Species-specific PCR-RFLP for identification of early life history stages of squid and other applications to fisheries research.

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Title: Species-specific PCR RFLP for identification of early life history stages of squid and other applications to fisheries research.

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Abstract

As cephalopods fulfil important roles in marine ecosystems and may be especially susceptible to overfishing the predicted expansion of cephalopod fisheries will require improved assessment and management of stocks to ensure ecosystem compatible exploitation. Genetic markers facilitating high throughput accurate species identification, particularly for early life history stages, would considerably benefit cephalopod fisheries research. Reported here is the development of a PCR-RFLP assay for identification of four squid species (*Loligo vulgaris*, *L. forbesi*, *Alloteuthis media* and *A. subulata*) of growing interest as fisheries resources. The assay was used to type morphologically indistinguishable paralarvae collected from Seine Bay and revealed 99% to be *A. media* despite *a priori* expectations that *L. vulgaris* (present at 1%) would predominate. As the method can be applied to various life history stages and tissue types it offers considerable potential for use in studies of life history, stock structure, reproduction, recruitment and abundance that are necessary for sustainable management.

Keywords: Cephalopoda – Loligo – Alloteuthis – species identification – sustainable – fisheries management

Running title: Molecular species identification of squid

1. Introduction

As many traditionally exploited fin fish stocks continue to decline there is growing interest in the expansion of cephalopod fisheries (Boyle 1990; Young et al. 2006). For example, they are becoming an increasingly important fisheries resource in the North East Atlantic (Sacau et al. 2005), an area where their exploitation was previously described as relatively low (Caddy & Rodhouse 1998). The typical short life cycle of cephalopods renders them vulnerable to overfishing (Bravo de Laguna 1989) and as they fulfil important roles in marine ecosystems improved assessment and management of stocks will be vital to ensure ecosystem compatible exploitation (Pierce et al. 1998; Young et al. 2006). Accurate, high throughput, species identification of early life history stages would greatly benefit essential studies of life history, stock structure, reproduction, recruitment and abundance; however, for many cephalopods such stages are morphologically indistinguishable. Species specific genetic markers may be applied to many areas of fisheries science (Teletchea 2009) including the identification of early life history stages (Fox et al. 2005).

Loligo forbesi and *L. vulgaris* are, from a fisheries viewpoint, the most important squid species in the northeast Atlantic (Boyle & Pierce 1994). Within the region their ranges are largely overlapping (Guerra & Rocha 1994), however, *L. forbesi* is more abundant in northern waters while *L.vulgaris* dominates the southern part of its range and is regarded as largely absent from Scottish waters (Pierce et al. 1994a, b). In the English Channel fishery landings data suggest that the life cycles of the two species are out of phase (Robin & Boucard-Camou, 1995), proposed to be a mechanism of for reducing competition. *Alloteuthis media* and its congener *A. subulata* are also of growing commercial interest with both species reported in abundance in the English Channel and Irish Sea (Hastie et al. 2009). The aim of this research was initially to develop a genetic assay permitting species identification of *L.vulgaris, L.forbesi* and *A. media,* and to validate it through analysis of

specimens of known type. The method is also predicted to distinguish *A. subulata* and although *A. subulata* control types were not available the RFLP information is also reported. The method was applied to the analysis of 96 wild caught paralarvae from the English Channel with results validated by DNA sequencing.

