Comparison of visual and molecular taxonomic methods to identify ichthyoplankton in the North Sea

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Abstract

The North Sea is an important spawning and nursery ground for many demersal and pelagic fishes whose spawning areas are largely overlapping in time and space. This makes ichthyoplankton visual identification from the various species particularly challenging. Despite historically intensive research in the area, detailed information on spawning sites and times for many taxa, are incomplete. To update and detail the mapping of fish spawning performance and distribution in the central and northern regions of the North Sea, the performance of a visual method and a molecular taxonomic approach used for taxonomic classification of ichthyoplankton was evaluated. Samples of fish eggs and larvae were collected regularly and in parallel at different latitudinal locations from the central to the northern North Sea, including a sample with 78 larvae used for direct comparison between both methods. A total of 5332 individuals were inspected and 36 different species were identified. The visual processing identified 89% of the collected larvae to species level, however, for the eggs the taxonomic resolution was lower with only 5% identified to species level. In comparison to visual identification, molecular barcoding gave higher precision of identification for larvae and especially for the eggs. For the larvae, 98% were assigned to species level, and for the eggs 94% were assigned to species level. We find that molecular barcoding is more effective and precise in taxonomic identification of both eggs and larvae to species level. However, visual identification is still needed to provide information on the developmental stages.

Monitoring of spawning areas is crucial for understanding the ecology of the early stages in fishes and development of marine management and conservation (Moser and Smith 1993). However, even in well-surveyed regions such as the North Sea, literature about fish spawning grounds and their spawning periods remain limited (Fox et al. 2008), and most of the studies and scientific information available concerning the biology and ecology of fish species mainly focus on commercially important species (Fox et al. 2008; Jansen et al. 2012; Nash et al. 2012). Considering the scarcity of data from well-chartered seas, it comes as no surprise that the deficit of knowledge about spawning patterns is a global ocean stewardship hindrance.

Historically, identification of spawning fish species in a location has been based on morphological identification of eggs and larvae by comparison with known characteristics (Ahlstrom and Moser 1980; Harada et al. 2015). A strong additional support for visual egg identification in a specific area is the presence of ripe and running individuals of the species,

particularly when spawning areas of the various species are spatially segregated. However, morphological taxonomy on fish eggs and larvae still remains challenging due to their small size, limited morphological development, scarcity of comparative material of known origin, and reliance on few characteristics such as egg diameter and presence of oil globules for species determination (Ahlstrom and Moser 1980; Moser 1996; Richards 2006; Richardson et al. 2007). Furthermore, some of the embryonic characters, such as pigmentations on the embryo, are only applicable in late stages of the egg development making earlier species determination difficult or impossible (Taylor et al. 2002; Valentin et al. 2013). In some cases, this have led to errors in the identification of commercially important fish species with consequences for management (Fox et al. 2005; Fox et al. 2008). As a response, researchers have turned toward alternative techniques such as molecular barcoding as a replacement for, or a complement to, traditional morphology-based taxonomic identification (Chapman et al. 2003; Hyde et al. 2005; Richardson et al. 2007; Lelievre et al. 2010; Gleason and Burton 2012; Ahern et al. 2018). In fact, many misidentifications were found when comparing visual and molecular methods to identify fish eggs at different locations (Lelievre et al. 2010; Ahern et al. 2018). In the North

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Sea, where spawning areas for the various species overlap to a large extent, molecular barcoding would have the potential to become a particularly valuable tool in future monitoring of spawning areas. The aim of this study was to evaluate the performance of two different approaches to identify egg and larval samples, the traditional morphophysiological taxonomic approach and DNA barcoding targeting the gene encoding for the mitochondrial Cytochrome c oxidase subunit I (COI), and suggest improvements for future methodology. Although in this study, samples were collected in the North Sea, our results can be applied in samples collected at other regions.

Materials and methods

Sample collection

A total of 361 samples were collected by four supply ships (Skandi Hugen, Esvagt Bergen, Stril Herkules, and Stril Merkul) between 56° and 61° N within the central and northern subregions of the North Sea (Fig. 1), using a WP2 net with a mesh size of 500 μm and a mouth opening of 0.57 m (0.25 m^2),

hauled vertically from 10 m above bottom to surface at a wire speed of 0.5 m sec^{-1}. Two hauls were performed at each sampling location, and the content of the net was poured from the cod-end through a 180 μm meshed sieve and fixated in a 100 mL plastic bottle. One haul was preserved in 96% ethanol for the molecular taxonomic analyses, the second haul was preserved in 4% formalin borax buffered seawater for the visual taxonomic identification. The samples were collected over a 28-month period from August 2017 to November 2019.

