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Development of a rapid genetic technique for the identification of clupeid larvae in the Western English Channel and investigation of mislabelling in processed fish products

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A novel genetic technique, involving real-time polymerase chain reaction (PCR) and high-resolution melt (HRM) analysis was developed, which successfully identified four species of clupeid larvae from the Western English Channel and revealed the presence of herring (*Clupea harengus*). This was unexpected in a context of global warming as herring are at the southern limit of their range off southwest England, where the larvae are generally very rare. The application of the method in cases of degraded DNA, such as in processed fishery products, was also assessed, revealing a low level of misidentification or equivocal identity (8.3%). Eight-year-old formalin-preserved samples were also analysed successfully. This highlights the potentially broad applications for HRM-based approaches to species identification in marine science.

Keywords: climate, *Clupea harengus*, *Engraulis encrasicolus*, high-resolution melt analysis, *Sardina pilchardus*, species identification, *Sprattus sprattus*.

Introduction

An important requirement for fishery management is accurate species identification (Teletchea, 2009). Traditional identification of fish species is based on external morphological features, such as body shape, colour, position of fins, and number of fin rays and gill-rakers (Strauss and Bond, 1990). Discrimination based on these characteristics may be time consuming or unreliable, especially when individuals have been damaged during collection or when the samples have been industrially processed (Durand *et al.*, 2010). The identification of egg and larval stages is equally essential for studies such as assessments of spawning grounds, early life-history dynamics, and environmental relationships (Teletchea, 2009); yet these early life stages can be especially difficult to identify (Fox *et al.*, 2005).

The problem of identification is particularly acute for larval clupeids, which are morphologically very similar and can require considerable effort and expertise to distinguish species in mixed samples. As a result, identification may be inferred from other clues, such as seasonal time of occurrence (Russell, 1935). Clupeid larvae include the families Clupeidae (herrings, shads, sardines, and sprats) and Engraulidae (anchovies), which together represent about one-third of the global seafood supply (FAO, 2010). They are small pelagic schooling fish, which are highly mobile, rely on plankton-based food chains, and have plasticity in growth, survival, and other life-history traits (Munk, 1991). These biological characteristics make them sensitive indicators of environmental change (Hunter and Alheit, 1995).

Fluctuations in abundance of herring (*Clupea harengus*) and sardine (*Sardina pilchardus*, also commonly known as pilchard) in the Western English Channel have been linked to changes in the climate and sea temperature at a range of time-scales, dating

back to at least the 16th century. More recently, changes in the relative abundance of these key ichthyoplankton species off Plymouth have also been linked to periods of warming (1921–1961; 1985–present) and cooling (1962–1980) (Southward *et al.*, 1988). The steady increase in sea temperature since the 1980s of ~0.2–0.6°C per decade (MCCIP, 2008) has been related to shifts in a range of a number of marine organisms (Beaugrand and Reid, 2003; Hawkins *et al.*, 2008; Beaugrand *et al.*, 2009), including the increased abundance and spawning of anchovy (*Engraulis encrasicolus*) in the North Sea (Beare *et al.*, 2004).

Techniques using molecular markers are being increasingly investigated to enable unequivocal species identification (reviewed in Teletchea, 2009; Lago *et al.*, 2011a, b). For example, using genetic identification, Fox *et al.* (2005) found that the majority of eggs in the Irish Sea, thought to be from cod (*Gadus morhua*), were actually from whiting (*Merlangius merlangus*), leading to a previous overestimation of cod stocks. These molecular techniques include polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) (Rehbein *et al.*, 1997), PCR-restriction fragment length polymorphism (RFLP) (Jérôme *et al.*, 2003a), and species-specific primers (Shivji *et al.*, 2002). However, such approaches involve gel electrophoresis as endpoints to species identification and exhibit one or more of the following shortcomings: they are time consuming, labour intensive, insufficiently sensitive, and have a substantial probability of cross-contamination. Direct sequencing of PCR products yields the most information, but is costly, requires more time and expertise, and may not be available at all laboratories (Rasmussen and Morrissey, 2009).

Real-time PCR coupled with high-resolution melt (HRM) curve analysis is a novel, rapid, and inexpensive method that can be used for species identification. HRM has distinct advantages over other PCR methods. It is a single-tube, high-throughput technique that can detect single base differences and requires no post-PCR manipulations, thereby reducing analysis time and minimizing the chances for cross-contamination and technical error (Berry and Sarre, 2007). TaqMan DNA technology is an alternative single-tube, real-time PCR-based method that has been used for the identification of fish and fish eggs (Taylor *et al.*, 2002; Fox *et al.*, 2005). However, assay development can be expensive and is somewhat limiting, as only the species for which the probes are designed can be identified (and any other species will give a null reaction).

