs through COI sequences and the confirmation of the reproductive cycle of these taxa in the summer and autumn in SDB. Of them, 35 taxa occurred in summer and 29 in autumn, while 13 occurred in both seasons. The study results indicated monthly variation in fish reproductive activities in the summer and autumn in Daya Bay, which had evident subtropical and tropical characteristics. Moreover, more than 60.8% of fish egg taxa could not be identified at the species level, indicating that there is an urgent need to develop a more reliable DNA library of adult fish in Daya Bay, which may aid in research work on the monthly changes in the reproductive cycle of fish in Daya Bay.

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1. Introduction

Understanding the early life histories of fishes improves the understanding of their recruitment and fluctuations in populations. Eggs provide valuable insights into fish reproductive biology, spawning times and locations, and recruitment success rates [1–4]. An understanding of the composition and spatiotemporal variation of fish eggs in plankton provides important information for fishery management that involves the selection of

areas for fishery closure, fishing moratoriums, fishery resource management strategies, identification of marine protection areas, and environmental evaluation of the effects of aquatic-related construction and engineering [2,5–7]. However, despite the importance of the accurate identification of fish taxa from their eggs, our understanding of the occurrence and distribution of fish eggs is limited, mainly because dynamic and complex ontogenetic changes in egg and larval morphology render accurate species identification difficult [1,3,8,9].

The use of molecular tools to identify fish eggs is now commonplace, and the cytochrome *c* oxidase subunit (COI) gene has proven to be particularly useful to achieve this. For example, using DNA barcodes, Leyva-Cruz et al. [10] identified 42 fish taxa from near Banco Chinchorro (Mexican Caribbean), Burrows et al. [9] identified 62 species from the Gulf of Mexico, Kerr et al. [11] identified 89 taxa from northwestern Cuba and across the Florida Straits, Hou et al. [12] identified 80 independent fish lineages in the eastern Beibu Gulf (China), and Breitbart et al. [13] identified 37 taxa from West Florida Shelf. DNA barcoding represents a valuable tool to accurately identify fish eggs and an aid in locating their spawning sites.

Daya Bay, in the north South China Sea (SCS), has a total sea area of 650 km^2. The environment throughout this bay is diverse and includes habitats such as coral reefs, mangroves, rocky reefs, beaches, and mudflats [14]. These habitats support a rich marine biodiversity, provide important ecosystem services for the region, and, in addition to supporting many commercially valuable fish and marine species, provide favorable conditions for fish spawning, growth, and nutrition [15]. Daya Bay is also considered one of China's aquatic resource breeding reserves, and the Daya Bay Aquatic Resources Provincial Nature Reserve is located here. However, this bay has been damaged by the combined effects of climate change, socio-economic development, and natural disturbances, which have worked in concert to decrease fishery resources. There is a clear trend toward less valuable and smaller fish being exploited [16,17], and a significant decline in biodiversity [18].

Daya Bay has the highest marine biodiversity in China, and represents an important spawning and nursery ground for many fish species. Research on fish eggs and larvae in this region extends back to 1984–1985, when 64 taxa were identified [19]. Wang [20] identified 36 taxa from fish eggs in two surveys from 1986 to 1987 and 1988 to 1989, wherein 23 taxa were identified to species. Lin et al. [21] analyzed seasonal variation in the numbers of eggs and species composition based on seasonal surveys from 2003 to 2005. Using morphology, Wang et al. [18] identified 19 taxa to the genus or family from fish eggs, and 18 taxa from larvae from Daya Bay. However, because of difficulties identifying fish eggs to species, information on fish spawning sites within Daya Bay remains limited.

It is increasingly important to accurately identify fish species during early-life-history stages, and to obtain information on their spawning activities for improved fishery management. We performed ichthyoplankton surveys in southwestern Daya Bay during the summer and autumn of 2021. Our objectives were to (a) identify fish species from their eggs, (b) evaluate the effectiveness of DNA barcoding to identify fish eggs in the study area, and (c) based on egg identifications, identify spawning periods of fish species during the sampling months for this region.

2. Materials and Methods

2.1. Sampling and Photography

In the summer (June–August) and autumn (September–November) of 2021, monthly surveys were performed in southwestern Daya Bay (22.53–22.66° N, 114.53–114.63° E) at 12 survey stations (Figure 1). Nets (80 cm diameter, 270 cm long, 505 μm mesh) with a cod-end container mesh of 400 μm were used to collect ichthyoplankton samples. At each survey station, four net deployments were performed: two horizontal tows at a fixed depth (10 min at 1.0–2.0 knots) and two vertical hauls (from the seabed to the surface at 1.5 m s^{-1}). Specimens from one horizontal and one vertical tow at each station were preserved in 75% ethanol–sea water solution and stored in cold storage (<0 °C). Other

samples were preserved in a 5% formalin solution. General oceanic flowmeters were attached to horizontal and vertical net hauls to estimate filtered water volumes.

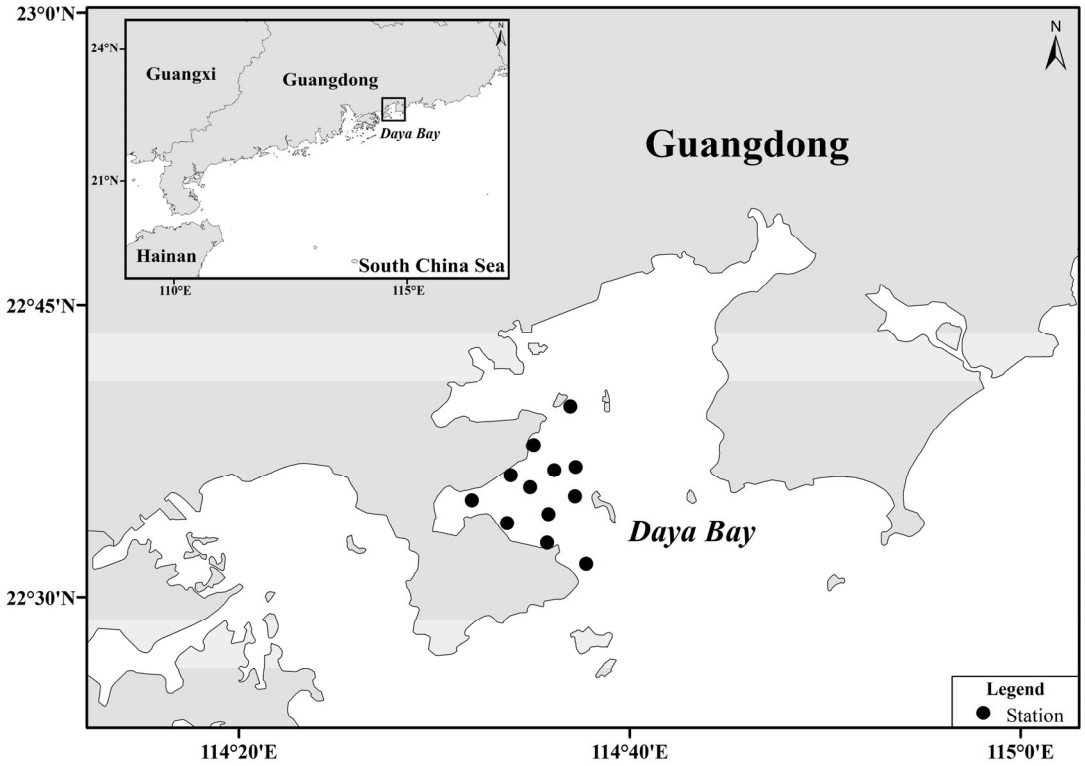

Figure 1. Survey stations of fish eggs in southwest Daya Bay.