An additional sample at 58° N was collected by the ship Kristine Bonnevie using a MIK net with a mesh size of 500 μm and a mouth opening of 2 m at a wire speed of 0.25 m sec^{-1}, to perform a direct comparison of both molecular and visual taxonomic approaches using the same individuals. The larvae present in the MIK sample was preserved in 96% ethanol.

Molecular taxonomic method

For DNA isolation, single eggs or the removed eyes of larvae were placed in individual wells of a 200 μL 96 well-plates (Axygen Scientific, CA, USA) containing 75 μL of a solution
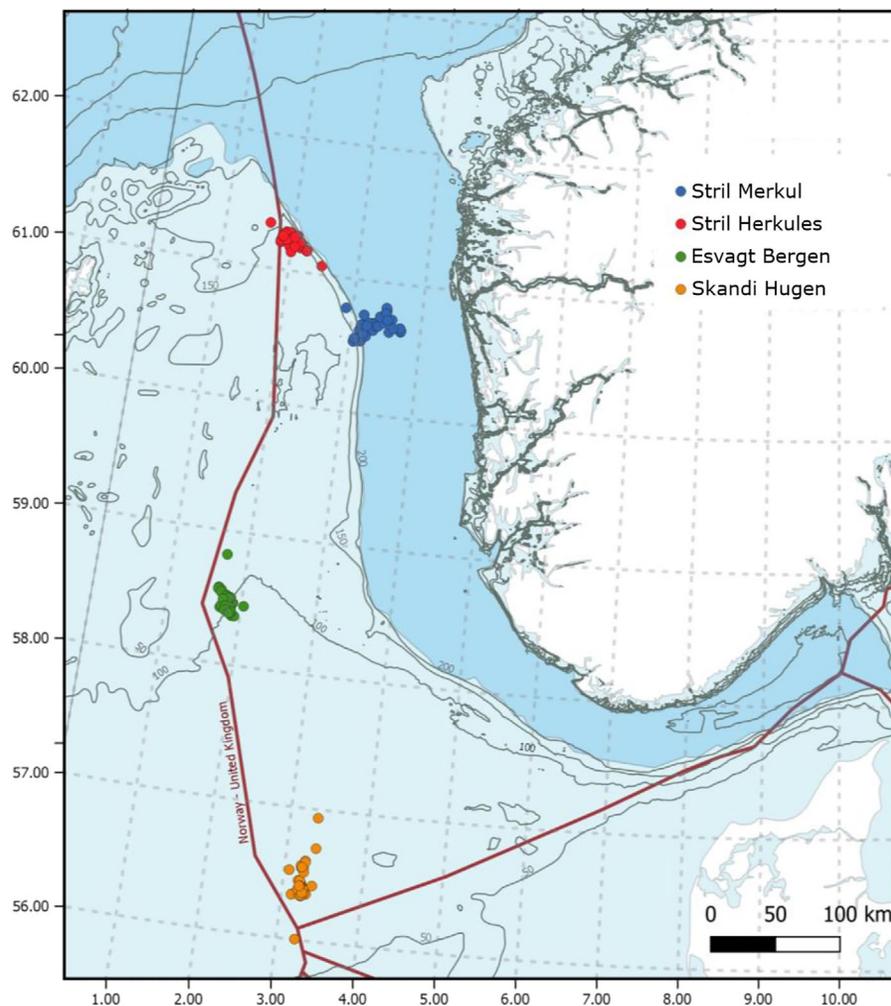
Fig. 1. Sampling sites in this study.

5% Chelex 100 Resin (BioRad, CA, USA) and 15 μL of Protein-ase K (Qiagen, Germany). The 96 well-plates were then incubated at 56°C for 1 h followed by 10 min at 96°C. After a brief centrifugation, the supernatant containing the nucleic acids was transferred into new 96 well-plates.

Following DNA isolation, PCR amplification targeting the mitochondrial COI gene was performed in 12 μL reactions containing 2.4 μL ×5 buffer, 1 μL of MgCl$_2$ [25 mM], 1.92 μL dNTPs [1.25 mM], 1.44 μL [10 μM] primer pair combination (LepF1_t1, VF1_t1, VF1d_t1, VF1i_t1, LepR1_t1, VR1d_t1, VR1_t1, VR1i_t1) with the M13F sequence incorporated in the forward primers (Ivanova et al. 2007), 0.07 μL GoTaq G2 DNA polymerase (Promega, WI, USA), 3.17 μL dH$_2$O, and 2 μL template DNA. The PCR conditions were (i) an initial denaturation of 2 min at 95°C, followed by (ii) 38 cycles of amplification (denaturation 30 s at 94°C, annealing at 52°C for 30 s and an extension of 1 min at 72°C), and (iii) a final extension of 10 min at 72°C. Clean-up of the PCR products was performed mixing 5 μL of the PCR product and 2 μL ExoSap-IT PCR product Cleanup (ThermoFisher, MA, USA) followed by an incubation at 37°C for 15 min and 80°C for 15 min. Finally, sequencing was performed using 1 μL of M13F primer (0.35 μM) at the sequencing facility at the University of Bergen (http://www.seqlab.uib.no). Sequence

Table 1. Number of eggs and larvae for each fish species identified in this study using the WP2 net.