Molecular markers are also increasingly being used to detect fish and seafood mislabelling, a major concern in both international and local markets worldwide (Logan *et al.*, 2008; von der Heyden *et al.*, 2010; Hanner *et al.*, 2011). Seafood mislabelling occurs when one species of fish, crustacean, or shellfish is sold as another species, often a lesser valued species sold as a higher valued species. For example, a molecular study revealed that three-quarters of fish sold in the USA as "red snapper" (*Lutjanus campechanus*) were mislabelled (Marko *et al.*, 2004). Furthermore, because of high demand, it has been suggested that anchovy semi-preserves may be susceptible to substitution using closely related species such as gilt sardine (*Sardinella aurita*), sprat (*Sprattus sprattus*), and sardine (Sebastio *et al.*, 2001).

The primary aim of the present study was to develop a novel and cost-effective molecular technique to allow identification of sardine, herring, sprat, and anchovy, these being the four clupeid species that regularly occur around the UK. The method was then applied to identify clupeid larvae from the Western English Channel in the context of response to climate change, particularly with respect to herring and anchovy larvae. In order to demonstrate the potentially wide suitability of HRM in identifying marine species, a preliminary assessment of the technique was also made in cases of degraded DNA, specifically in formalin-preserved samples as well as processed clupeid products from across Europe.

Table 1. Adult fish sampled.

Species	Origin	Collectors[a]
Engraulis encrasicolus	Western English Channel	MBA, UK
Engraulis encrasicolus	Western Mediterranean	ICM, Spain
Engraulis encrasicolus	South Atlantic Ocean (Gansbaai)	DAFF, South Africa
Engraulis encrasicolus	Aegean Sea	HCMR, Greece
Engraulis encrasicolus	Adriatic Sea	ISPRA, Italy
Engraulis encrasicolus	North Atlantic Ocean (Cádiz)	IPIMAR, Portugal
Clupea harengus	Western English Channel	MBA, UK
Clupea harengus	North Sea	FRS Marine Laboratory, UK
Clupea harengus	Baltic Sea	IFM-GEOMAR, Germany
Clupea harengus	Kattegat	DTU Aqua, Denmark
Clupea harengus	Norwegian Sea	DTU Aqua, Denmark
Clupea harengus	North Atlantic Ocean (Iceland)	MRI, Iceland
Sardina pilchardus	Western English Channel	MBA, UK
Sardina pilchardus	Western Mediterranean	ICM, Spain
Sardina pilchardus	Aegean Sea	HCMR, Greece
Sardina pilchardus	Adriatic Sea	ISPRA, Italy
Sardina pilchardus	North Atlantic Ocean (Cádiz)	IPIMAR, Portugal
Sprattus sprattus	Western English Channel	MBA, UK
Sprattus sprattus	North Sea	FRS Marine Laboratory, UK
Sprattus sprattus	Adriatic Sea	ISPRA, Italy
Sprattus sprattus	Bristol Channel	CEFAS, UK

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Methods

Development of the molecular assay

Adult individuals of the four species were caught off Plymouth and used to test the extraction protocol and suitability of primers. Further tissue samples from adults across the target species' geographic range were also obtained from research institutes across Europe (Table 1). In total, 108 adult fish were analysed.

Primer design and HRM

The mitochondrial genes cytochrome *b* (cyt *b*), cytochrome oxidase I (COI), and 16S rRNA (16S) have all been successfully used in previous studies on distinguishing clupeid species (Rehbein *et al.*, 1997; Jérôme *et al.*, 2003a, b). Three sets of primers used in the above studies and seven newly designed degenerate primers (Supplementary material, Table S2) were tested with known reference specimens (Table 1) to assess their ability to distinguish the four species. The primer sets varied in their ability to discriminate between species (Supplementary material, Figure S1). The primer set CluCOI4F/R was selected as it was the most successful at separating the four species, as demonstrated by the HRM of the four target species from across their geographical range (Figure 2a). There was a slight variation in melting temperature (T_m) between runs; however, this was overcome by including a known standard for all four species. The variation across runs was adjusted to the standards, and a temperature range for each species was generated from the adult samples (Supplementary material, Table S3). This provided a baseline of melting temperatures, against which any unidentified samples could be referenced. Sixty-seven samples were run twice to verify the repeatability of the method, and every sample was identified consistently across both runs. The temperature range demonstrated by the anchovy was quite broad, and the T_m from a small number of individuals ($n = 5$) from the Western Mediterranean and Aegean Sea overlapped with that shown by herring. This high intraspecific variation may be due to the complex genetic population structure of anchovy. Indeed, in the Mediterranean and northeastern Atlantic, both allozyme and mitochondrial DNA (mtDNA) assays have demonstrated genetic subdivisions between sea basins (e.g. Bembo *et al.*, 1996; Magoulas *et al.*, 2006). Therefore, the diagnostic range of T_m for the anchovy was set with reference only to those collected within the Atlantic (i.e. excluding individuals from the Mediterranean). This provided a non-overlapping range of diagnostic T_m for all four species: anchovy (79.50–79.75°C), herring (79.85–80.35°C), sprat (80.40–80.85°C), and sardine (81.25–82.00°C). Additionally, when considering larvae sampled in the English Channel, anchovy and herring spawn at different times of the year, providing an additional mechanism for distinguishing between these two species in any larvae with intermediate herring and anchovy T_m.