2. Materials and methods

Cytochrome oxidase I (COI) sequences available for the four species on GenBank together with additional sequences collected as part of ongoing squid population genetic research within our group, were aligned using BioEdit (Hall 1999). The alignment was used to identify suitable primer sites for cross species PCR amplification of a section of the COI region and locate restriction enzyme cleavage sites within amplicons using NEBcutter (Vincze et al. 2003). Patterns of cleavage site presence were compared across sequences to identify potential species-specific diagnostic enzyme combinations for RFLP genotyping. Restriction digests were performed individually for enzymes following manufacturers (New England Biolabs) recommendations with products separated on 2% agarose gels and visualised by Gel-Red staining. To validate the PCR-RFLP assay it was performed on control specimens of adult *L. vulgaris* (Bay of Biscay [n = 16]; English Channel [n = 24]; Irish Sea [n = 8]), *L. forbesi* (Irish Sea [n = 6]; Moray Firth [n = 12]) and *A. media* (English Channel [n =19]; Moray Firth [n =7]) that had been morphologically classified and preserved in absolute ethanol. DNA was extracted from control specimens following Winnepenninckx et al., (1993). The PCR-RFLP assay was then employed to genotype paralarvae (*n* = 96) sampled from Seine Bay as part of the JUVECEPH 2011 survey. DNA was extracted from individual paralarvae using the Chelex-Proteinase K method in McKeown & Shaw (2008).

3. Results and Discussion

The primers CRESH-F (5'-GAGCAGGCTTAGTTGGTACTTC-3') and CRESH-R (5'-ATGGCTCCAGCTAACACAGG-3') permitted PCR amplification of a 544bp fragment of the COI gene in all individuals tested. PCR's were performed in 25µl total reaction volumes consisting of 1X PCR Buffer, 2mM MgCl2, 200 µM of each dNTP, 0.2 units of Taq (BioLine), 0.5 µM of each primer, and 2 µl of DNA template. Amplifications involved an initial denaturation step (95 ^{o}C for 3 min) followed by 35 cycles of 30 s at 95 ^{o}C, 30 s at 52 ^{o}C and 30 s at 72 ^{o}C. Based on sequence comparisons the combination of SfcI and BccI restriction enzymes was predicted to reciprocally differentiate *L. vulgaris, L. forbesi* and *Alloteuthis* spp. by a minimum of two site differences (Table 1) with BccI predicted to distinguish *A. media* and *A. subulata* by a single site difference (Table 1). Analysis of control specimens for the three species produced clear restriction patterns with fragment sizes corresponding to expectations from sequences (Fig. 1). The method could not be empirically tested for *A. subulata* due to the unavailability of control samples, however, the absence of any nucleotide differences within the primer sites, and conservation of restriction enzyme cleavage sites among GenBank sequences support the likelihood of the predicted species specific RFLP. Furthermore, the PCR-RFLP assay successfully excluded *A. subulata* from the paralarvae samples (described below). Although dependent on a small number of site differences, analysis of reference individuals and GenBank sequences spanning a wide geographical range did not indicate any ambiguity due to intraspecific polymorphisms. However, as the main focus of this research was the English Channel further analysis of individuals from throughout the respective ranges of each species is recommended, particularly for *A. media* and *A. subulata* as some localised interspecific haplotype sharing has been reported (Lefkaditou et al. 2011).

Unambiguous genotypes were obtained for all paralarvae (i.e. there were no reaction failures or unrecognised genotypes). Of the 96 paralarvae, 95 were classified as *A. media* and one individual was identified as *L. vulgaris*. The validity of species assignments was confirmed by DNA sequencing of the COI amplicon (using the PCR primers) and use of BLASTn for a subset of individuals. Inaccurate species identification of early life history stages has been reported to severely compromise estimates of stock biomass in other commercially important taxa (e.g. Cod, Fox et al. 2005) and the species ratios reported here were surprising as *L. vulgaris* was expected to predominate. However, when early development stages are correctly identified they can be used to estimate stock sizes and accurate estimates of sustainable catches. Egg identification in plankton surveys is carried out using molecular markers for stock assessment in cod, whiting and haddock in the southern North Sea (Taylor et al. 2002). Species identification of early life history stages can also be used to construct spatial/temporal spawning maps (Fox et al. 2008; Munk et al. 2009). This is highly relevant to cephalopods as widespread human activities, particularly bottom fishing operations, but also shipping, oil exploration and production have been highlighted as potentially damaging to cephalopod spawning areas (Hastie et al. 2009).

