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Integrating molecular identification of pelagic eggs with geostatistical mapping to improve the delineation of North Sea fish spawning grounds

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Abstract :

Maps of the spawning grounds of commercially important fishes are necessary when assessing the level of connectivity between life stages of fishes and for identifying ecologically valuable marine areas. A first step toward mapping the spawning grounds is a reliable and rapid species identification of pelagic fish eggs to assess the spatio-temporal distribution of spawning aggregations. As many species have similar egg sizes and morphology, the molecular validation of visually identified eggs is often essential for the use of such data in fisheries management. In the present study, we developed a rapid 16S rRNA PCR-restriction fragment length polymorphism (RFLP) assay to distinguish between formalin-fixed fish eggs of dab *Limanda limanda*, flounder *Platichthys flesus* and pout *Trisopterus* spp., which were collected during the 2008 International Bottom Trawl Survey in the Eastern English Channel and southern North Sea. A comparison of the rapid 16S rRNA PCR-RFLP method with initial visual identification revealed 93% of correct identifications for dab, 90% for pout, but only 64% for flounder, representing an overall error rate of 17%. Visual misidentification occurred mainly between dab and flounder and between flounder and pout. Egg abundance and the relative proportions of each species were subsequently analysed geostatistically. Molecular identifications were incorporated to obtain corrected interpolated distribution maps, taking into account the results from molecular identifications as a correction factor. This highlighted the distinct spawning grounds for the 3 studied taxa and facilitated the identification of regions of high conservation value for these species.

Keywords : Fish eggs ; PCR-RFLP ; 16S rRNA ; Geostatistical analyses ; Distribution ; Spawning grounds

1. Introduction

Synergy between environmental changes and increasing anthropogenic pressure has led to a dramatic decline in biodiversity in the world's oceans and coastal areas. The obvious over-exploitation of several fish stocks has prompted authorities to act on an urgent improvement of species, biodiversity and marine habitat management and protection. A global strategy, advocated by the scientific community, is the instigation of ecosystem-based, sustainable sea use management (Jennings *et al*., 2001). Such an approach necessitates spatially explicit management decisions that focus on a specific ecosystem and the range of anthropogenic and natural factors affecting it. Marine protected areas (MPAs) have received great attention because of their potential benefits for the conservation of biodiversity and are increasingly being studied for the purpose of fishery management. In order to preserve the resilience of fish populations to natural or human disturbances and to promote their regeneration capacity, efficient MPA networks need to be designed to match the populations' larval dispersal patterns (Hilborn *et al*. 2004). Hence, a detailed knowledge of spawning aggregations (goal of the study) and larval transport rates may improve the spatial management of these species (Christensen *et al*. 2009).

The Dover Strait, a narrow corridor at the junction of the English Channel and the North Sea, is experiencing heavy shipping traffic and fishing pressure. The local commercial fishery, mainly operated by France, the United Kingdom, Germany, The Netherlands and Belgium, benefits from a high species richness and fish abundance. It has resulted in a serious overexploitation of stocks over the last decades (Daan, 1997; Greenstreet *et al.,* 1999; Kjærsgaard *et al*., 2009). Consequently, the exploitation of most major commercial species stocks in the North Sea is now mainly based on the first-year classes of the population. Yearly fluctuations in recruitment have a high impact on the demographic stability of harvested fish populations and recruitment success is becoming a fundamental parameter for fisheries management. The dispersive egg and larval stages are critical phases in the fish life cycle, subjected to high mortality due to predation and unpredictable environmental conditions. As a result, spawning grounds are considered sensitive habitats, both in terms of ecosystem functioning and fishery activities. Hence, it is essential to identify, investigate, and subsequently protect spawning grounds of high ecological value, to improve the management of fish stocks and to maintain appropriate population levels. The reliable identification of early life stages is pivotal to monitoring the spatio-temporal species composition and to map the relative densities of egg/larval aggregations during a spawning season. This has unfortunately been impossible because of the similarity of egg sizes and morphology. Thus, molecular validation of visually identified eggs is required to exploit such data in fisheries management.

Many fish species spawn during winter in the North-East Atlantic; such as for instance the Gadids cod (*Gadus morhua*), whiting (*Merlangius merlangus*), three species of *Trisopterus* (*T. luscus, T. minutus* and *T. esmarkii*), and the flatfishes dab (*Limanda limanda*) and flounder (*Platichtys flesus*).

Fish eggs are generally identified by morphological criteria under a binocular microscope. Many eggs, however, such as those of dab and flounder, have the same range of size. Early stages are therefore very difficult to identify visually (Taylor *et al.*, 2002; Bayha *et al.,* 2008). A range of molecular tools (mainly used for the identification of processed fish products) have often proven useful for the identification of fish. (for a complete review, see e.g. Teletchea, 2009; Puyet and Bautista, 2010, Rasmussen and Morrissey, 2011). These molecular tools allow for a rapid and reliable identification of all fish life-history stages from eggs to adults (Karaiskou *et al*., 2007). While later life stages can be recognized easily under a microscope, such methods are particularly useful for the identification of fish eggs, where size and shape can greatly overlap at sampling.