Latin name	Common name	Molecular taxonomic approach		Visual taxonomic approach	
		Eggs	Larvae	Eggs	Larvae
Argentina silus	Greater argentine				1
Argentina sphyraena	Lesser argentine	10	1		2
Arnoglossus laterna	Scaldfish	1			
Benthosema glaciale	Glacier lantern fish		2		
Brosme brosme	Tusk	5	1		
Callionymus lyra	Dragonet		1		
Callionymus maculatus	Spotted dragonet	74	18		
Crystallogobius linearis	Cristal goby	1	1		1
Enchelyopus cimbrius	Four-bearded rockling	39	7		1
Eutrigla gurnardus	Gray gurnard	93	7		4
Gadiculus argenteus	Silvery pout	9			
Gadus morhua	Cod	14	8		
Gaidropsaurus vulgaris	Three-bearded rockling	4			
Glyptocephalus cynoglossus	Witch	34	6		7
Hippoglossoides platessoides	Long-rough dab	72	28	36	16
Lepidorhombus boscii	Four-spot megrim	2			
Lepidorhombus whiffiagonis	Megrim	4	6		
Limanda limanda	Dab	810	82		110
Maurolicus muelleri	Pearlside	135	2	76	
Melanogrammus aeglefinus	Haddock	35	20		18
Merlangius merlangus	Whiting	163	34		23
Merluccius merluccius	European hake	72	4		6
Microstomus kitt	Lemon sole	43	6		3
Molva molva	Ling	81	23		3
Phrynorhombus norvegicus	Norwegian topknot	12	1		
Pleuronectes platessa	Plaice	3			3
Pollachius pollachius	Pollack	14			3
Pollachius virens	Saithe	92	16		4
Scomber scombrus	Mackerel	321	56	7	74
Sebastes fasciatus	Acadian redfish	1			
Sebastes viviparus	Norway redfish		2		
Scophthalmus maximus	Turbot	1			
Trachurus trachurus	Atlantic horse mackerel	1			
Trisopterus esmarkii	Norway pout	54	47		16
Trisopterus minutus	Poor cod	4	5		

Table 2. Group and stages of the eggs analyzed following the visual taxonomic approach using the WP2 net.

Group	Egg stages				
	I	II	III	IV	V
Large eggs with large perivitelline space					
Hippoglossoides platessoides	6	8	7	10	5
Unassigned			1		
Small eggs with sculptured membrane and no oil globule					
Callionymus spp.	42	23	10	1	
Eggs with several oil globules and yolk with peripheral segmentation					
Eggs with several oil globules and unsegmented yolk					
Eggs with one oil globule and segmented yolk					
Maurolicus muelleri	65	4	11	6	
Unassigned				1	
Eggs with one oil globule and unsegmented yolk					
Scomber scombrus		1	2	3	1
Unassigned	227	33	104	112	39
Eggs without oil globules and with segmented yolk					
Eggs without oil globules and with unsegmented yolk					
Unassigned	663	108	223	244	83
Pelagic oval eggs					
Unknown	99	13	18	6	8

Table 3. Number of eggs and larvae identified at a family level in this study using the WP2 net.

Family name	Molecular taxonomic approach		Visual taxonomic approach	
	Eggs	Larvae	Eggs	Larvae
Ammodytidae				1
Callionymidae			76	8
Gadidae				9
Gobidae				3
Phycidae				2
Pleuronectidae				4
Triglidae			1	3

analysis was performed in Geneious v8.0.5 (Kearse et al. 2012). The resulting sequences were used as queries for BLASTn (Altschul et al. 1990) in GenBank (www.ncbi.nlm.nih.gov/genbank) using the default parameters and the Barcode of Life Data System (BOLD; www.boldsystems.org) databases and compared to reference sequences to determine its taxonomic identity. A threshold of a >99% match to assign sequences to species has been used in this study.