PCRs were conducted in 10 µl volumes following Griffiths *et al.* (2010), with annealing temperatures following Supplementary material, Table S2. Real-time PCRs were conducted on a Rotor-Gene 6000 real-time machine (Corbett Research, Sydney, Australia) and results analysed using Rotor-Gene 6000 Series (Software 1.7). The 10 µl reactions consisted of 1 µl of DNA, 1 µl of each primer (10 mM), 2 µl of MilliQ-H_2O, and 5 µl of SYBR Green SensiMix (Bioline, UK). Cycling conditions consisted of 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55–65°C (depending on primers) for 15 s, 72°C for 15 s. Immediately following PCR, a melting curve was performed by raising the incubation temperature from 70 to 90°C in 0.5°C increments with a 2 s pause after each step.

Sample collection

Local samples of clupeid larvae were obtained from routine plankton sampling at station L4 (50°15'N 04°13'W, Figure 1), situated ~ 10 nautical miles southwest of Plymouth. Sampling was by Young Fish Trawl (YFT), using a nominal 700 µm × 0.9 m^2 inlet area net with a filtering codend, on a double oblique tow at ~ 2 knots towing speed to ~10 m above the seabed. Water depth at station L4 is 55 m, and the site is influenced by seasonally stratified waters and estuarine flow from Plymouth Sound (Pingree and Griffiths, 1978). A range of physical, chemical, and biological measurements, notably zooplankton and phytoplankton species' composition, has been carried out at L4 on a weekly basis since 1988 (Smyth *et al.*, 2010). *In situ* near-surface temperature measurements at L4 (5 m depth, CTD SeaBird19) were collated from the Western Channel Observatory (WCO) database to investigate any differences in temperature between 2009 and 2011 and the previous 15-year period (from 1993 to 2008).

In order to test the utility of HRM analysis in cases where DNA has become degraded, 48 processed clupeid products purchased across Europe were analysed (Supplementary material, Table S1), including 4 herring, 3 sprat (marketed under the name "brisling"), 11 anchovy, and 30 sardine products. The technique was also applied to seven formalin-preserved larvae, which were made up of a 2009 Continuous Plankton Recorder sample and YFT samples collected in the spring of 2003 (five individuals captured at L5, see Figure 1) and February 2005 (one individual from station E1).

Analysis of the samples

One YFT sample was selected each month from September 2009 to August 2010 to examine the annual cycle of clupeid abundance; all larvae were analysed by real-time PCR and species identified by HRM curve profiling (Figure 2b). Samples were initially stored in 4% formalin and transferred to 97% ethanol 1–3 d after sampling. Larvae analysed ranged in total length from 4 to 34 mm; most were in post-larval stages of development but all are referred

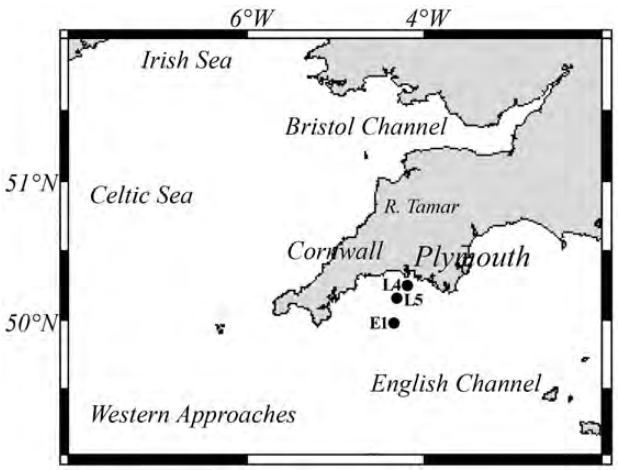

Figure 1. Location of stations L4, L5, and E1 in the Western English Channel (modified from Smyth *et al.*, 2010, by permission of Oxford University Press).