The main problem regarding the genetic identification of fish eggs is the preservatives used for the fixation and conservation of egg samples. Many preservatives may be used, but to be visually identified, eggs are usually fixed and preserved in formaldehyde. As reported by many authors (Chang and Loew 1994; Skage and Schander, 2007; Sawada *et al*., 2008; Akimoto *et al.,* 2002), this method of fixation may cause fragmentation or alteration of the DNA, thus endangering DNA

analysis. Moreover, the duration of formalin fixation may also affect the quality of the extracted DNA, especially for pelagic eggs at the earliest developmental stages. This becomes clear in the studies of Akimoto *et al.* (2002), who only managed to amplify 6 out of 10 formalin-fixed pelagic eggs belonging to the genus *Beryx,* on a shorter region of 125 bp for the 16S rRNA gene. Similarly, when Karaiskou *et al.* (2007) used a PCR-RFLP method based on a 370-bp fragment of cytochrome b to identify the eggs of European horse mackerel species, they reported that egg identification was more successful for ethanol-preserved eggs compared to formalin-preserved eggs. However, Perez *et al*. (2005) demonstrate the possibility of DNA extraction with formalin fixed hake (*Merluccius merluccius*) and megrim (*Lepidorhombus whiffiagonis* and *Lepidorhombus boscii*) eggs.

In the present study, the fixation solution was shown to be efficient at an improved percentage of formalin below 1%. The storage solution for formalin-fixed eggs did not contain formalin at all in order to avoid DNA damage. Thus, chub and spotted mackerel formalin-fixed eggs were successfully identified based on a 320 bp region of the cytochrome *b* gene (Sezaki *et al.*, 2001) and using a PCR-RFLP approach. A PCR-RFLP method on a cytochrome *b* fragment gene was also recently specifically developed for formalin-fixed eggs to distinguish cod, haddock and whiting, while comparing results with visual identifications (Lelièvre *et al.*, 2010). In the latter study, eggs were formalin-fixed in a less than 1% formalin buffer and stored after 2 to 3 weeks of fixation in a storage buffer without formalin to perform further DNA based analysis. Based on this latter study, we chose to preserve fish eggs in these fixation and storage buffers in our study to visually pre-sort eggs before performing DNA-based egg species identification. So, in this present study, we initially focused on the reliable identification of formalin-fixed eggs of dab, flounder and a group of three *Trisopterus* species whose eggs are very similar and difficult to distinguish visually. We first designed a PCR-RFLP assay based on a 16S rRNA gene region to identify a representative sub-sample of the species' egg. After evaluating the species identification success for both the visual and the molecular methodology, we used the inferred relative proportion of each species to perform geostatistical analyses to obtain accurate interpolated maps of spawning grounds. Maps based solely on molecular data were used to improve the accuracy of the maps obtained by visual identifications and to enable a final comparison of spawning habitat using all collected samples.

2. Material and methods

2.1. Data collection and sampling

The present study is based on data collected in 2008 (Fig. 1). Fish egg samples were collected during the International Bottom Trawl Survey (IBTS) organised during the first quarter (January-February) of the year by the International Council for the Exploration of the Sea (ICES). The goal of this survey was to estimate the abundance and recruitment level of the main commercial fish species exploited in the North Sea. The French survey was organised by Ifremer in the Southern part of the North Sea. During this survey, muscle tissue of adult fishes of the target species was also collected (stored in 70% ethanol) from bottom trawl samples to obtain standard references with reliable identification and to assess the likelihood of that specific species spawning in the analysed region.

Fish egg samples were collected using a Continuous Underway Fish Eggs Sampler (CUFES, Checkley *et al.* 1997; Checkley *et al.*, 2000; Zwolinski *et al.,* 2006), a sampling method proven to be efficient in the North Sea during winter (Lelièvre *et al*., in press). During sampling, water was pumped continuously from 5 meters below the surface and filtered over a mesh size of 500 μm. Samples were collected every 30 minutes. For each sample, the volume of filtered water was determined using both sample duration and pump flow. Samples were fixed in a 0.9% buffered formalin seawater solution and transferred to a storage solution without formalin after two to three weeks of fixation (see Lelièvre *et al.,* 2010 for details). A spatially representative subset of 38 samples was selected for molecular identification and validation. Where there were too many eggs per sample a maximum of 10 were selected for analysis.

2.2. Microscopic identification of fish eggs

Fish eggs were identified at the species level under a binocular microscope. The microscopic identification was based on variables such as size, the presence of oil globules and the degree of pigmentation (Russell, 1976; Munk and Nielsen, 2005). The absence of sexually mature adults of a given species in neighbouring trawl hauls helped rule out specific species during the taxonomic identification of eggs in the CUFES samples. If a species was not present in the nearby trawls, the presence of this species' eggs was considered unlikely. Different developmental stages could be identified. Corresponding to stages IA and IB, there was no embryo at stage 1. At stage 2, which corresponds to stages II and III, an early embryonic development could be observed. At stage 3, which corresponds to stages IV and V, a well-developed embryo could be found, as described in Thompson and Riley, 1981. Only eggs of the youngest stage 1 were used in the present study to reliably identify the location of the spawning grounds after their recent laying.

Given that many species spawning in the Eastern English Channel and the southern North Sea exhibit the same egg size range during the studied period (Table 1), we have grouped the different sampled eggs into three groups based on morphological traits. Also, no misidentification of the egg species was possible between groups but very likely within each group: group 1 includes eggs of three rocklings species (*Ciliata mustela*, *Ciliata septentrionalis* and *Enchelyopus cimbrius*); group 2 includes eggs of other Gadidae species such as cod, haddock and whiting, which were the subject of a previous study (Lelièvre *et al.*, 2010). Group 3, including dab (*Limanda limanda*), flounder (*Platichthys flesus*) and *Trisopterus sp*., is the topic of this paper.