Visual taxonomic method

Larval identification followed the procedures described in Russell (1976) and Munk and Nielsen (2005), using a Leica M80 stereomicroscope with focusing arm (Leica Microsystems,

Germany). Larval measurements were recorded using a millimeter paper. Fish eggs were photographed under an Olympus SZX16 stereomicroscope (Olympus, Japan) with 0.4575 pixels μm^{-1} resolution and subsequently measured using Image J (https://imagej.nih.gov/ij/) with the plugin ObjectJ (https://sils.fnwi.uva.nl/bcb/objectj/) and the Cindy's Fish Eggs project (https://sils.fnwi.uva.nl/bcb/objectj/examples/CindysFisheggs/Manual-Cindy-6.htm). Pictures of the fish eggs were used for identification following the methods described in Russell (1976), in combination with the two main categories described in (Ahlstrom and Moser 1980). Briefly, the eggs were classified according to: (i) independent characters of the embryo (i.e., presence or absence of oil globules, egg size, egg shape, character of yolk, and width of the perivitelline space); and (ii) dependent characters of the embryo (mainly pigment patterns). Egg staging was determined following the criteria described in Riley (1973) and Thompson and Riley (1981).

Results

A total of 5332 individuals were collected, 5254 using the WP2 net and 78 individuals (all larvae) using the MIK net.

WP2 net

The 5254 individuals collected using the WP2 net were divided into 2734 (consisting of 2342 eggs and 392 larvae) for the molecular analyses and 2520 individuals (consisting of 2187 eggs and 333 larvae) for the visual taxonomic analyses. In total 35 species were identified, all of them except one, the

Table 4. Number of larvae identified in this study using the MIK net.

Larvae	Molecular taxonomic approach	Visual taxonomic approach
Callionymidae		4
Callionymus lyra	1	
Callionymus maculatus	4	
Clupea harengus	22	22
Gobiidae		2
Crystallogobius linearis	28	29
Microstomus kitt	21	20
Unknown	2	1

greater argentine (*Argentina silus*), were identified using the molecular approach (Table 1).

Using molecular barcoding, 2204 of the 2342 eggs collected were assigned into 31 different species, implying that 94.1% were assigned to species level, while 138 eggs were not possible to be identified. The effectiveness of the molecular method when applied to the larvae was slightly higher with 384 of the 392 larvae collected (97.9%) assigned to 25 different species (Table 1). Three species were only identified as larvae. Norway redfish (*Sebastes viviparus*) (also termed Norway haddock) has internal fertilization/egg incubation and releases first the hatched larvae into sea. Glacier lantern fish (*Benthosema glaciale*) is a deep-water species with its habitat confined to the adjacent Norwegian Trench (Kloppmann and Ellis 2015), hence, the sampling locations are outside, or at best at the fringe of its spawning areas. The third species, dragonet (*Callionymus lyra*), is known to spawn in the Southern Bight (Daan 2015). A single larva was identified in this study, demonstrating that main spawning areas are outside the sampling locations.

In contrast, the eggs collected for the traditional visual taxonomic approach were photographed and measured allowing not only detailed scrutiny but also determine their stages (Table 2). However, we were only able to identify the eggs of two species with a high degree of confidence; long-rough dab (*Hippoglossoides platessoides*) and pearlside (*Maurolicus muelleri*). In addition to these, only a few eggs of mackerel (*Scomber scombrus*) were identified but at much lower numbers than the eggs found using the molecular taxonomic method (Table 1). Therefore, only 129 of the 2187 fish eggs collected (5.8%) for the visual taxonomic were identified to species level. While 76 eggs (3.5%) could be assigned to the family *Callionymidae* (Table 2).

Nonetheless, the visual approach performed better when applied to the larvae. Of the 333 larvae analyzed, 295 (88.6%) were assigned to 18 different species (Table 1). In addition, 30 larvae (9%) could be assigned to 7 different families (*Ammodytidae, Callionymidae, Gadidae, Gobiidae, Phycidae, Pleuronectidae, Triglidae*) as deeper taxa identification was not possible (Table 3).

MIK net

The results from the 78 larvae collected with the MIK net followed the same pattern as the individuals collected using the WP2 net. Using molecular barcoding, 76 of the 78 individuals were assigned to species level, while only 71 of the 78 individuals were assigned to species level using the visual taxonomic approach (Table 4). Although the same taxa were found using both methods, the visual taxonomic method could not identify the larvae from the family *Callionymidae* and some *Gobiidae* at a deeper level, while the molecular barcoding could differentiate two different *Callionymidae* species (*C. lyra* and *C. maculatus*). Furthermore, all the species found in the larvae collected using the MIK net except one, herring (*Clupea harengus*), were also found with the using the WP2 net.