All fish eggs collected from each station were examined by a stereomicroscope. A subset of eggs was randomly selected for molecular identification because it was impractical to amplify and sequence all the eggs. At each station, when there were >100 eggs, ≤100 eggs were randomly selected and photographed; when there were fewer than 100 eggs, all eggs were selected. Among them, photographed eggs with different morphological features were first examined and selected for molecular identification. We also selected eggs with differing morphologies if they were not represented in the random egg sample (to identify more fish species or taxa). Finally, the minimum number of eggs from one station in October (n = 4) and the maximum number from one station in September (n = 14) were selected. In total, 7792 eggs were examined; among them, 567 eggs were selected and photographed, and then prepared for the following molecular analysis.

2.2. Molecular and Data Analyses

In the present study, primer treatment processes and DNA extraction methods followed Hou et al. [22]. Each egg was numbered, cleaned in hydrogen peroxide for 5–8 min, and photographed beneath a Zeiss microscope (Axioplan 2 imaging E; ZEISS, Göttingen, Germany) at magnifications of 7.5–150×. The egg was then put in a cleaned centrifuge tube, dried in the air, and quickly punctured by a sterilized needle. Using an Axygen DNA Extraction Kit (Axygen, Shanghai, China), total genomic DNA was extracted. Universal primers FishF1 and FishR1 were used to amplify a partial fragment of the 5′-end of COI sequences (~648 bp) [23]. The polymerase chain reaction (PCR) conditions for COI sequences involved the following: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min [22]. The amplified DNA was fractionated by electrophoresis using 1% low-melting agarose gels. Successful amplification bands were isolated, then purified with DNA Gel Extraction Kit, and sequenced bidirectionally on an

ABI 3730 XL DNA system, following the manufacturer's protocols (PerkinElmer Applied Biosystems, Foster, CA, USA).

The sequences obtained from tracer files were first checked and assembled using SEQMAN (DNASTAR Inc., Madison, WI, USA), following Ahern et al. [7] and Hou et al. [3,22]. High-quality sequences were then aligned and manually edited with MEGA v7.0 [24]. Fish egg identification was performed using the Barcode of Life Data System (BOLD v4). The COI sequence of each egg was compared with sequences in the BOLD database to determine similarities and species identity [25]. Reference sequences of the best and second-best interspecific matches from BOLD were retained, and percentages of sequence similarity were recorded. Following Hubert et al. [26] and Hou et al. [12], we applied three similar criteria: (i) if the similarity between the COI sequence and the best sequence match was >98%, the similarity with the nearest neighbor species was <98%, and there was a genetic divergence >2% between the sequence and nearest neighbor species, then it was considered to belong to the matched species. The sequence was delimited to the matched species if all of the top 99 matches belonged to a single species within the specified threshold (Case I). (ii) Sequences were delimited to the genus if the genetic divergence was >98% similarity or the threshold <2% to the best-matched species and nearest neighbor species, and the two matched species were congeneric. Otherwise, sequences were identified to the family or order (Case II). (iii) Sequence matching <98% was considered unidentifiable (Case III). Genetic distance was calculated based on the Kimura 2-parameter model (K2P, [27]) using MEGA v7.0 [24]. To illustrate lineage diversity via phylogenetic topology, a neighbor-joining (NJ) tree was constructed based on the K2P model with 1000 bootstrap replicates using MEGA v7.0.

To describe egg morphology, egg and oil globule diameters were measured to the nearest 0.001 mm, and the number of oil globules were counted. To evaluate sampling effort adequacy to describe species richness, species accumulation curves were created for each survey using the "random" method as an accumulator function in the "vegan" package implemented in R version 4.2.3 [28]. The means and standard deviations of species accumulation curves were determined from random data subsampling without replacement [29].

3. Results

3.1. Sequence Analysis

In total, 52,735 alcohol-preserved fish eggs were examined in summer (June–August) and autumn (September–November). Among them, in summer, 32,479 eggs were obtained from horizontal trawls and 633 from vertical trawls; in autumn, 18,924 eggs were obtained from horizontal trawls and 699 from vertical trawls. The highest mean total egg abundance in horizontal tows occurred in June (4113.46 ind. 100 m^{-3}) and the lowest in November (114.72 ind. 100 m^{-3}). For vertical hauls, the highest mean total abundance occurred in September (1562.59 ind. 100 m^{-3}), and the lowest in November (30.02 ind. 100 m^{-3}) (Supplementary Figures S1 and S2). We obtained 498 high-quality sequences (87.8%) from 567 fish eggs. Because of challenges posed by the COVID-19 outbreak on laboratory analysis, high-quality sequences were not obtained from 69 (12.2%) eggs. After the alignment and trimming of noisy sites, a length of 636 nucleotides was obtained and subjected to analyses. Egg sequences identified to species were deposited in BOLD.

3.2. Molecular Identification of Fish Eggs

Using 2% genetic divergence and 98% similarity thresholds to represent species boundaries in the BOLD analysis, 96.4% of egg sequences (480/498) had a match between 98% and −100%. Among these 498 egg sequences, 116 sequences (23.3%) were assigned to 20 species (Case I) (Table 1), 364 (73.1%) had ambiguous species delimitation and were identified to 26 taxa (Case II) (Table 2), and 18 (3.6%) could not be identified (Case III) (Figure 2, Supplementary Table S1). Case I species belonged to five orders, 13 families, and 18 genera; the most abundant families were Engraulidae (four species, 30 individuals), Pempheridae

(one species, 16 individuals), and Leiognathidae (one species, 15 individuals). These Case I species also included the commercially important fishes *Carangoides praeustus* (Anonymous [Bennett], 1830), *Cynoglossus joyneri* Günther, 1878, *Sillago lutea* McKay, 1985, *Sillago sihama* (Forsskål, 1775), *Heteromycteris japonicus* (Temminck and Schlegel, 1846), and *Nibea albiflora* (Richardson, 1846). Based on adult fish habitat data for species from Fishbase, six Case I species were associated with reefs, nine were demersal, three were pelagic–neritic, one was benthopelagic, and one was pelagic–oceanic (Table 1). Case II taxa were categorized to 6 orders, 16 families, and 16 genera.

Table 1. The fish eggs identified according to the species based on molecular analyses.