Overall 1,050 station samples (with approximately 52,000 dab and 4,107 flounder eggs at stage 1 and 212 *Trisopterus* species eggs at later stages respectively) were identified by binocular microscope during the 2008 IBTS sea survey. A subset of 353 eggs originating from 38 samples were analysed genetically after visual identification. 226 eggs were initially labelled as *Limanda limanda*, 64 eggs as *Platichthys flesus* and 63 eggs as *Trisopterus* sp.

2.3. DNA extraction and amplification

DNA from adult fish muscle tissue was extracted using a modified phenol/chloroform/isoamyl (PCI) alcohol extraction method (Jérôme *et al.*, 2003). The low DNA yield of this extraction method on the fish eggs required the use of a second method based on magnetic bead technology (ChargesSwitch® Forensic DNA Purification Kit, Invitrogen, Carlsbad, USA). Eggs were labelled after microscope identification and the total DNA was extracted from individual egg tissue as proposed in Lelièvre *et al.* (2010).

The 16S rRNA gene fragment from each species was amplified using two primers 16S-F (5' CAAGCAGAGAAGACCCTATGG) (C. Lemaire, unpublished) and 16S-R-univ (5'GGTAACTCGGTTCGTTGATCGG), specifically designed for this study. The PCR reactions were carried out in a total volume of 50 µL including a 1.25 µL DNA template, a 0.2 mM dNTP (Interchim), a 2 mM $MgCl_2$, a 10 µL 5X buffer, 1.25 units of GoTaq® polymerase (Promega), 0.2 mM of each primer, molecular biology grade water (Interchim) was added to adjust to the final volume. The Polymerase Chain Reactions were performed in a MyCycler™ thermocycler (Biorad). The PCR cycle consisted of a five-minute preheating step at 95 °C, 35 cycles of amplification (95°C for 30 s, 48°C for 30 s, 72°C for 40 s) and a final seven-minute extension step at 72°C.

The DNA amplification was checked on a 1.5% agarose gel (Interchim) using a TAE buffer (2 mM EDTA, 40 mM Tris acetate, pH 8.5). Band visualization was carried out using GelRedTM (Interchim) via ultraviolet trans-illumination (Image Master VDSCL, GE Healthcare) and 1D Elite software (GE Healthcare). The size of the expected PCR products was estimated using the MassRuler™ 100 bp DNA ladder range (MBI Fermentas).

2.4. DNA sequencing of reference adult DNA

Before sequencing, double-stranded PCR products were purified by a simple bind-wash-elute procedure on a silica membrane (*QIAquick* PCR Purification Kit, Qiagen) according to the manufacturer's protocol. PCR fragments were used for direct cycle sequencing with the dye terminator cycle sequencing kit (Beckman) following the manufacturer's protocol. A Beckman Coulter CEQ 8000 DNA sequencer was used for sequencing analysis in both directions with the primers used for PCR amplification. The DNA sequences were edited with BioEdit software (Hall, 1999). The same primers and amplification conditions were used for all fish eggs before RFLP analysis.

2.5. RFLP of the 16S rRNA gene fragment

Restriction maps of a total of 40 16S rRNA gene sequences (31 sequences retrieved from the GenBank and new sequences obtained from 2 individuals per species, except for *T. esmarkii*, for which only one individual was sampled in this study) were generated using the FastPCR software (Kalendar *et al*., 2009). The AciI restriction enzyme (whose recognition site is 5'C/CGC) was selected, based on its ability to generate characteristic restriction profiles for each species with band sizes easily distinguishable on agarose gels. Restriction was carried out at 37°C for 4 h with a 20 µL reaction mixture containing 10 µL of PCR products and two units of the AciI enzyme in the buffer recommended by the manufacturer (Ozyme). The reaction was stopped by heating at 65°C for 20 min. The DNA restriction fragments were separated in 50 minutes by electrophoresis at 90V, on 3% agarose gels as described above. The DNA fragments with sizes below 50 bp generated in the restriction digestion were not used for the identification because they could not be reliably scored.

2.6. Geostatistical analyses and interpolated maps

Many fish populations exhibit particular distribution and aggregation patterns at different times of the year as well as in different phases of their life cycle (Mello and Rose, 2005; Petitgas, 1993; Petitgas, 2001, Vaz *et al*., 2005). Geostatistics, which represent a suite of methods for analysing spatial data, were used to identify and describe quantitatively the spatial distribution of each species' eggs (Matheron, 1962; Webster and Oliver, 2001). Geostatistics can also be applied to the estimation of values of variables of interest at non-sampled locations from more or less sparse sample data points based on spatial auto-correlation between these points. Geostatistical estimation is known by the general term kriging.

In order to enable distance calculation from angular coordinates, the longitude correction ($\text{longitude}\times\cos((\text{latitude}\times\pi)/180)$) was used to transform decimal degrees of longitude into decimal degrees of latitude, which are of constant distance.

Geostatistics does not require full normality, but it performs better if the distribution approaches normality as much as possible. Egg abundance data were tested for normality using histograms, skewness and kurtosis. The data were transformed when the skewness value exceeded |1| and/or kurtosis exceeded 1 and when a normalising function improving the data distribution was found. As a result, egg abundances were log-transformed ($\log 10(x+1)$, with x being the abundance), to reduce data skewness.