At present, for *L. vulgaris* and *L. forbesi*, fishery statistics do not distinguish between the two species since they are of similar appearance and equal commercial value (Chen et al. 2006). In the case of *A. media* and *A. subulata* commonly used morphometric characters (e.g. relative fin length) have been shown to provide inaccurate species identification of adults (Anderson et al. 2008). Species misidentification within fishery landings or stock assessments can severely compromise stock sustainability (Garcia-Vazquez et al. 2012). Application of species specific genetic markers in catch estimates and population surveys should therefore be considered for more accurately estimating stock sizes of the two congeners studied here,

particularly in light of recent range shifts reported for *Loligo* (Chen et al. 2006) and suggested for *Alloteuthis* (Anderson et al. 2008) that may rapidly alter mixed fisheries.

DNA based species identification methodologies can be applied to all the different life stages of a marine species and a range of sample types (e.g. whole individuals, biopsies, processed and dried tissue) and thus offer considerable potential to fisheries science. The integration of such methodologies has often been hampered by relatively high per sample costs and by the time and specialist skills needed (Lindeque et al. 2006). By omitting the need for sequencing the PCR-RFLP method described here represents a simple, cost effective means for high throughput screening. Furthermore, although details for two enzymes are reported, digestion with BccI alone is sufficient to discriminate among the four species. The PCR-RFLP method may also be extended to other cephalopod species through judicious selection of cutting enzymes that may be identified from analysis of sequences on GenBank and/or by exploratory sequencing.

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References

Anderson FE, Pilsits A, Clutts S, Laptikhovsky V, Bello G, Balguerias E,Lipinski M, Nigmatulin C, Pereira JMF, Piatkowski U, Robin JP, salman A, Tasende MG (2008) Systematic of Alloteuthis (Cephalopoda:Loliginidae) based on molecular and morphometric data. J Exp Mar Biol Ecol 364, 99-109.

Boyle PR (1990) Cephalopod biology in the fisheries context. Fish Res 8, 303-321.

149 Boyle PR, Pierce GJ (1994) Fishery biology of northeast Atlantic squid: an overview. Fish
150 Res 21, 1-15.

151 Bravo de Laguna J (1989) Managing and international multispecies fishery: the Saharan trawl
152 fishery for cephalopods. In Marine Invertebrate Fisheries: Their Assessment and
153 Management, J.F. Caddy (ed.). New York: Wiley, 591-612.

154 Caddy JF, Rodhouse PJ (1998) Cephalopod and groundfish landings: evidence for ecological
155 change in global fisheries? Rev Fish Biol Fisher 8, 431-444.

156 Chen CS, Pierce GJ, Wang J, robin JP, Poulard JC, Pereira J, Zuur AF, Boyle PR, Bailey N,
157 Beare DJ, Jereb P, Ragonese S, Mannini A, Orsi-Relini L (2006) The apparent disappearance
158 of Loligo forbesi from the south of its range in the 1990s: trends in Loligo spp. Abundance in
159 the northeast Atlantic and possible environmental influences. Fish Res 78, 44-54.

160 Fox, CJ, Taylor MI, Pereya R, Villasana MI, Rico C (2005). TaqMan DNA technology
161 confirms likely overestimation of cod (Gadus morhua L.) egg abundance in the Irish
162 Sea:implications for the assessment of the cod stock and mapping of spawning areas using
163 egg-based methods. Mol Ecol 14, 879-884.

164 Fox CJ, Taylor M, Dickey-Collas M, Fossum P, Kraus G, Rohlf N, Munk P, van Damme JG,
165 Bolle LJ, Maxwell DL, Wright PJ (2008) Mapping the spawning grounds of North sea cod
166 (Gadus morhua) by direct and indirect means. Proc Royal Soc Biol Sci 275, 1543-1548.

167 Garcia_vazquez, Machado-Schiaffino G, Campo D, Juanes F (2012) Species
168 misidentification in mixed hake fisheries may lead to overexploitation and population
169 bottlenecks. Fish Res 114, 52-55.