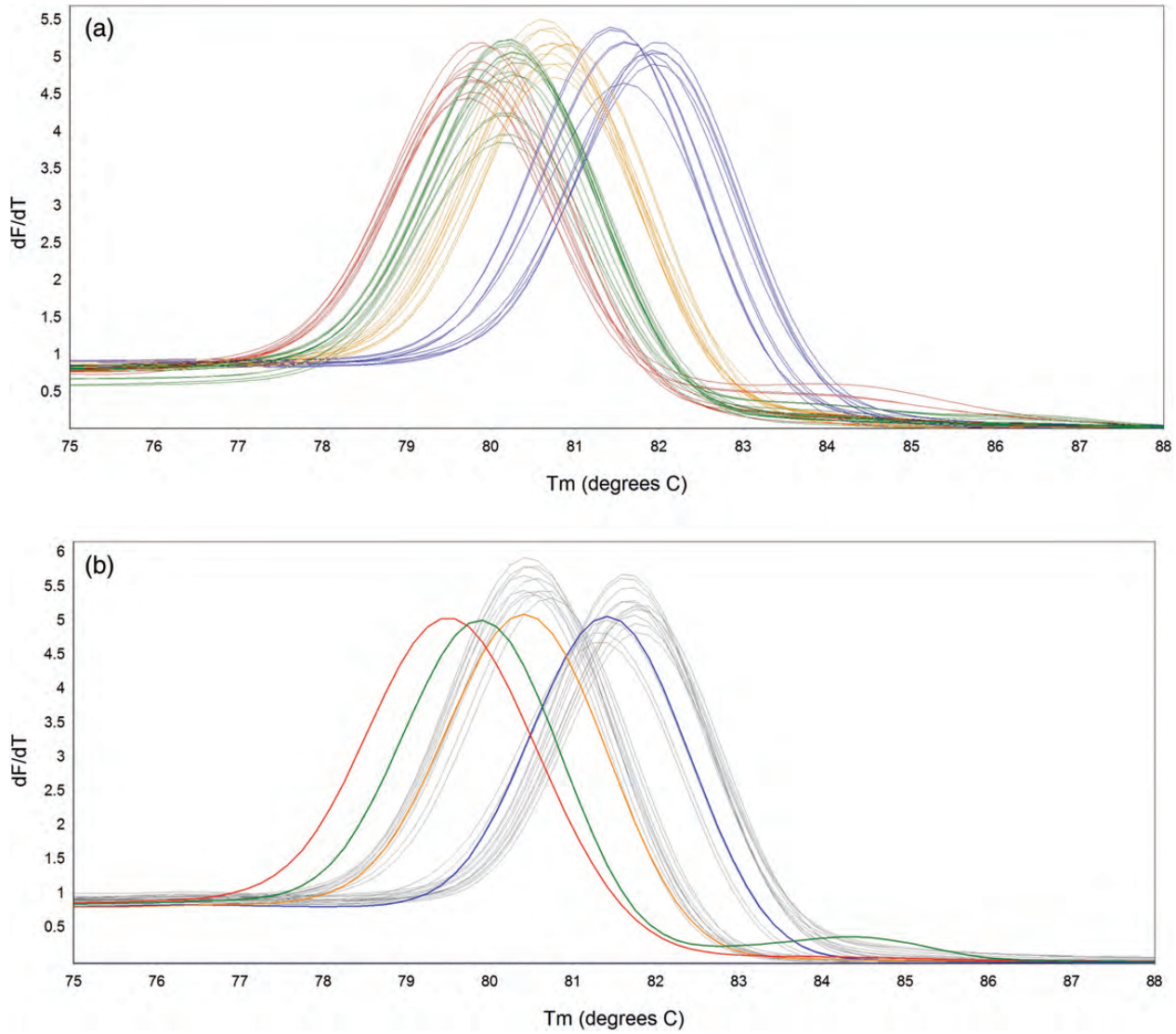

Figure 2. (a) High-resolution melt (HRM) curve analysis for the four target clupeid species from across a broad geographical range and (b) example of a real-time PCR analysis of unidentified clupeid larvae (in grey) compared with targets (blue = sardine, green = herring, orange = sprat, red = anchovy). The y-axis, dF/dT, denotes the rate of change of fluorescence relative to temperature.

to generically as "larvae". A total of 277 clupeid larvae were analysed. Although larvae were collected in November 2010, the sample was discarded due to excessive damage; a late October and early December sample was used as a substitute. To investigate the pattern of abundance further, additional samples in summer/autumn (July–October) and winter (December–February) from 2009, 2010, and 2011 were also analysed.