Family	Species	Common Name	Number of Specimens Identified by COI	Egg Diameter/mm	Oil Diameter/mm	Summer	Autumn	Habitat
Carangidae	*Carangoides praeustus*	Brownback trevally	2	0.686–0.691	0.194–0.255	+		Demersal
Clupeidae	*Hilsa kelee*	Kelee shad	10	0.964–1.078	0.051–0.111	+		Pelagic–neritic
Cynoglossidae	*Cynoglossus joyneri*	Red tonguesole	1	0.723	0.045–0.073		+	Demersal
Engraulidae	*Encrasicholina heteroloba*	Shorthead anchovy	7	1.019–1.135 × 0.542–0.665		+	+	Reef-associated
Engraulidae	*Stolephorus commersonnii*	Commerson's anchovy	8	1.318–1.444 × 0.586–0.752	0.064–0.091	+	+	Pelagic–neritic
Engraulidae	*Thryssa mystax*	Moustached thryssa	7	0.950–1.048		+		Pelagic–oceanic
Engraulidae	*Thryssa setirostris*	Longjaw thryssa	8	0.968–1.031		+		Pelagic–neritic
Labridae	*Halichoeres nigrescens*	Bubblefin wrasse	14	0.600–0.658	0.130–0.178	+	+	Reef-associated
Leiognathidae	*Leiognathus ruconius*	Deep pugnose ponyfish	15	0.627–0.687	0.124–0.179	+		Demersal
Mugilidae	*Plicomugil labiosus*	Hornlip mullet	2	0.905–0.932	0.473	+		Reef-associated
Pempheridae	*Pempheris schwenkii*	Silver sweeper	16	1.147–1.383	0.261–0.375	+	+	Reef-associated
Platycephalidae	*Inegocia japonica*	Japanese flathead	1	0.799	0.165		+	Demersal
Platycephalidae	*Thysanophrys celebica*	Celebes flathead	4	0.942–1.026	0.081–0.092	+		Demersal
Sciaenidae	*Nibea albiflora*	Yellow drum	7	0.726–0.772	0.215–0.230		+	Benthopelagic
Scorpaenidae	*Tetraroge barbata*	Bearded roguefish	1	0.950			+	Reef-associated
Sillaginidae	*Sillago lutea*	Mud sillago	2	0.661–0.679	0.124–0.239	+	+	Demersal
Sillaginidae	*Sillago sihama*	Silver sillago	8	0.670–0.716	0.122–0.164	+	+	Demersal
Soleidae	*Heteromycteris japonicus*	Bamboo sole	1	1.004	0.024–0.053		+	Demersal
Soleidae	*Pardachirus pavoninus*	Peacock sole	1	1.464	0.079–0.117		+	Reef-associated
Soleidae	*Solea ovata*	Ovate sole	1	0.877	0.026–0.111		+	Demersal

Table 2. Community composition of fish eggs, occurrence numbers and times of the identified taxa between June and November based on molecular analyses.

Family	Species	Total	June	July	August	September	October	November
Synodontidae	*Synodus* sp.	1		1				
Synodontidae	*Trachinocephalus* sp.	4	1	2	1			
-	Clupeiformes sp.	22		4	4	7	4	3
Clupeidae	*Hilsa kelee*	10	10					
Clupeidae	*Sardinella* sp.1	15		15				
Clupeidae	*Sardinella* sp.2	1	1					

Table 2. *Cont.*

Family	Species	Total	June	July	August	September	October	November
Engraulidae	*Encrasicholina heteroloba*	7		3	2	2		
Engraulidae	*Encrasicholina* sp.	38	1	9	5	22		1
Engraulidae	Engraulidae sp.1	1		1				
Engraulidae	Engraulidae sp.2	1	1					
Engraulidae	Engraulidae sp.3	1			1			
Engraulidae	*Engraulis* sp.	1		1				
Engraulidae	*Stolephorus commersonnii*	8	1	1	2	4		
Engraulidae	*Stolephorus* sp.	100	20	21	3	20	6	30
Engraulidae	*Thryssa mystax*	7	7					
Engraulidae	*Thryssa setirostris*	8	5	3				
Mugilidae	Mugilidae sp.	1				1		
Mugilidae	*Plicomugil labiosus*	2		2				
Callionymidae	*Callionymus* sp.	1						1
Carangidae	*Alepes* sp.	5	1	2	2			
Carangidae	*Carangoides praeustus*	2		2				
Gerreidae	*Gerres* sp.1	10				10		
Gerreidae	*Gerres* sp.2	32	5	5	5	17		
Haemulidae	Haemulidae sp.	2				2		
Labridae	*Halichoeres nigrescens*	14	5	1	5		2	1
Labridae	*Stethojulis* sp.	1	1					
Leiognathidae	*Equulites* sp.	1	1					
Leiognathidae	Leiognathidae sp.	110	16	22	11	24	34	3
Leiognathidae	*Leiognathus ruconius*	15	12	3				
Lutjanidae	*Lutjanus* sp.	1					1	
Pempheridae	*Pempheris schwenkii*	16	2	9		2	3	
Sciaenidae	*Johnius* sp.	4					1	3
Sciaenidae	*Nibea albiflora*	7				1	2	4
Serranidae	*Cephalopholis* sp.	7		7				
Sillaginidae	*Sillago lutea*	2		1			1	
Sillaginidae	*Sillago sihama*	8	2		1		4	1
Sillaginidae	*Sillago* sp.	1			1			
Cynoglossidae	*Cynoglossus joyneri*	1						1
Cynoglossidae	*Cynoglossus* sp.	2					2	
Soleidae	*Heteromycteris japonicus*	1					1	
Soleidae	*Pardachirus pavoninus*	1				1		
Soleidae	*Solea ovata*	1						1
Platycephalidae	*Inegocia japonica*	1						1
Platycephalidae	*Thysanophrys celebica*	4		2	2			
Scorpaenidae	Scorpaenidae sp.	1				1		
Scorpaenidae	*Tetraroge barbata*	1					1	
Unidentified	Unidentified species 1	4	2		1	1		
Unidentified	Unidentified species 2	6	1	1	1	3		
Unidentified	Unidentified species 3	3		3				
Unidentified	Unidentified species 4	4					2	2
Unidentified	Unidentified species 5	1		1				