The variogram, the central tool of geostatistics, is a function that measures the relation between pairs of observations, which may be any distance apart. It is a model of the spatial auto-correlation pattern of the variable of interest that summarises how the variance of a variable changes as the distance and direction separating any two points varies. Therefore, the variogram is a plot of the mean variance of each pair of points against their separation distance, often referred to as the

experimental variogram. Typically, variance increases with the separation distance between the observations until it reaches a maximum called “sill variance” which corresponds to the maximum variance of the data. The lag distance at which the sill is reached (the range) marks the limit of spatial dependence and indicates the average diameter of patches (Webster and Oliver, 2001). The variogram has a positive intercept on the ordinate axis, called “nugget variance”, which is the amount of variance not explained by the spatial model which mostly arises from variation occurring over distance smaller than the sampling interval. An experimental variogram was computed for each fish species and for both visual and molecular identification data. These experimental variograms were fitted by non-linear models.

Four variogram models (exponential, circular, spherical and pentaspherical) were visually and statistically adjusted to the experimental variogram using the least-square regression. The one with the best visual and statistical fit with the experimental variogram was retained as the theoretical variogram and was used to estimate the nugget, sill and range parameters that characterise the shape of the variogram. The model retained may therefore differ for each dataset.

Local trend or drift violates some basic assumptions of geostatistics because the values change in a smooth and predictable way. Variables were examined for trend at the outset by fitting a low-order polynomial (linear or quadratic regression) on the spatial coordinates (Webster and Oliver, 2001). When the fitted function accounted for over 20% of the variance, the variogram was computed using the abundance data residuals and compared to the variogram of the original data.

Furthermore, the amount of spatial structuring (Q) explained by the variogram was estimated as the variance attributable to spatial dependence (C) divided by the sill variance (i.e. the maximum variance in the data calculated as the sum ($C + C_0$), C_0 being the nugget variance). The nugget was the amount of variance not explained by the spatial model and arising mostly from variation occurring over distances smaller than the sampling interval. High values of Q (up to a maximum of 1) indicated that the variable distribution was strongly structured in space. Finally, the estimated nugget, sill and range parameters were used to interpolate egg abundances on the mesh of a regular grid by using interpolation methods known as ordinary kriging or universal kriging in the case of a spatial drift.

Geostatistical analyses were performed using GenStat software (GenStat Release 7.1., 2004). The resulting interpolated values were imported into a geographical information system using the ArcMap 9.1 (ESRI) software. The program was used to produce continuous grids of 0.008 decimal degree resolution, displaying the spatial patterns of each variable. Extrapolated areas (located outside the study area) were discarded from the maps.

Egg abundance was transformed into its original units to enable the computation of the total egg abundance maps. The total abundance maps were multiplied with molecular maps to have final corrected maps for each species. The resulting maps were then log-transformed again for mapping purposes. This approach has already been successfully used for cod, haddock and whiting (Lelièvre *et al.*, 2010).

3. Results

3.1. PCR-RFLP analysis

The amplification of the 16S rRNA gene produced a fragment of 254 bp for dab and flounder and a fragment of 238 bp for pout species due to a 16-nucleotide deletion. Sequence analysis of 9 sequences obtained from this study from reference samples (2 adults specimens per species except for *T. esmarkii*) (data not shown) combined with 31 sequences retrieved from the GenBank (11 dab sequences, 14 flounder sequences and 6 pout species sequences) has indicated that this fragment was polymorphic enough to discriminate between dab, flounder and pout after digestion with only the AciI enzyme. And the DNA fragments generated after the digestion (114, 81 and 59

bp for *Limanda limanda,* 173 and 81 bp for *Platichtys flesus* and, 126 and 112 bp for *Trisopterus sp)* were compatible with the expected size on the basis of the restriction map (Fig. 2). The three *Trisopterus* species could not be distinguished with AciI on the studied 16S rRNA gene fragment, as they all displayed the same restriction profile with this enzyme except for 3 *Trisopterus minutus* GenBank sequences (individuals sampled in the Mediterranean). Since in term of abundance and commercial interest the three pout species are less important species than dab and flounder, we focused on the distinction of dab, flounder and the whole *Trisopterus* genus.

3.2. Molecular analyses validation

Among the 353 eggs extracted, 164 were successfully amplified (46%). Ten (out of 38) sites from the study area were not analysed due to a low DNA yield or a lack of amplification from egg extracts. All stations analysed were spread evenly across the study area, with the exception of the Belgian coast where no samples of sufficient quality could be analysed. In total, we discovered 28 visual identification errors out of the 164 eggs analysed (17%), spread across 11 sites (39% of the analysed stations) (Fig. 3). Of the 107 eggs visually identified as dab based on morphological criteria, 99 eggs were confirmed as dab by molecular analyses (92.5% correct identification, Table 2). Of the 47 visually identified flounder eggs, 30 were correctly identified by molecular analyses, while five were in fact *Trisopterus* sp. and 12 were dab eggs (Only 64% correctly identified). Of the ten *Trisopterus* sp. eggs analysed by molecular analyses, seven were correctly identified by visual method and three were in fact flounder eggs (70% correct identification). These results revealed that visual identifications of flounder and possibly Trisopterus *sp*. may be problematic and effetively require validation by molecular technique.

The relative error ratio per station was analysed for each species. It confirmed a good visual identification for dab and *Trisopterus sp*. with a mean error of 0.04 and a median of 0. For the latter species, however, these results should be regarded with caution because of the low number of eggs analysed. Flounder presented a higher error rate with a mean of 0.37 and a median of 0.29. Most of the time errors in flounder identification were due to the confusion with dab (71%), compared to a 29% confusion with *Trisopterus* sp. However, no confusion between dab and *Trisopterus* sp. was found to have been made.