170 Guerra A, Rocha F (1994) The life history of Loligo vulgaris and Loligo forbesi
171 (Cephalopoda: Loliginidae) in Galician waters (NW Spain). Fish Res 21, 43-70.

172 Hall TA (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis
173 program for windows 95/98/NT. Nuc Acid Symp Ser 41, 95-98

174 Hastie LC, Pierce GJ, Wang J, Bruno I, Moreno A, Piatkowski U, Robin JP (2009)
175 Cephalopods in the north-eastern Atlantic:species, biogeography, ecology, exploitation and
176 conservation. Oceanogr Mar Biol 47, 111-190.

177 Lefkaditou E, Tsigenopoulos CS, Alidromiti C, Haralabous J (2012) On the occurrence of
178 Alloteuthis subulata in the eastern Ionian Sa and its distinction from the sympatric Alloteuthis
179 media. J Biol Res-Thessalon 17, 169-175.

180 Lindeque PK, Hay S, heath MR, Ingvarsdottir A, Rasmussen J, Smerdon GR, wanier JJ
181 (2006) Integrating conventional microscopy and molecular analysis to analyse the abundance
182 an distribution of four Calanus congeners in the North Atlantic. J Plankton Res 28, 221-238.

183 McKeown NJ, Shaw PW (2008) Single paternity within broods of the brown crab (Cancer
184 pagurus L.); a highly fecund species with long term sperm storage. Mar Ecol Prog Ser 368,
185 209-215.

186 Munk P, Foc CJ, Bolle LJ, van Damme CJG, Fossum P, Kraus G (2009) Spawning of North
187 Sea fishes linked to hydrographic features. Fish Oceanogr 18, 458-469.

188 Pierce GJ, Boyle PR, Hastie LC, Key L (1994a) The life history of Loligo forbesi
189 (Cephalopoda: Loliginidae) in Scottish waters. Fish Res 21, 17-41.

190 Pierce GJ, Boyle PR, Hastie LC, Shanks AM (1994b) Distribution and abundance of the
191 fished population of Loligo forbesi in UK waters: analysis of fioshery data. Fish Res 21, 193-
192 216.

193 Pierce GJ, bailey N, Stratoudakis Y, Newton A (1998) Distribution and abndance of the
194 fished population of Loligo forbesi in Scottish waters: analysis of research cruise data. ICES
195 J Mar Sci 55,14-33.

196 Robin JP, Boucaud-Camou E (1993) Proportion of squid Loligo forbesi and Loligo vulgaris
197 in French landings of the Channel bottom trawl fishery:sampling scheme of the Port-en-
198 Bessin landings and preliminary results, International Council for the Exploration of the Sea,
199 ICES CM 1993/K:20. ICES: Copenhagen.

200 Sacau M, Pierce GJ, Stowasser G, Wang J, Santos MB (2005) An overview of cephalopods
201 relevant to the SEA6 Area. Department of trade and industry report. Available HTTP:
202 http://www.offshore-sea.org.uk/consultations/SEA_6/SEA6_Cephalopods_UOA.pdf.

203 Taylor MI, fox C, Rico I, Rico C (2002) Species-specific TaqMan probes for simultaneous
204 identification of (Gadus morhua L.), haddock (Melanogrammus aeglefinis L.) and whiting
205 (Merlangius merlangus L.). Mol Ecol Notes 2, 599-601.

206 Teletchea F (2009) Molecular Identification methods of fish species: reassessment and
207 possible application. Rev Fish Biol Fisher 19, 265-293.

208 Vincze T, Posfai J, Roberts RJ (2003) NEBcutter: a program to cleave DNA with restriction
209 enzymes. Nucleic Acids Res 31, 3688-3691.

210 Winnepenninckx B, Backeljau T, De Wachter R (1993) Extraction of high molecular weight
211 DNA from molluscs. Trends Genet 9, 407.