DNA isolation

Total genomic DNA extraction from adult fish was performed according to the Promega (Madison, WI, USA) Wizard kit, following the manufacturer's guidelines. For larvae, a 10% Chelex DNA extraction (Estoup *et al.*, 1996) protocol was chosen for its simplicity and rapidity. Whole larvae were cut into small pieces and placed in 250 μl of 10% Chelex 100 solution with 10 μl (20 mg ml^{-1}) of proteinase K (Fisher Scientific) and incubated at 56°C for ~4 h or overnight at 37°C. After digestion, the samples were placed at 100°C for 15 min. Undiluted DNA was used for subsequent PCR amplification. For processed products, the above DNA extraction method was used with an additional "defatting" step, where the tissue was placed in distilled H_2O and dried on tissue paper to remove oil before being placed in 500 μl of Chelex solution.

Sequencing

In order to provide a DNA sequence to match the T_m of the known adult reference specimens, one adult of each species was sequenced with the CluCOI4F/R primers used in the melt curve analysis. In 11 cases (five adults and six larvae), the melt curve analysis gave intermediate/equivocal results (see the Results section), and sequencing was employed to resolve the species identification. PCRs were performed in 25 μl reactions according to Griffiths *et al.* (2010). PCR products were cleaned and directly sequenced by Macrogen (The Netherlands). Electropherograms were checked

by eye, edited, and a BLAST search was carried out on GenBank (Bethesda, MD).

Statistical analysis

Long-term (1993–2008) monthly sea temperatures were compared with monthly temperatures in 2009, 2010, and 2011. Differences were considered significant when greater than one standard deviation (s.d.). Data are presented in SigmaPlot 11.0 (CA, USA).

Results

DNA sequence validation

Larvae were identified based on the HRM diagnostic temperature range defined by the adult samples (excluding the anchovy samples from the Mediterranean Sea). However, in a very small number of cases, the results of the HRM required further investigation. Two larvae showed an intermediate T_m of 80.9 and 81.0°C, i.e. between the ranges defined for sardine and sprat, and the species identity remained uncertain. Four larvae caught in March, May, June, and July displayed a herring T_m, which was inconsistent with this species' spawning time (as herring typically spawn in December to February). Sequencing resolved the HRM identity of the above six larvae as sardine and sprat. The DNA sequencing also supported the identification of the five anchovy adults from the Western Mediterranean/Aegean that had a T_m more typical of herring (see the Methods section above). These results, combined with the sequences from the reference specimens collected in the Western English Channel, all demonstrated >95% homology with the four target species (although the 100 bp sequence did not provide sufficient resolution to distinguish between a number of closely related anchovy species).

Species composition of larvae from station L4

The method allowed clear and rapid identification in 271/277 of samples. The proportion of species through 2009–2010 (Figure 3) shows a progression from sardine, which were present in their autumn spawning period from September to early December, then to herring from December until February. This was followed by sprat until May when sardine again appeared during their spring/summer spawning season, co-occurring with sprat until August. No anchovy larvae were identified.

Temperature trends

Temperatures over the sampled 12-month period (September 2009–August 2010) were colder by 0.5°C than the long-term (1993–2008) mean temperature (Figure 3). The months in which herring larvae were present (December 2009 and January 2010) were also colder than the long-term mean (11.1 and 8.8°C compared with long-term means of 11.7 and 10.0°C, respectively); the difference was significant for January (−1.17°C). The months of September 2009, February 2010, March 2010, and August 2010 were also significantly colder at −0.61, −1.14, −1.23, and −0.62°C, respectively. In contrast, November 2009 was significantly warmer (+0.84°C).

Additional summer/autumn and winter samples revealed the presence of herring larvae in January and February 2011 (Figure 4). Temperatures in 2010–2011 were slightly colder than the long-term mean (−0.2°C). January was significantly colder (−0.9°C), whereas February was also colder (−0.77°C) but the difference was not significant.

Processed clupeid products

The assay generated unequivocal results for 38 out of the 48 processed clupeid products. All four herring products were correctly labelled, and all except one anchovy product were also correctly labelled, the latter being identified as *S. pilchardus*. Seven out of 11 anchovy samples produced a T_m which overlapped that of herring. Thus these samples were run with a second set of primers, Clucytb4F/R, which can distinguish herring from other species (Supplementary material, Figure 1Sb). Twenty-eight out of 30 sardine and two out of three sprat samples were correctly labelled. The remaining three samples labelled "sardines", "Portuguese sardines", and "brisling" could not be identified as their T_m range was entirely different from that of the four target species (Figure 5). In total, 4 out of 48 (8.3%) processed clupeid products were found to be either mislabelled or of equivocal identity.