3.3. Interpolated map

Geostatistical analyses were applied to both egg abundance data (for each species identified morphologically using all available samples i.e. 1,050 observations) and egg relative abundance data (for each species identified genetically in each sample using a subset of 28 successfully analysed observations) (Table 3). Generally, the extent of the spatial structure (range) was comparable for each species based on visually identified data (about 1° i.e. approximately 115 km). However, the molecular data revealed a much more structured spatial distribution (larger Q values) and more variable patch extents (from 0.6° for flounder to 1.7° for pouts) (Fig. 4). The relative occurrence maps (Fig. 5) of each species were multiplied with abundance maps of the considered group. These were obtained by visual identification in order to produce distribution maps of the spawning ground for each species in the entire studied area (Fig. 6).

Although the three species covered a large proportion of the studied area, molecular analyses revealed that each species seemed to have more distinct spawning grounds. Corrected maps (Fig. 6) showed that dab spawned over a large extent mainly in the centre and offshore parts of the studied area, whereas flounder seemed to prefer more coastal areas near the French, Belgian and Dutch coasts. The distribution of the *Trisopterus* species was unclear because of the very low sampling size, pointing to a lack of large spawning aggregations at the time of sampling. Still, an aggregation of *Trisopterus* eggs was observed near the Belgian coasts and another one was observed offshore near the Dogger Bank. However, due to the lack of appropriate samples along the Belgian coast, the distribution patterns in this area are based on interpolation from adjacent areas and probably need verification. Corrected maps were very similar to maps from visual data

(not shown) confirming the relatively reliable identification by microscope. Generally, however, molecularly corrected maps showed that fish egg distribution was much more restricted than visual data maps had illustrated. They revealed that the distribution of each species' spawning grounds was much more spatially restricted than initially expected.

4. Discussion

The detailed mapping of breeding grounds for exploited fish species is a prerequisite for sustainable management practices and for the protection of ecologically important marine areas. The reliable assessment of species diversity within and connectivity patterns between spawning grounds requires fine scale sampling efforts and powerful pelagic egg identification methodologies. Additionally, the integration of such data into geostatistical mapping analyses enables the identification of temporally stable spawning aggregations requiring protection and good management. The problem of visual fish egg identification can easily be solved through molecular techniques (but at a cost), resulting in high confidence about the timing and location of (over) exploited fish species' spawning grounds. Our results demonstrate that a limited number of DNA-based egg identifications is sufficient to enable large sample sizes and good geographical coverage of sampling due to the integration of molecular data and geostatistical analysis.

4.1. Molecular identification and technical issues in fish egg identification

For formalin fixed samples containing a low concentration of template DNA, mitochondrial DNA is often used in molecular analyses since it is more resistant to deterioration than nuclear DNA, due to its small size, circular structure and the protection offered by the cellular compartment of the mitochondrion. Moreover, near one hundred mitochondrial genomes are present in eggs, each containing around ten copies of the DNA (Hunter *et al.*, 2010). The mitochondrial markers mostly used for species identification are the genes encoding cytochrome *b* and 16S rRNA. Due to their high nucleotidic substitution rate enabling rapid identification, both markers have often been used for the differentiation of fish eggs (Aoyama *et al.,* 2000; Karaiskou *et al.*, 2007).

Identification based on RFLP is however highly dependent on the distinctness and intensity of the PCR amplification products, the electrophoresis gels. The PCR must be optimised to lead to sufficiently discriminating restriction profiles after enzymatic digestion. In this study, some difficulties were encountered during the amplification of fish egg DNA, and particularly first stage (earlier egg without embryo) egg DNA. The variability of amplification yield between eggs could thus be mainly due to the low quantity of DNA in some eggs. Furthermore, the high lipid content of fish eggs could reduce the DNA retrieval and increase residual substances after the DNA extraction which could inhibit PCR amplification (Aranishi, 2005). The use of buffered formalin, known to induce DNA fragmentation and nucleotide alterations may explain the observed PCR yield variability (Chakraborty *et al*., 2006; Palmer, 2009). However in our study, eggs were only fixed in buffered formalin (< 1%) for 2-3 weeks. The eggs were then, stored in a conservation solution without formalin. This method of fixation and storage had been applied successfully before (Lelièvre *et al*., 2010). Overall, the observed identification errors were mainly due to egg size and spawning area overlapping between species. Therefore, the PCR-RFLP technique applied on the 16S rRNA gene provided an accurate and reliable method of identification of dab, flounder and pout species' fish eggs.

4.2. Comparative mapping of the spawning grounds of dab, flounder and pout

This study shows that dab and flounder exhibit two distinct spawning areas. Our results are concordant with earlier studies documenting the spawning grounds of dab by Van der Land (1991) and Rijnsdorp *et al.* (1992). Dab spawning occurs throughout the south-eastern North Sea with offshore concentrations of eggs in the German Bight, north of the Frisian Islands, along the

southern edge of the Dogger Bank and northeast of Flamborough Head (Harding and Nichols, 1987). These patterns were confirmed by the present results and were also in agreement with data from Ehrenbaum (1905-1909) and Bohl (1957). It can be concluded that dab has a strong spawning site fidelity. Flounder is located mainly inshore, near the Dutch coasts but also near German and Danish coasts. Van der Land (1991) found that most flounder eggs were observed in the area west and northwest of the Dutch west coast, the eastern English Channel and the area northwest of Helgoland, confirming that flounder has a well-defined spawning period and area. Following Quéro and Vayne (1997), *Trisopterus luscus* and *T. minutus* seem to be located inshore, in the English Channel but mainly near the French Atlantic coasts, between the Gironde Estuary and North of the Loire River, as well as along the Vendée coast. The southern distribution border of *Trisopterus esmarkii* lies around the Dogger Bank (Hessen *et al*., 2005). However, the low densities observed in our survey, restrains us from drawing any conclusions on the distribution of *Trisopterus* spawning grounds. Overall, our results highlight the importance of the south-eastern part of the North Sea as the main spawning ground for the species investigated in this study as well as many more (Lelièvre *et al*, 2010).