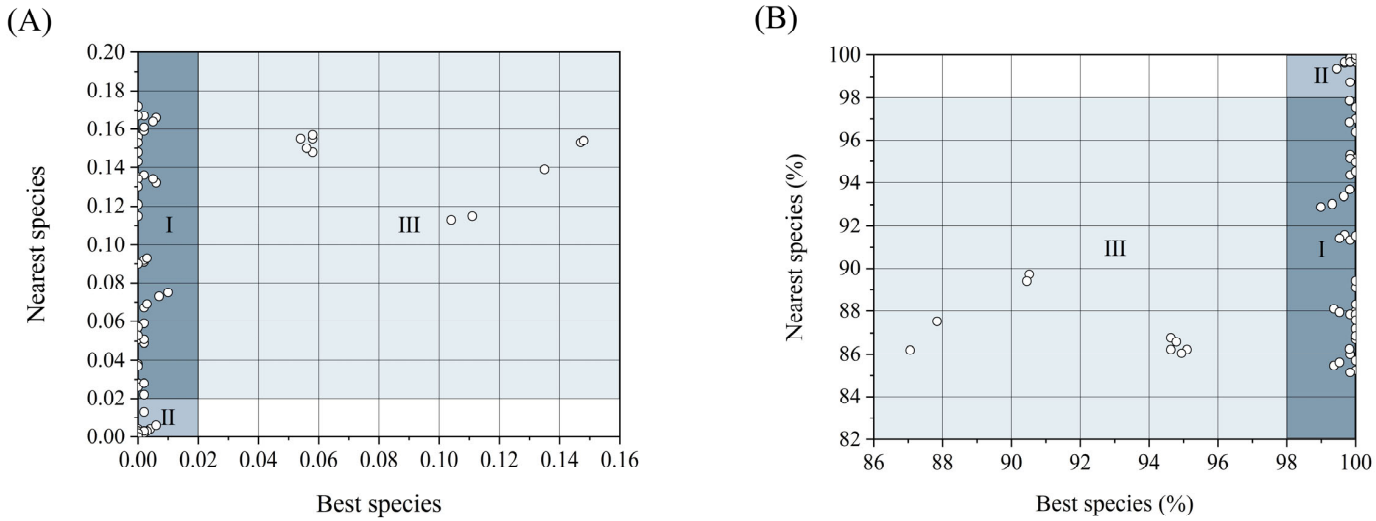

Figure 2. The best match compared with the nearest neighbor for each specimen. (**A**) Genetic distance; (**B**) similarity percentage. Case I, delimited to the species level; Case II, delimited to the genus or uncertain; Case III, unidentified.

The NJ tree of the COI sequences revealed identifiable fish eggs to form 51 independent lineages, suggesting that at least 51 distinct species/taxa of fish occurred in these waters (Figure 3). Of them, 35 taxa occurred in summer and 29 in autumn, while 13 occurred in both seasons; 22 taxa occurred only in summer and 16 in autumn (Table 2). Each month, 13–25 fish egg taxa were collected, with the lowest number (13) occurring in November and the highest (25) in July. Two taxa (*Stolephorus* sp. and Leiognathidae sp.) occurred each month, and Clupeiformes sp., *Encrasicholina* sp., and *Halichoeres nigrescens* (Bloch and Schneider, 1801) occurred in five months, indicating that these fish species had long spawning periods in southwestern Daya Bay waters (Table 2).

Fish egg species accumulation curves were non-asymptotic, suggesting that more taxa would be collected with increased sampling effort (Figure 4).

3.3. Fish Egg Morphology

The diameters of 498 fish eggs identified by COI sequences were measured. Most eggs (99.6%) were <1.500 mm in diameter (56.2% were ≤1.000 mm, and 43.4% were 1.001–1.500 mm. Only 0.4% were >1.500 mm (Figure 5, Supplementary Table S2). Most eggs had a transparent and smooth chorion. Of 20 Case I species, the eggs of 18 were spherical and those of 2 were elliptical (Figure 6). For spherical eggs, nine fish egg species had one oil globule (Figure 6A–I), and 6 had two or more oil globules (Figure 6J–O); eggs of *Thryssa mystax* (Bloch and Schneider, 1801), *Thryssa setirostris* (Broussonet, 1782), and *Tetraroge barbata* (Cuvier, 1829) lacked oil globules (Figure 6P–R). For elliptical eggs, *Encrasicholina heteroloba* lacked oil globules, and *Stolephorus commersonnii* (Lacepède, 1803) had one oil globule (Figure 6S,T). Egg diameters ranged 0.600–1.464 mm among species and families. The smallest egg diameters of identified species were those of *H. nigrescens* (0.600–0.658 mm) and the largest was *Pardachirus pavoninus* (Lacepède, 1802) (1.464 mm). At the family level, the smallest eggs belonged to Labridae, and the largest to Soleidae (Figure 7). Most fish species produced small eggs with average egg diameters of 12 species (60.0%) and 9 families (69.2%) were ≤1.000 mm (Figure 7).

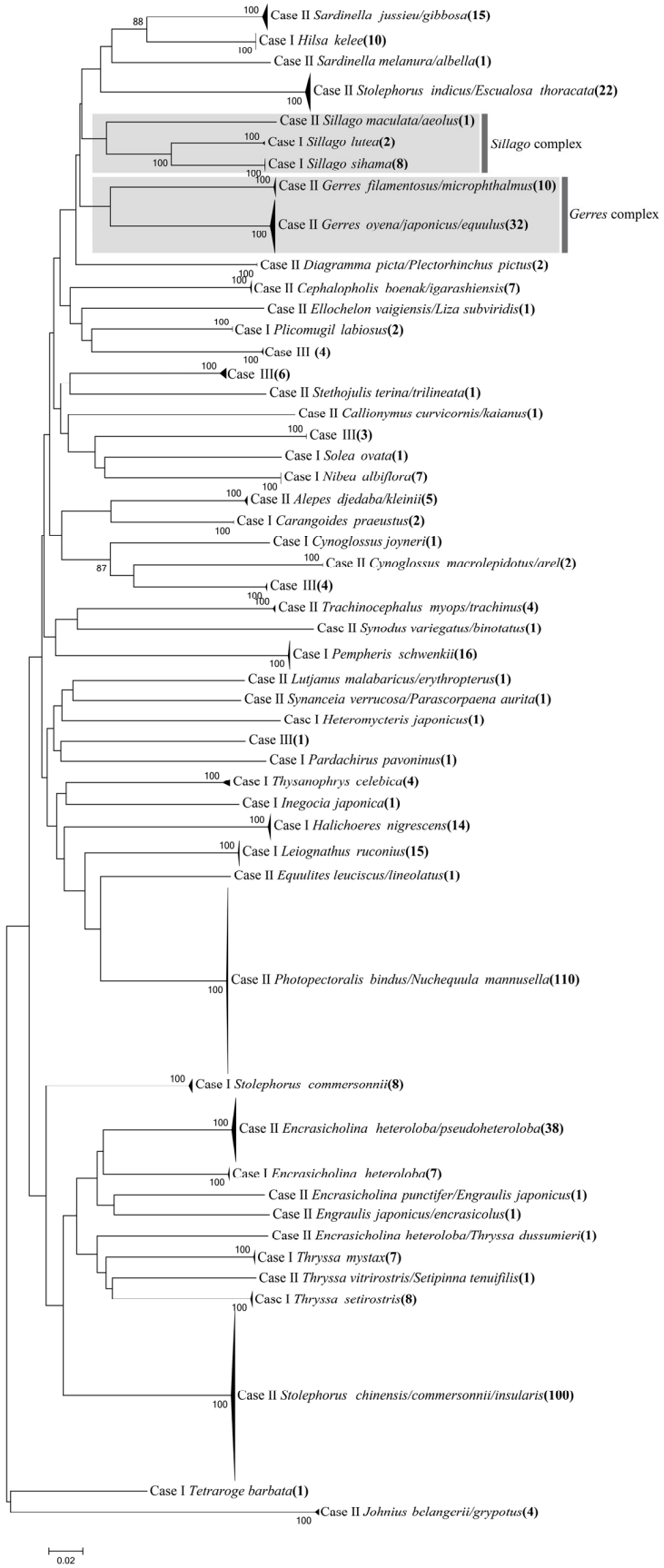

Figure 3. The neighbor-joining tree of the COI sequences of fish eggs. The bootstrap values > 70% are given at the nodes.