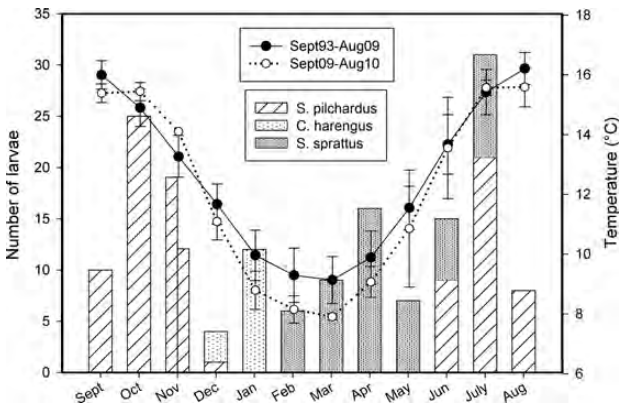

Figure 3. Monthly species number and composition from September 2009 to August 2010 (bars) showing corresponding monthly mean temperature compared with long-term (1993–2009) mean temperature. Data are shown as the mean ± 1 s.d. "November" is composed of a late October and early December sample.

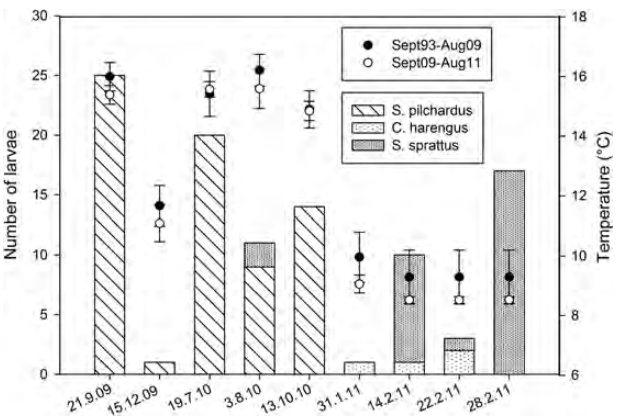

Figure 4. Sample composition (bars) in September and December 2009, July–October 2010, and January–February 2011, mean monthly temperature for 2009–2011, and long-term monthly temperature. Data are shown as the mean ± 1 s.d.

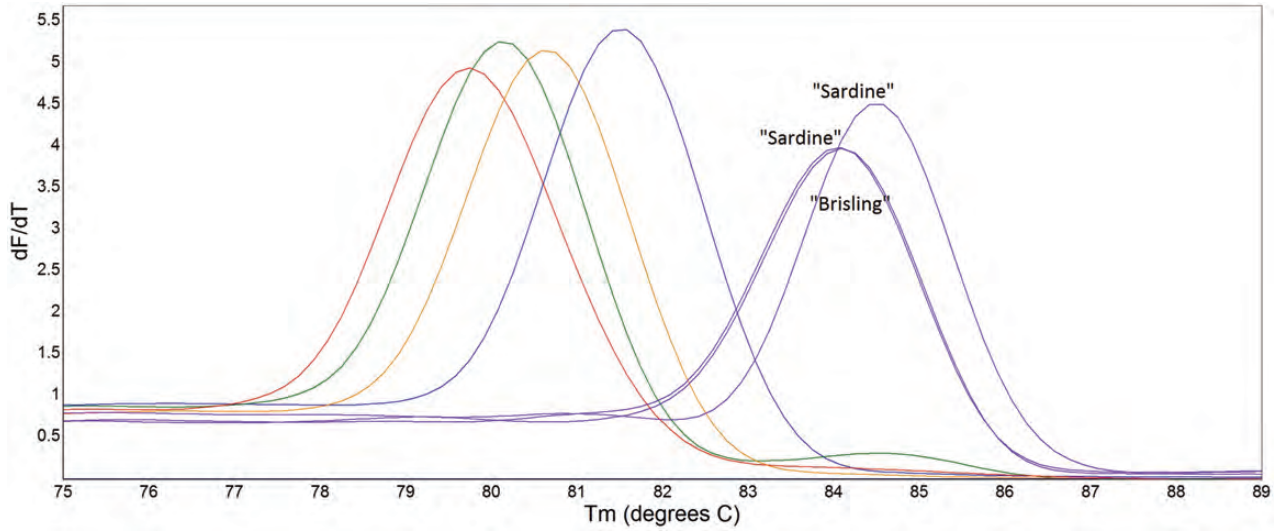

Figure 5. Melt curve analysis showing the four target reference specimens (blue = sardine, green = herring, orange = sprat, red = anchovy) and three processed products, labelled "sardine" and "brisling". The y-axis, dF/dT, denotes the rate of change of fluorescence relative to temperature. Note the high T_m out of the range of either sprat or sardine.