4.3. Reliable ichthyoplankton surveys

Maps of fish spawning grounds can be based on various sources of information including the occurrence of mature fish in trawl surveys (Hedger *et al*., 2004) and for some species acoustic records of calls made during courtship (Širovic *et al*., 2009; Hawkins *et al*., 2002). For pelagic spawners, plankton surveys provide another useful source of information (Checkley *et al*., 1997; Fox *et al*., 2008). Since eggs will diffuse away from spawning sites, it is desirable to identify the species at their earliest developmental stages. Ichthyoplankton surveys are a powerful tool to monitor spawning areas when taxonomic identification is reliable enough (Fox *et al*., 2005). Although many eggs can be visually identified to the species level based on their morphology, this is not always the case as several co-spawning species produce eggs of similar appearance and size. Recent advances in applying molecular genetics to plankton samples, including the present study, have however largely overcome this limitation (Fox *et al*., 2008; Goodsir *et al*., 2008, Lelièvre *et al*., 2010).

Given the cost involved in molecular analysis, such analyses should only be performed after a validation test on eggs with uncertain identification and on a sub-set of collected samples. Such an approach, ensuring that visual identification is fine-tuned and corrected with molecular results, ensures a cost effective and accurate use of these data.

To achieve the objective of international and European conservation of the marine environment, countries must implement a coherent network of marine protected areas (MPAs) in their territorial waters by 2012. In Canada, marine protected areas (MPAs), are considered to be an important tool for fisheries management (Côté and Finney, 2006). Indeed, several studies have shown that MPAs can achieve the objectives of conservation and fisheries management by increasing biodiversity, restoring population structure and the dynamics of stocks and by facilitating the establishment of stable and productive ecosystems (Fisher and Frank, 2002, Gell and Roberts, 2003, Roberts *et al*. 2005, Kraus *et al*. 2009). The closure of Georges Bank (1994) was an example of a measure directed at protecting the yellowtail flounder (*Yellowtail founder, Limanda ferruginea*) and other Gadidae species. This closure has resulted in an increase of cod biomass and also affected other species such as scallops for which biomass has also increased. The protection zone had a positive effect on the habitat and the productivity of the ecosystem by reducing mortality due to fishing. Indeed, after 4 years of protection, in 1998, the scallop population had multiplied by 14; Individuals' biomass and size had also increased. The cod stock increased by 18%, that of yellowtail flounder by 80% and that of the haddock was completely restored.

Some MPAs were designed to protect spawning areas where spawning aggregations are the main target of commercial fisheries. However, in the evaluation of areas for protection it seems important to take into account not only the spawning grounds but also migration and nursery. A detailed knowledge of the aggregation and transport processes of larvae may therefore improve

the design of marine protected areas (Christensen *et al*. 2009) for the ultimate benefit of the fishery industry.

Thus, the knowledge on spawning grounds can directly contribute to the sustainable management of fisheries' resources and managers may decide to prohibit the use of a given area to protect spawning grounds in order to maintain productive fishery. But also, fisheries management can limit trawl mesh size to protect adults and to ensure they have the opportunity to reproduce at least once (Fuiman and Werner, 2002).

5. Conclusion

The various phases of a fish life cycle are distributed across different spatial structures and scales. Indeed, reproducing adults are encountered at specific and distinct areas, differing from the spatial distribution of eggs, larvae and juveniles, mainly found in nursery areas.

Spawning areas are essential and of special interest because they are considered a key habitat for a species' resilience to harvesting. Moreover, suitable spawning habitats will strongly affect the recruitment success (Borja *et al*., 1998, 2008) and, consequently, the demographic stability of a population. (Bellier *et al*., 2007). Hence, the location, quality and size of spawning grounds are essential factors influencing the spatial and temporal dynamics of fish populations. We have shown that the application of molecular analyses on fish eggs exponentially increases the accuracy of spawning maps and, ultimately, will enhance our understanding of the fish population's spatial dynamics. Such knowledge is necessary to implement a spatially explicit, ecologically sound protection scheme. Indeed, the delineation and size of MPAs, which may be defined by the habitat needed for spawning or larval settlement, would require such information. This would soon have positive effects on targeted populations (Fuiman and Werner, 2002). Managers could then easily decide to close a given area or season to fishing in order to protect spawning and nursery grounds which would ultimately benefit the sustainability of the fishery.