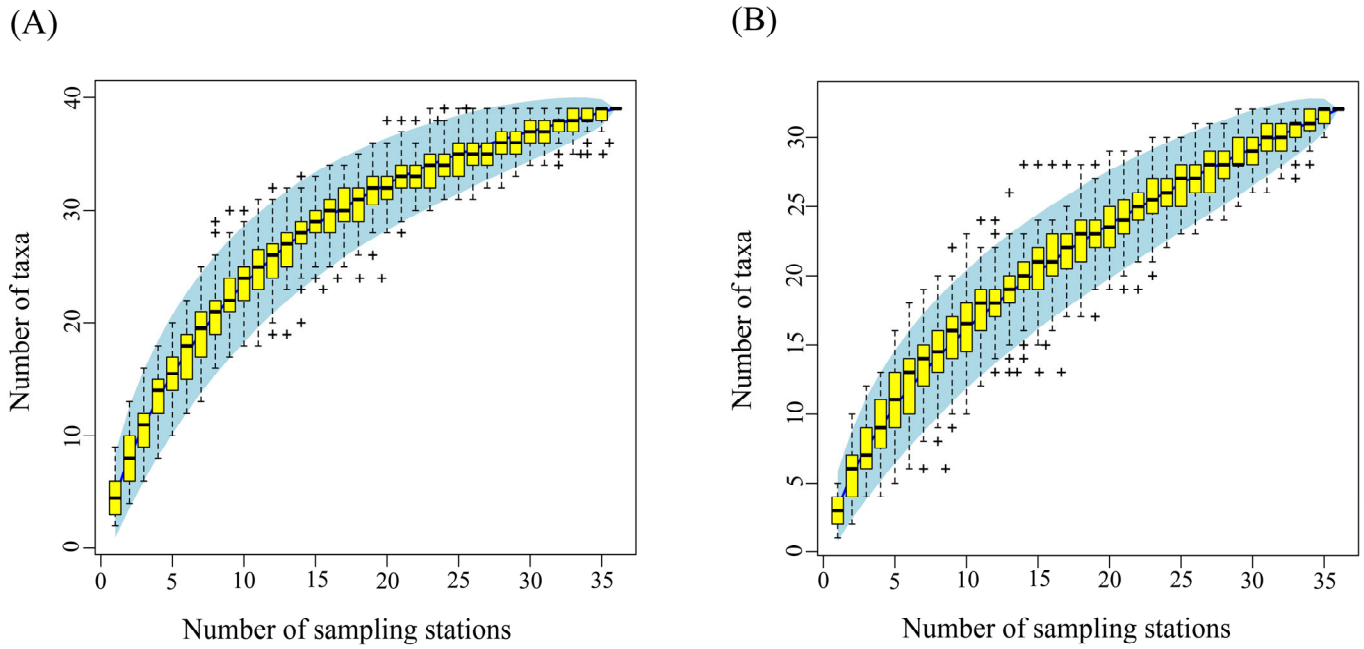

Figure 4. Species accumulation curves of fish eggs taxa identified by molecular methods in the southwest Daya Bay. (**A**) Summer; (**B**) autumn. The light blue area indicates 95% confidence intervals. Yellow boxes are the interquartile ranges, the central bold mark on each box indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points not considered outliers, and the outliers are plotted individually using the "+"symbol.

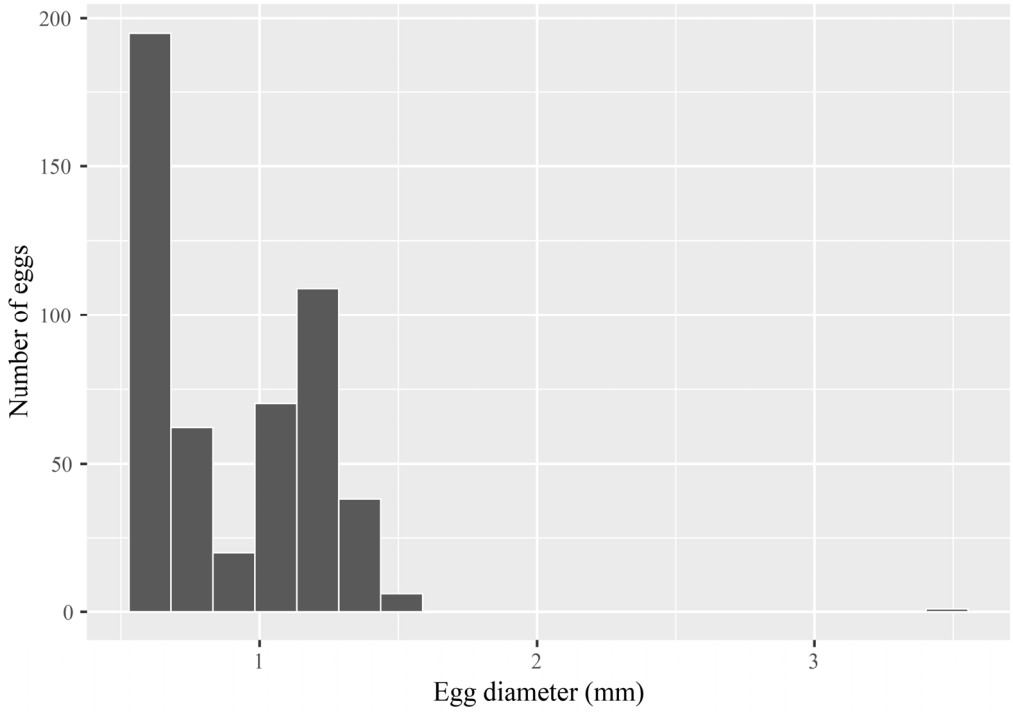

Figure 5. The egg diameter distribution of identified fish eggs based on COI sequences in the southwest Daya Bay.