212 Young IAG, Pierce GJ, Stowasser G, Santos MB, Wang J, Boyle PR, Shaw PW, Bailey N,
213 Tuck I, Collins MA (2006) The Moray Firth directed squid fishery. Fish Res 78, 39-43.

214

215 **Table 1.** Location of restriction enzyme cleavage sites and expected fragment sizes for each

216 enzyme/species combination.

217

Species	Sfc I **Cleavage sites –** *Fragment sizes*	Bcc I **Cleavage sites –** *Fragment sizes*
Loligo vulgaris	**277, 352 –** *277,192,75*	**None**
Loligo forbesi	**277 –** *277,267*	**385 –** *385,159*
Alloteuthis media	**None**	**254 –** *290 254*
Alloteuthis subulata	**None**	**254, 436 –** *254, 182, 108*

218

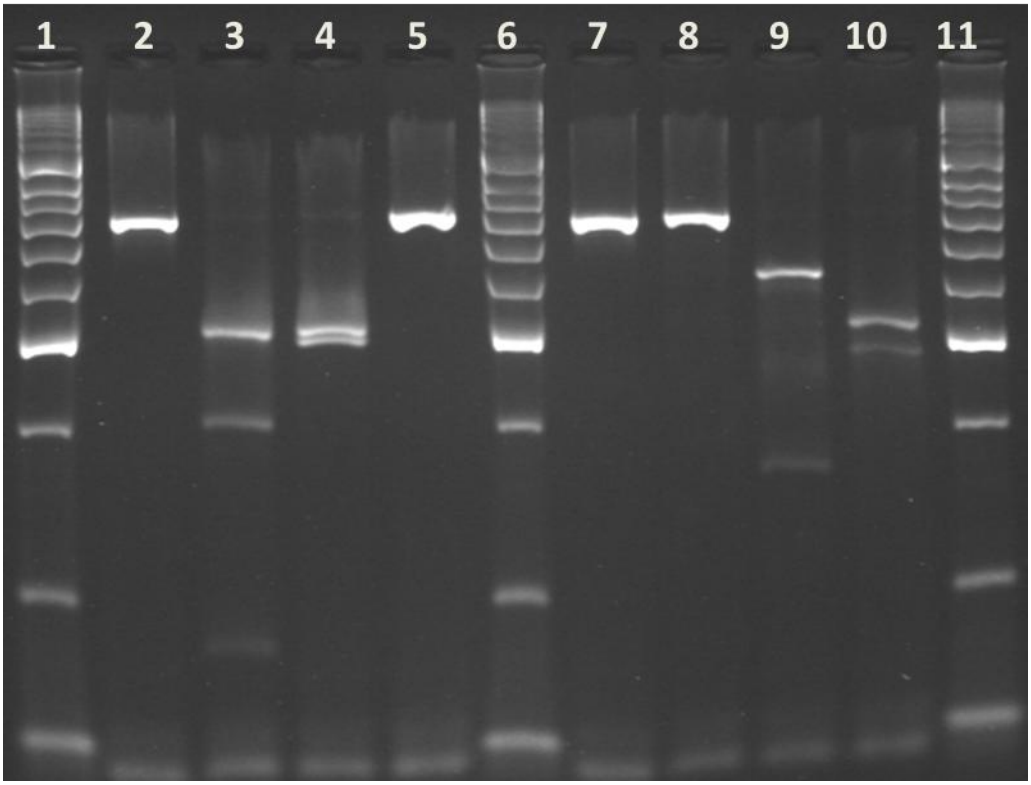

Figure 1. PCR-RFLP patterns for digestion with Sfc I (3 = *L.vulgaris*, 4 = *L. forbesi*, 5 = *A. media*) and Bcc I (8 = *L.vulgaris*, 9 = *L. forbesi*, 10 = *A. media*) and unrestricted PCR products (2 & 7) and molecular weight marker Bioline Hyperladder II (1,6,11).