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Tables

Table 1. Limanda limanda, Platichthys flesus and Trisopterus spp. Scientific and common names of target species, egg diameter size and spawning period

Scientific name	Common name	Egg size diameter (mm)	Spawning period
Limanda limanda	Dab	0.66–0.92	Jan–Jun
Platichthys flesus	Flounder	0.80–1.13	Jan–Jul
Trisopterus minutus	Poor cod	0.9–1.23	Feb–Jul
T. luscus	Pouting	0.9–1.03	Jan–Sep
T. esmarkii	Norway pout	1.00–1.19	Jan–Apr

Table 2. Limanda limanda, Platichthys flesus and Trisopterus spp. Absolute and proportionally correct identification

Microscope identification	Molecular identification				% of correct identification
	Dab	Flounder	Pout spp.	Microscope sum	
Dab	99	8	0	107	92.5
Flounder	12	30	5	47	63.8
Pout spp.	0	3	7	10	70
Molecular sum	111	41	12	164	

Table 3. Limanda limanda, Platichthys flesus and Trisopterus spp. Geostatistical analyses on visual and molecular data for each species. Trend fit: variation explained by the quadratic trend and removed before variographic analysis; model: variogram model retained; Q: amount of spatial structuring explained by the variogram; nugget: amount of variance not explained by the spatial model; sill: maximum variance of the data; range: approximate average diameter of patches in decimal degrees of latitude.

Species	Transformation	Trend fit (%)	Model	Q (%)	Nugget	Sill	Range (°lat)
Visual identification data (n = 1050)							
Dab	$\log_{10}(x+1)$	51.4	Pentaspherical	87.2	0.076	0.518	1.251
Flounder	$\log_{10}(x+1)$	19.6	Pentaspherical	60.7	0.096	0.148	1.013
Pout spp.	$\log_{10}(x+1)$	0.2	Circular	45.5	0.012	0.010	1.099
Molecular identification data (n = 28)							
Dab	None	8.3	Pentaspherical	70.8	0.354	0.860	1.297
Flounder	None	32	Circular	100.0	0.000	0.037	0.636
Pout spp.	None	1.6	Spherical	89.7	0.013	0.113	1.701

Figures

Figure 1. Location of the CUFES (Continuous Underway Fish Egg Sampler) samples during the IBTS (International Bottom Trawl Survey) 2008

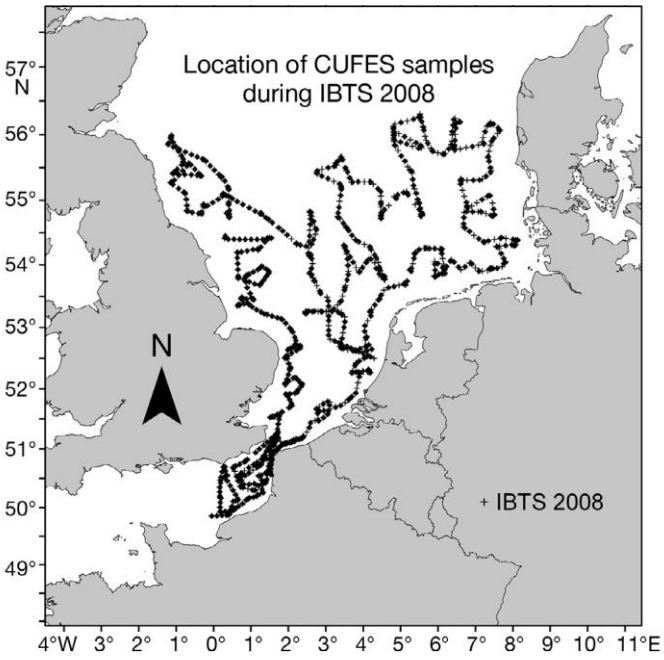

Figure 2. Limanda limanda (Ll), Platichthys flesus (Pf) and Trisopterus spp. (Tsp). Synthetic 3% agarose gel electrophoregram of DNA fragments after digestion by the AciI restriction enzyme. MW: 100 bp standard molecular weight

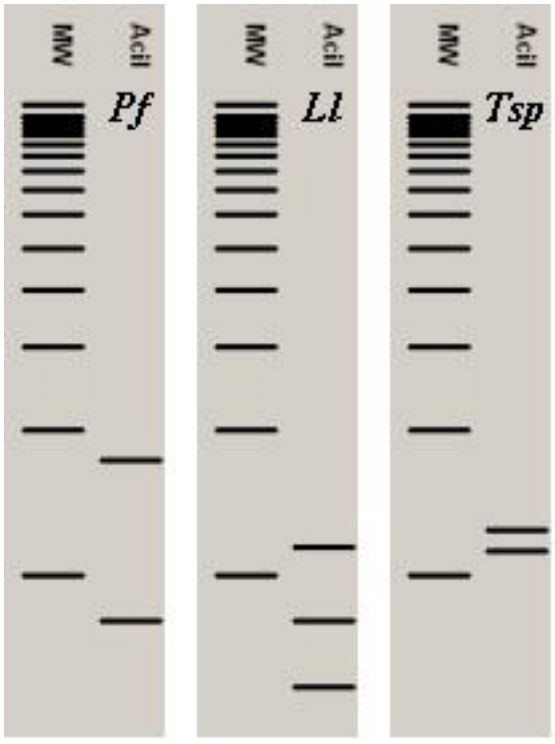

Figure 3. Limanda limanda, Platichthys flesus and Trisopterus spp. Localisation of global error identification for all species of the group studied