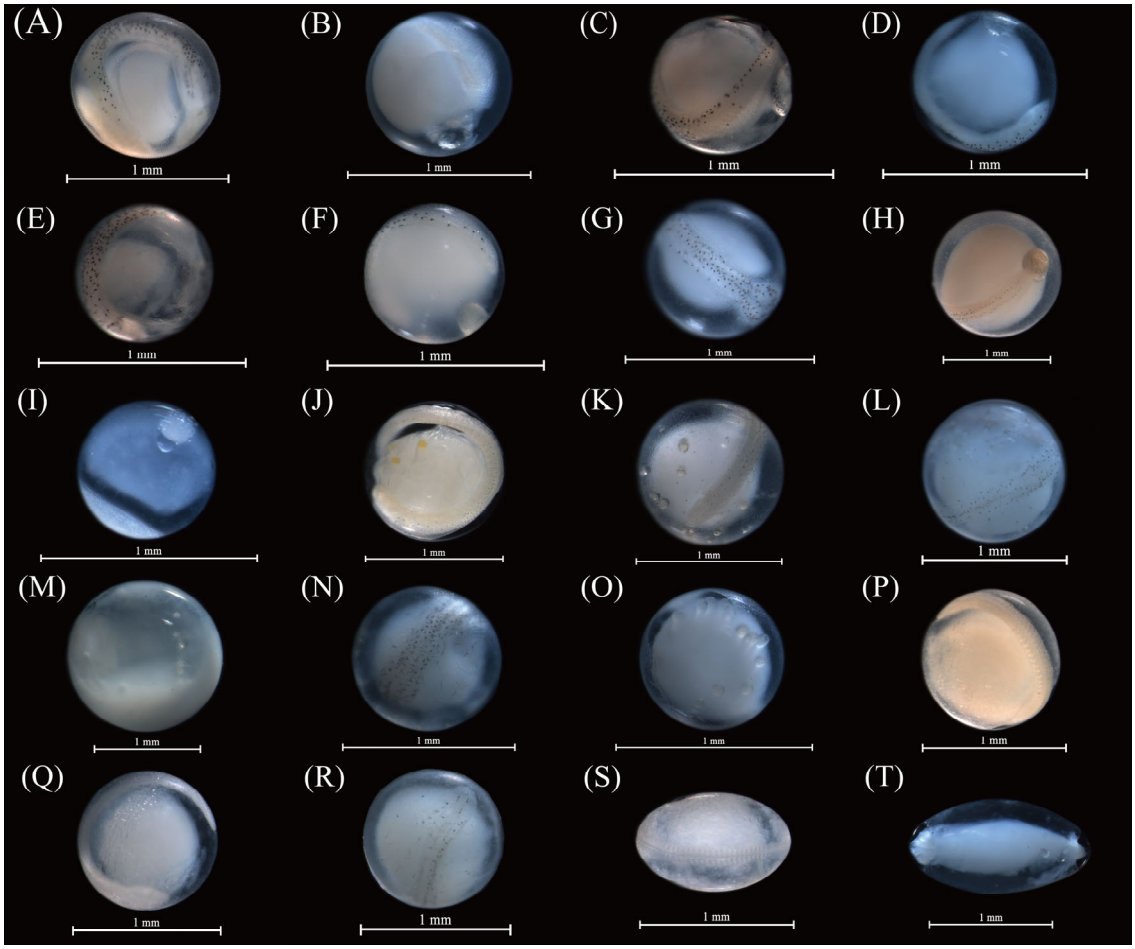

Figure 6. Photographs of the egg specimens that were identified to species level by COI sequences in the southwest Daya Bay. (**A**) *Plicomugil labiosus*, GDYH16882, 0.932 mm; (**B**) *Inegocia japonica*, GDYH17775, 0.799 mm; (**C**) *Sillago lutea*, GDYH16999, 0.679 mm; (**D**) *Sillago sihama*, GDYH17725, 0.700 mm; (**E**) *Carangoides praeustus*, GDYH16911, 0.686 mm; (**F**) *Leiognathus ruconius*, GDYH16781, 0.647 mm; (**G**) *Nibea albiflora*, GDYH17588, 0.746 mm; (**H**) *Pempheris schwenkii*, GDYH16900, 1.219 mm; (**I**) *Halichoeres nigrescens*, GDYH17805, 0.626 mm; (**J**) *Hilsa kelee*, GDYH16783, 1.034 mm; (**K**) *Thysanophrys celebica*, GDYH17108, 1.026 mm; (**L**) *Heteromycteris japonicus*, GDYH17718, 1.004 mm; (**M**) *Pardachirus pavoninus*, GDYH17626, 1.464 mm; (**N**) *Solea ovata*, GDYH17759, 0.877 mm; (**O**) *Cynoglossus joyneri*, GDYH17772, 0.723 mm; (**P**) *Thryssa mystax*, GDYH16851, 0.978 mm; (**Q**) *Thryssa setirostris*, GDYH16811, 0.972 mm; (**R**) *Tetraroge barbata*, GDYH17702, 0.950 mm; (**S**) *Encrasicholina heteroloba*, GDYH16897, 1.019 × 0.654 mm; (**T**) *Stolephorus commersonnii*, GDYH17667 1.418 × 0.752 mm.

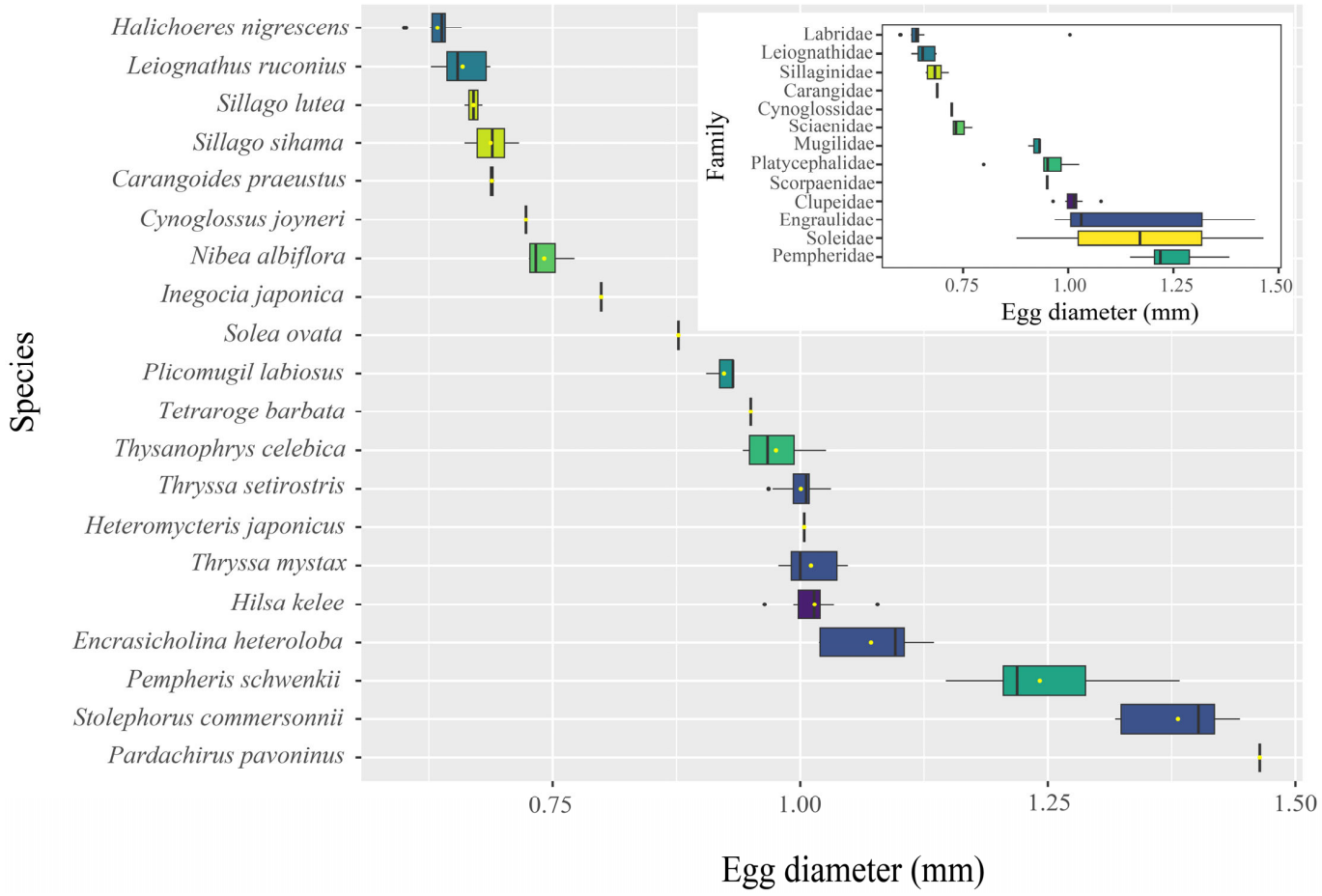

Figure 7. The egg diameter of identified fish eggs at the species level based on COI sequences.