Formalin-preserved larvae

The method identified more than half of the formalin-preserved samples (4/7). The CPR sample and the two April 2003 larvae were identified as sprat, whereas the February 2005 individual was sardine. These results are consistent with the expected species progression through the year (Figure 3). All three samples from May 2003 failed to amplify. The duration of formalin preservation for the above samples was 2, 8, and 6 years respectively.

Discussion

Performance of high-resolution melt analysis

This study successfully developed an mtDNA technique using real-time PCR and HRM analysis to identify species of clupeid larvae collected from the Western English Channel. The method allowed clear and rapid identification in >97% (271/277) of samples. The slight between-run variation in the melt range of each species highlights the importance of including known reference individuals; however, the results were repeatable and species identification was consistent between runs. Furthermore, the method successfully identified 8-year-old formalin-preserved samples. Three samples failed to amplify, which may have been due to poor fixation.

There were some limitations with the protocol, and some issues remain with identifying species based on their T_m alone. First, anchovy from the Mediterranean and the Aegean Sea exhibited high intraspecific DNA sequence variation, which was revealed by their broad range of melting temperatures that overlapped with herring T_m in a small number of cases. However, anchovy and herring spawn at markedly different temperatures and hence different times of the year (Munk and Nielsen, 2005), and are thus unlikely to occur concurrently in plankton samples from the English Channel. Second, a very small number of sardine and sprat larvae analysed from the Western Channel produced a T_m that was either outside the diagnostic range for the target species or actually within the diagnostic range defined for herring, which meant that further analysis was required to resolve their identity. This issue was only associated with a minority of cases, so is unlikely to be problematic in ichthyoplankton studies, particularly as any ambiguous results can be reassigned in proportion to the unambiguous identities. Future investigation would benefit from the inclusion of blind control standards in the sample series to report the true error rate of the analysis.

Larvae from the Western English Channel

The results validated the expected seasonal occurrence of sprat and sardine (Kanstinger and Peck, 2009) in the Western English Channel, but also revealed the presence of herring larvae and absence of anchovy larvae. This region represents the approximate geographical boundary between the distribution of cold-water herring and warm-water sardine. The border between these two species has shifted northwards and southwards on a decadal scale since the 15th century in relation to changing temperature (Southward *et al.*, 1988). During the last few decades of generally increasing sea temperatures, larvae of the cold-water-adapted herring have been rare in routine plankton samples taken off Plymouth (Coombs and Halliday, 2011, and data referred to therein). To investigate whether the presence of herring larvae in the present study could be related to temperature, we compared long-term monthly temperatures at station L4 with monthly temperatures in the 12-month study period. It is interesting to note that in a context of warming, the last 2 years have in fact been colder, with winter 2009/2010 the coldest since 1978/1979 (http://www.metoffice.gov.uk). Individual month comparisons lacked a consistent relationship. For example, December 2010, the month preceding the appearance of larvae in 2011, was particularly cold; however, the same does not hold true for 2009/2010 where November was warmer by nearly 1°C. It is also possible that herring larvae have actually not been as rare in the routine plankton samples taken off Plymouth as previously believed. In the past, clupeid larvae have been grouped together with identification to species not routinely attempted. However, it has been presumed that the presence of any, or at least significant

numbers of, herring larvae would generally be picked up because during their early development they are noticeably morphologically distinct from the other clupeids; this may not be a valid assumption.

Anchovy spawn at slightly higher temperatures (14–18°C; Motos *et al.*, 1996) than sardine (14–15°C; Coombs *et al.*, 2006), with spawning taking place in regions adjacent to Plymouth, habitually in the bay of Biscay (Motos *et al.*, 1996) and recently in the North Sea, in response to recent rising temperatures (Beare *et al.*, 2004). Although there have been no records of anchovy eggs or larvae off Plymouth, the adults are present; it might be speculated that the continued warming trend would lead to spawning here. Furthermore, hydrographic conditions off Plymouth are similar to those of other areas favoured for anchovy spawning, e.g. a variety of estuarine, coastal, and oceanic areas (Palomera and Sabates, 1990), often influenced by river plumes in salinities of ~32–35 (Motos *et al.*, 1996). Station L4 off Plymouth exhibits a similar salinity range as well as being influenced by the outflow from the river Tamar (Pingree and Griffiths, 1978). Suitable spawning temperatures between 14.1 and 17.6°C (even up to 18.3°C on one occasion) have been regularly recorded from mid/late June to late October/early November every year between 2002 and 2011, but no anchovy larvae were identified in the present study.