Discussion

The evaluation of performance of the two approaches used for ichthyoplankton taxonomic identification demonstrate that DNA barcoding was more precise for larval fish and particularly more precise for egg identification than morphometrically based visual analysis. Although most of the larvae could be assigned to species level using the visual taxonomic assignment, the molecular approach achieved a slightly higher degree of identification. The improved identification was brought about by identification of members from the family *Callionymidae* that could not be identified to species level using the visual approach. As a result, seven more larval species were identified when the molecular method was used. However, the most striking difference between the two approaches was in the degree of identification of fish eggs. Using the molecular taxonomic approach 94% of the eggs could be assigned to species level, which is congruent with previous studies using similar methods (Gleason and Burton 2012; Lewis et al. 2016). DNA barcoding is highly dependent on the sequences deposited in the databases, but in our study, for all barcodes DNA sequence similarities were >99%. On the other hand, the eggs analyzed using the visual taxonomic method were grouped according to the different classifying parameters such as egg size or presence and number of oil globules. Yet, only the eggs of *H. platessoides*, *M. muelleri*, and the family *Callionymidae* could be identified to species and family level. This is due to their unique morphometric features that stand out from other eggs, such as the particular large perivitelline space and the large egg diameter in long-rough dab, and the special sculptured chorion with one oil globule in pearlside (Munk and Nielsen 2005). Additionally, although some of the *S. scombrus* eggs could be identified using the visual taxonomic method, the considerable higher number obtained in the molecular barcoding samples indicate that it is likely that most of the *S. scombrus* eggs remained unidentified using the visual taxonomic approach. Therefore, it was possible to group the eggs according to their characteristics, but it was not possible to assign them to any of the several species present

within each group. The identification limitations are due to overlapping in one or several of the classifying parameters both in different species and families (Markle and Frost 1985).

Traditionally, the main criticism of using the DNA sequencing technology for ecological applications has been associated with high economic laboratory cost and protracted analyses time. In our study, using the molecular taxonomic method, we identified between 100 and 150 individuals per working day (one individual identified every 3–4 min), which agrees with previous findings (Richardson et al. 2007). While using the visual taxonomic approach, we identified one individual every 2–3 min on average. Furthermore, during recent years molecular barcoding methods have developed toward becoming both more time and cost efficient (Gleason and Burton 2012; Nobile et al. 2019). In fact, the costs per individual analyzed in our study were similar to figures found in recent literature, estimated to be around $4 (Gleason and Burton 2012). However, it should be noted that the sequencing step in this study was performed at an external facility which increases the cost.

Even if visual methodology allows determination of ichthyoplankton developmental stages, sample processing requires taxonomists with sufficient level of competence only achievable through considerable time and training. In some cases, it takes years of experience, to specialize in a specific genus or family to identify ichthyoplankton. In contrast, molecular analyses are more generic and amenable to automation, making access to personnel who can undertake the analyses less challenging. Despite molecular barcoding only revealing taxonomic identity, it has been proven to be an invaluable technique to assign newly discovered fish eggs to their species (Rathnasuriya et al. 2019).

Ideally, samples should be staged by visual taxonomic analysis and determined to species using molecular barcoding. Unfortunately, samples analyzed by the molecular method are most often preserved in ethanol which is efficient for preserving DNA but unfortunately causes pigmentation loss and shrinkage in eggs and larvae, making them unsuitable for morphometric analysis (Goodsir et al. 2008; Espeland and Sannæs 2018). Likewise, samples for visual identification are generally preserved in formalin which do not modify the eggs or larvae size, but it is harmful for human health and damages the DNA, preventing the use of the samples for molecular barcoding (Skage and Schander 2007; Sawada et al. 2008). To overcome this issue, a previous study used a modified formalin solution to preserve the samples. Although, a positive amplification of up to 80–85% of eggs for restriction fragment length polymorphism (RFLP) analysis was obtained, it was restricted to only three different Lotidae and Gadidae species (Lelievre et al. 2010).

Overall the results clearly demonstrate the advantages of molecular barcoding over the traditional visual approach for identifying ichthyoplankton to species level. Even though the molecular method exhibited a higher number of different species for larval identification, this advantage become considerably greater when fish eggs are taken into consideration. The results

from this study can be extrapolated to any other region as long as a reliable barcode database has been developed. In studies where developmental stage is desired, and in regions lacking comprehensive barcode database coverage, or where the taxonomic rank can be higher than species rank, the traditional taxonomy is invaluable. However, we strongly recommend implementing DNA barcode analyses as a routine for at least a subset of the samples in ichthyoplankton monitoring programs, especially when focusing on fish eggs, to provide a more accurate species identification.

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