4. Discussion

4.1. Fish Egg Amplification and Sequencing Success Rates

In the present study, we extracted and amplified 567 ethanol preserved fish eggs and successfully obtained 498 high-quality sequences among them (87.8% of the samples). Normally, the eggs preserved in high ethanol concentration can obtain high success rates of the amplification and sequencing of fish eggs. However, this may reduce the morphological quality of early-life stages of fishes, especially the egg stages [30]. In our previous study, we proposed preserving ichthyoplankton samples immediately in a 4% neutral formalin solution and then transferred to a 75% ethanol solution, which can ensure obtaining a certain proportion of molecular sequences, with a relatively high morphology quality pictures [31]. However, if DNA is not extracted and amplified quickly, of the fish eggs transferred from a formalin-fixed solution to an ethanol solution, i.e., within two/three months, the success rate decreases sharply to <20% (and nears 0%). This proposal is not suitable for many of the monthly survey samples conducted in Daya Bay. Therefore, we fixed eggs in a combination of 75% ethanol–seawater, a solution we hoped would enable egg identification using both morphology and sequencing. We report a sequencing success rate of 87.8%. Other studies have reported variable sequencing success rates for fish eggs. Ahern et al. [7] sequenced 2354 of 6422 eggs (COI/16S fragments, 36.66%), Liu et al. [32] obtained 7933 *cytb* sequences from 8983 drifting eggs (88.31%), Chen et al. [33] obtained 397 high-quality sequences from 641 eggs (61.93%), and Hou et al. [12] obtained 541 COI high-quality sequences and 41 *cytb* sequences (66.67%) from 873 eggs. Our low success rate was most likely because of the long delay between egg collection and molecular analysis. Previous studies have suggested that a lower ethanol concentration and long storage periods (>6 months) can negatively affect DNA stability and result in its degradation [34–36]. However, a higher

ethanol concentration (95–100%) can induce albefaction, brittleness, oil globule dissolution, and morphological damage [36,37]. Therefore, timely DNA extraction and amplification from fish eggs fixed in lower-strength alcohol are recommended to improve sequencing success rates.

4.2. Performance of Fish Egg Identification Using DNA Barcodes

We identified 51 distinct taxa using COI sequences, of which 20 taxa (39.2%) were identified to species. However, 25 taxa (49.0%) were ambiguous and could only be identified to the genus or family, and 1 taxon (2.0%) could be identified to the order only. The rate of successful identification to species was similar to that in studies of fish eggs in the eastern Beibu Gulf [12]. This may be because of misidentification and incompleteness of DNA barcode reference libraries for ocean fishes from Daya Bay and adjacent sea areas in the northern SCS. The high proportion of ambiguous identifications limited our ability to identify fish eggs from this region using the BOLD database platform [38,39].

Misidentifications in the DNA barcode library of fish may occur because of phenotypic plasticity, the existence of new species, cryptic diversity, genotypic variation, different life stages, and the varied systematic competencies of persons responsible for making identifications. We report ambiguous delineation of taxa in the genera *Synodus*, *Trachinocephalus*, *Sardinella*, *Encrasicholina*, *Engraulis*, *Stolephorus*, *Callionymus*, *Alepes*, *Gerres*, *Stethojulis*, *Equulites*, *Lutjanus*, *Johnius*, *Cephalopholis*, *Sillago*, and *Cynoglossus*, the families Engraulidae, Mugilidae, Haemulidae, Leiognathidae, and Scorpaenidae, and in the order Clupeiformes (Table 2). Among these, misidentifications, cryptic diversity, and new species were prevalent [40–50]. Reliable DNA barcodes for these taxa are necessary to ensure accurate species identification through morphology or using multiple DNA fragments [51,52].

4.3. Species Composition Variation

During surveys in 1984–1985, 64 fish taxa were identified from fish eggs and larvae, including 42 to species, and 22 to the genus and family level [19]. In 1986–1987 and 1988–1989, 36 taxa were identified from fish eggs (23 taxa to species, and 13 taxa to genus or family) [20]. More than 30 years later, on the basis of all fish egg samples, we identified 46 taxa (20 to species, 25 to genus or family, and 1 to order) (Table 2). Among these taxa, 10 belonged to the Engraulidae (21.7%) and 3 each (6.5%) to the Clupeidae, Leiognathidae, Sillaginidae, and Soleidae, indicating that these 5 families were dominant taxa in southwestern Daya Bay. Compared with the results of surveys from 1984 to 1985, the number of taxa identified from fish eggs have decreased by nearly 20%. We believe the main reason for this is that we only collected samples over six months (in summer and autumn), so some species that spawn in winter and spring were excluded. Future surveys should collect samples during winter and spring. However, it is also possible that some change has occurred in fish communities in Daya Bay over the last 30 years. In 1980–1990, dominant species were *Konosirus punctatus* (Temminck and Schlegel, 1846), *Leiognathus rivulatus* (Temminck and Schlegel, 1845), *Trichiurus japonicus* Temminck and Schlegel, 1844, and *Pampus argenteus* (Euphrasen, 1788) [53], whereas *K. punctatus*, *Sardinella zunasi* (Bleeker, 1854), *S. commersonii*, and *Evynnis cardinalis* (Lacepède, 1802) were dominant in 2004–2005 [16]. In 2016–2017, dominant speices were *Evynnis cardinalis* (Lacepède, 1802), *Callionymus richardsoni* Bleeker, 1854, *Clupanodon punctatus* (Temminck and Schlegel, 1846), *T. japonicus*, *Thamnaconus hypargyreus* (Cope, 1871), *L. brevirostris*, and *Apogon lineatus* (Temminck and Schlegel, 1843) [54]. On the basis of trawl-survey data, the number of fish species decreased from 180 in 1987 to 127 in 2015 [55]. Corresponding to significant shifts in fish community structure in Daya Bay, changes also occurred in fish egg composition. Xu et al. [19] reported dominant eggs to belong to the Leiognathidae (50.8%), Clupeidae (25.2%), Engraulidae (8.7%), Sparidae (4.4%), and Cynoglossidae (2.9%) in 1984–1985. In 1986–1987, dominant eggs included those of the genera *Sardinella* (28.0%), *Thryssa* (20.5%), and the family Leiognathidae (17.8%); from 1988 to 1989, dominant eggs belonged to the family Leiognathidae (66.9%), and the genera *Sardinella* sp. (19.0%), and *Thryssa* sp. (6.8%), with a sharp decline in the eggs of

Clupeidae, Engraulidae and Sparidae [20]. For 2003–2004, Lin et al. [21] reported that dominant fish eggs from this region belonged to the Leiognathidae (74.1%), *Sebastiscus marmoratus* (7.0%), Clupeidae (4.8%), Engraulidae (3.0%), and Sparidae (1.7%), and for 2004–2005, Leiognathidae (50.3%), Clupeidae (23.8%), Engraulidae (6.3%) and *S. marmoratus* (3.4%). For 2015, Wang et al. [18] reported dominant egg taxa to include *Sillago* sp. (73.03%), Mugilidae sp. (6.71%), and Sparidae sp. (5.64%). For 2017–2018, Zhang [56] reported Clupeidae (38.8%), Nemipteridae (13.0%), Leiognathidae (12.5%), Mugilidae (8.2%), and Engraulidae (5.5%). For 2020, Tan et al. [57] reported Leiognathidae (63.1%), Sparidae (25.8%), Engraulidae (3.5%) and Clupeidae (2.5%) (Figure 8 and Supplementary Table S3). We report the most abundant families to be Engraulidae (34.5%), Leiognathidae (25.3%), Gerreidae (8.4%), and Clupeidae (5.2%).