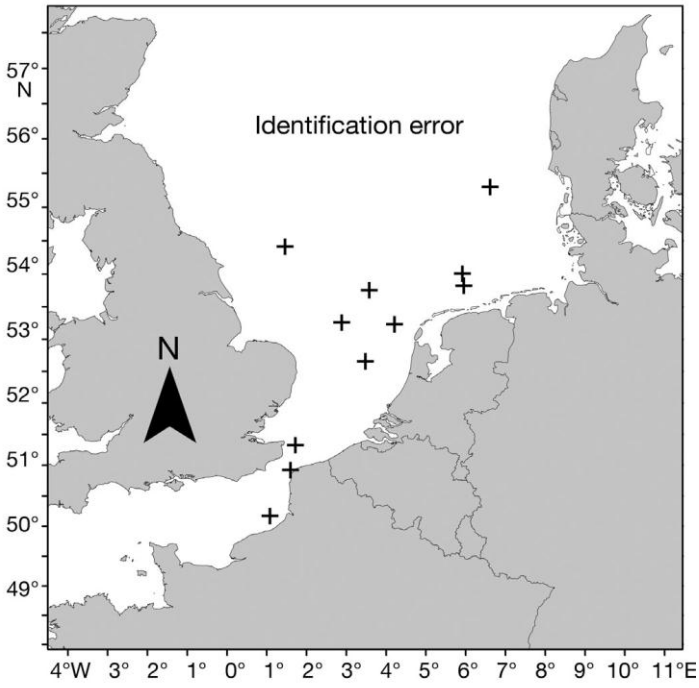

Fig.4. Limanda limanda, Platichthys flesus and Trisopterus spp. Location of eggs identified by molecular analyses

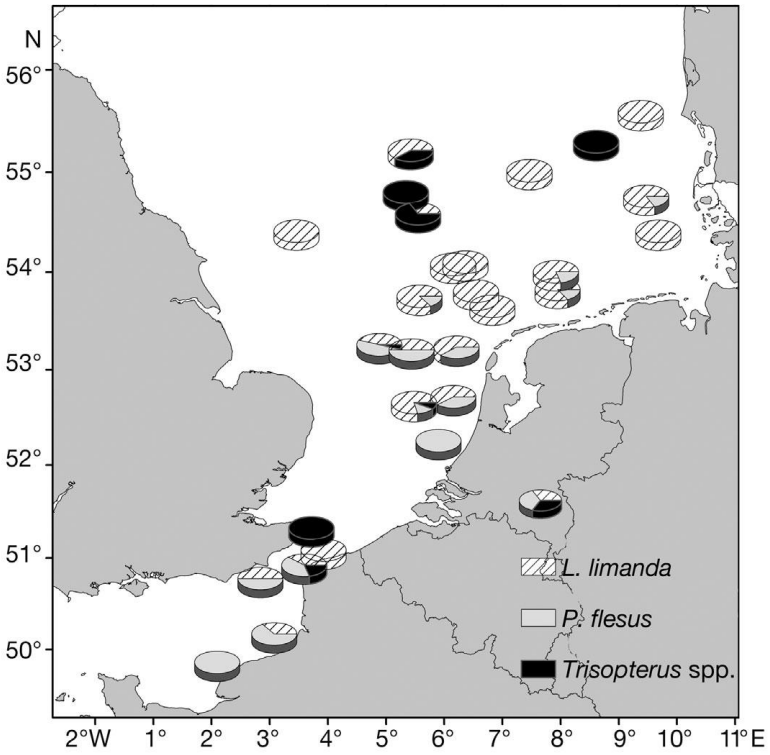

Figure 5. Relative abundance distribution of a) dab (*Limanda limanda*), b) flounder (*Platichthys flesus*) and c) *Trisopterus* species eggs identified by molecular analyses. The maps represent the interpolated relative amount of eggs of each species found in each genetically analysed sample.

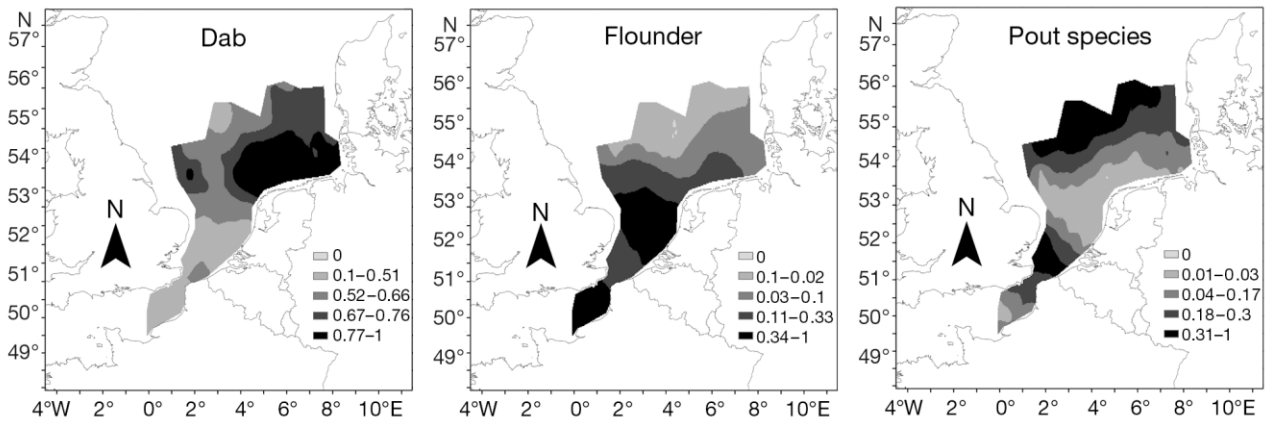

Figure 6. Egg distribution corrected by molecular analyses a) dab *(Limanda limanda)*, b) flounder *(Platichthys flesus)* and c) *Trisopterus* sp. The scale represents the log-transformed egg densities per $20m^3$