In light of the above results, further investigation of the abundance and composition of clupeid larvae off Plymouth in relation to temperature, e.g. particularly cold or warm years, would be of interest to clarify the longer term incidence of herring larvae and to monitor for appearance of anchovy larvae. While recent climatic warming has led to potentially favourable temperatures for spawning of anchovy in the Western English Channel, other suitable ecological conditions may not be present. Responses to climate change can be complex, and the absence of anchovy larvae in the present study may be explained by a lag in their response to warming or by an increase in their abundance, but without an associated range expansion as shown by Simpson *et al.* (2011).

Processed products

Three products labelled as "sardine", "Portuguese sardine", and "brisling" (i.e. sprat) were rejected by the melt curve analysis as being *S. pilchardus* and *S. sprattus* (Figure 5), although they could not be identified as alternative species. There is evidence that one of the most common substitute species for canned *S. pilchardus* is *S. aurita*, followed by *Sardinella longiceps* (Lago *et al.*, 2011b), but neither of these species was included in the analysis and hence this could not be confirmed. However, as the melting temperatures of these three samples were considerably different from those of either *S. sprattus* or *S. pilchardus*, it seems unlikely that the species originate from the Clupeidae. This observation highlights the advantages of using a generic primer assay as opposed to a species-specific assay (e.g. Morgan *et al.*, 2011). Indeed, these authors used HRM to identify species of blacktip reef sharks; however, the melt profiles were too similar to develop a generic-primer assay; instead three pairs of species-specific primers were developed. Using a more generic primer assay, as applied in the present study, allows samples comprising species outside the reference baseline to be identified from their atypical T_m values, e.g. samples with high T_m in Figure 5. Although the exact species cannot be determined, it is nevertheless possible to state with some confidence that they are not consistent with sardine or sprat (in this case), and are therefore likely to be mislabelled. In contrast, a species-specific primer assay will yield a null result and no additional information.

In the case of anchovy, mislabelling was found in one out of 11 products, where the species was identified as *S. pilchardus*. Anchovy may be more prone to substitution by other species, such as *S. pilchardus*, because of its higher commercial value compared with other clupeids. The comparatively low level of misidentification is surprising in light of recent studies. For example, an investigation of cod and haddock (*Melanogrammus aeglefinus*) products from supermarkets in Ireland revealed a high level of fraud, with 25% of fresh and 80% of smoked samples genetically identified as different species (Miller and Mariani, 2010). The T_m of a number of anchovy products overlapped that of herring; however, their identity was easily resolved using a second set of primers. Although the technique developed in this paper is not a complete substitution for sequencing or other DNA techniques that can detect seafood fraud, its application to seafood products can, nevertheless, be used as a rapid and inexpensive preliminary tool capable of screening many samples at once.

Conclusions

This paper developed a real-time PCR–HRM assay to identify clupeid species and presented applications in two diverse fields: plankton ecology and food mislabelling. HRM analysis is less expensive and more rapid than full sequencing since gel electrophoresis, PCR clean-up, sequencing reactions, chromatogram editing, sequence alignment, and analyses are not required (Morgan *et al.*, 2011). The real-time PCR–HRM assay is a useful diagnostic tool if large numbers of samples need to be identified and when it is impractical or impossible to conduct traditional morphological identification. For example, Kanstinger and Peck (2009) were unable to identify clupeid larvae to species in 56% of their samples due to excessive damage caused by the net.

This real-time PCR–HRM assay was successful at discriminating species of clupeid larvae in the Western English Channel, although a small minority of samples were not identified correctly on the basis of T_m alone. Despite this limitation, it was reproducible and revealed the unexpected presence of herring larvae, in a region close to the southern limit of their range that, given global patterns of warming, could have become less hospitable to this cold-adapted species. HRM is likely to be applicable across taxa due to the relative ease with which the PCR reagents, enzymes, and conditions can be adjusted, and to the extremely small amount of tissue necessary. DNA was isolated and amplified successfully from 8-year-old formalin-preserved samples, without the need for a lengthy phenol–chloroform extraction protocol. The HRM approach holds great potential for future investigations.

Supplementary material

Supplementary material is available at the *ICESJMS* online version of the paper. Table S1 shows details of processed products. Table S2 gives the primer sequences for PCR amplification of cytb, 16S, and COI genes. Figure S1 shows the melt curve analysis of target species, red = anchovy, orange = sprat, green = herring, blue = sardine, with primers (A) 16S1F/Rb and (B) Clucytb4F/R. The y-axis, dF/dT, denotes the rate of change of fluorescence relative to temperature. Table S3 shows the melt temperatures for adults of each species in every run. Values are shown as minimum–standard–maximum. The last row shows the

temperature range that defines each species adjusted to the standards across all runs. Adjusted values are shown as standard ± °C.

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