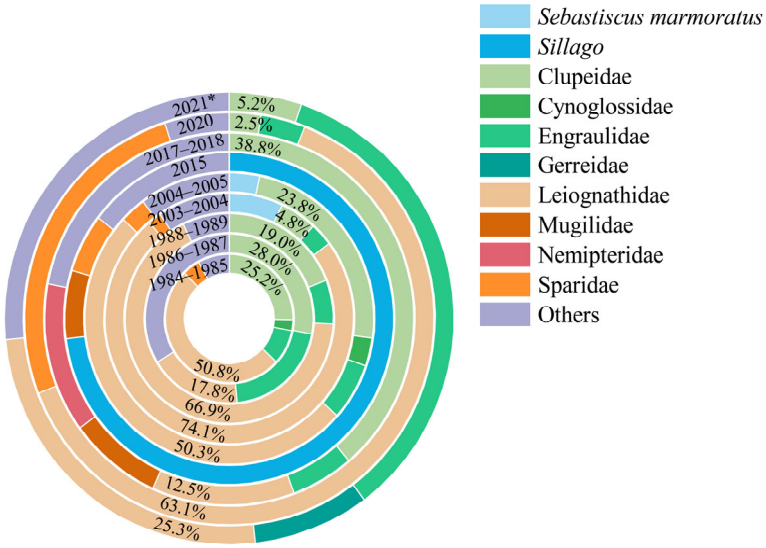

Figure 8. Historical data of fish egg survey in Daya Bay. * indicate the present study.

Compared with dominant fish eggs in the past 30 years, the eggs of Leiognathidae varied from 12.5% to 74.1% of all eggs, and their proportional contribution to the egg pool has declined by 25.5% from 1984 to 2021. The proportional contribution of eggs of Clupeidae varies from 2.5% to 38.8% of all eggs, and has declined by 20% from 1984 to 2021. The eggs of commercially important fish taxa (Sparidae) were dominant in surveys between 2003 and 2020, but their proportional contribution increased considerably in 2020 (mostly *Acanthopagrus schlegelii* Bleeker, 1854, but several other species were present) (Figure 8). Our results are inconsistent with previous surveys in which eggs were identified based on morphology, wherein the eggs of *Pempheris schwenkii* and *Halichoeres nigrescens* were not identified from this region; these two species are associated with reefs, making them difficult to observe and capture. These two non-economic fish species may have been overlooked by commercial fishers, explaining their absence from fishery resource surveys. We also report the eggs of six economically fish species: *C. praeustus*, *C. joyneri*, *H. japonicus*, *N. albiflora* (all rarely caught in previous fishery resource surveys), *S. lutea*, and *S. sihama*. We demonstrate our method to be capable of detecting species in an egg pool that are otherwise retained in fishery surveys as adults in this region. Additionally, because we limited our investigation to southwestern Day Bay waters, collected only drifting and suspended eggs using zooplankton nets, and subjected only a portion of samples to molecular identification, it is likely that the true egg-pool species diversity for this region is underestimated.

4.4. Daya Bay Summer and Autumn Spawning Periods

Fish eggs directly indicate fish spawning activity and spawning period. An improved understanding of spawning periods can support monitoring efforts. Because spawning times tend to be species- and region-specific, it is important to determine them for as

many species as possible for a region. It is also difficult to schedule sampling during peak reproductive periods because environmental variation may lead to different estimates of stock abundance. Thus, monthly surveys are needed to monitor the stock status of fish during their early life stages. Most of the few studies that have examined fish eggs in Daya Bay did so almost 30 years ago, and these surveys mostly identified eggs to genus and family level using their morphology [18–21]. Consequently, many species were not detected. In the present study, we detected the spawning time of 46 identified fish egg taxa over a 6-month period in summer and autumn; thus, the spawning periods identified in this study might apply to fish species in Daya Bay.

Of the 46 identified fish egg taxa, 11 occurred in both summer and autumn, 20 occurred only in summer, and 15 occurred only in autumn. Annual water temperatures in Daya Bay ranged 16.8–30.9 °C, and averaged 29.2 °C during high-temperature periods and 20 °C during low-temperature periods [58]. Our survey area was near the Daya Bay Nuclear Power Plant, from which thermal discharge may elevate adjacent water temperatures by 4–7 °C [59]. Water temperature plays an important role in fish egg hatching and development, with the hatching time of fish eggs in different sea areas having species- and region-specific characteristics. In the northern SCS, pelagic fish eggs typically hatch within 18–70 h after being spawned [60], while increased water temperature may cause some ill-adapted fishes to leave the area to spawn, they may shorten the incubation time of others, potentially affecting species occurrence, early recruitment, and community [61,62]. After nearly 30 years of operation of powerplant operation, some fish have adapted to new habitat in this environment. Therefore, those fish eggs group that we identify can inform us of changes in fish spawning activity in the vicinity of the Daya Bay Nuclear Power Plant.

While planktonic fish eggs normally disperse in ocean currents, Daya Bay is semi-enclosed and in our sampling area water flow is relatively slow [63]. Thus, hydrological conditions do not transport fish eggs long distances before they hatch. With increased water temperatures, fertilized fish eggs also hatch faster [64]. Consequently, we predict that most eggs reported in our study originated from local spawning events, indicating that the spawning grounds of these species are nearby.

5. Conclusions

We identified the fish fauna of southwestern Daya Bay on planktonic egg samples collected in monthly surveys over summer and autumn. The DNA barcoding of eggs allowed us to infer the spawning periods of these species. From 498 high-quality COI sequences, 51 fish taxa were identified to 6 orders, 19 families, and 30 genera, including 20 to species, 25 to genus or family, and 1 to order. Among the 46 identified taxa, 11 occurred in both summer and autumn, 20 occurred only in summer, and 15 occurred only in autumn. We also present the images of fish eggs to facilitate their identification in subsequent surveys that use egg morphology to identify eggs. These insights into fish spawning periods in Daya Bay will inform conservation and fishery management. Because 31 taxa (60.8%) could not be identified to species, a more reliable DNA barcode library for fishes from Daya Bay is required, as is increased systematic work on the fish fauna of this region.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9120510/s1. Table S1: The blast results of fish egg sequences in the BOLD system. Table S2: The egg diameters of fish eggs in Southwest Daya Bay. Table S3: Historical composition of fish eggs in Daya Bay. Figure S1: Fish egg abundance in summer. Figure S2: Fish egg abundance in autumn. References [18,21,56,57] are cited in the Supplementary Materials File.

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