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

2   other applications to fisheries research.

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9   **Abstract**

10   As cephalopods fulfil important roles in marine ecosystems and may be especially susceptible

11   to overfishing the predicted expansion of cephalopod fisheries will require improved

12   assessment and management of stocks to ensure ecosystem compatible exploitation. Genetic

13   markers facilitating high throughput accurate species identification, particularly for early life

14   history stages, would considerably benefit cephalopod fisheries research. Reported here is the

15   development of a PCR-RFLP assay for identification of four squid species (*Loligo vulgaris,*

16   *L. forbesi*, *Alloteuthis media* and *A. subulata*) of growing interest as fisheries resources. The

17   assay was used to type morphologically indistinguishable paralarvae collected from Seine

18   Bay and revealed 99% to be *A. media* despite *a priori* expectations that *L. vulgaris* (present at

19   1%) would predominate. As the method can be applied to various life history stages and

20   tissue types it offers considerable potential for use in studies of life history, stock structure,

21   reproduction, recruitment and abundance that are necessary for sustainable management.

22   **Keywords:** Cephalopoda – Loligo – Alloteuthis – species identification – sustainable –

23   fisheries management

24   **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

50 specimens of known type. The method is also predicted to distinguish *A. subulata* and

51 although *A. subulata* control types were not available the RFLP information is also reported.

52 The method was applied to the analysis of 96 wild caught paralarvae from the English

53 Channel with results validated by DNA sequencing.

54

2. Materials and methods

56 Cytochrome oxidase I (COI) sequences available for the four species on GenBank together

57 with additional sequences collected as part of ongoing squid population genetic research

58 within our group, were aligned using BioEdit (Hall 1999). The alignment was used to identify

59 suitable primer sites for cross species PCR amplification of a section of the COI region and

60 locate restriction enzyme cleavage sites within amplicons using NEBcutter (Vincze et al.

61 2003). Patterns of cleavage site presence were compared across sequences to identify

62 potential species-specific diagnostic enzyme combinations for RFLP genotyping. Restriction

63 digests were performed individually for enzymes following manufacturers (New England

64 Biolabs) recommendations with products separated on 2% agarose gels and visualised by

65 Gel-Red staining. To validate the PCR-RFLP assay it was performed on control specimens of

66 adult *L. vulgaris* (Bay of Biscay [n = 16]; English Channel [n = 24]; Irish Sea [n = 8])*, L.*

67 *forbesi* (Irish Sea [n = 6]; Moray Firth [n = 12]) and *A. media* (English Channel [n =19];

68 Moray Firth [n =7]) that had been morphologically classified and preserved in absolute

69 ethanol. DNA was extracted from control specimens following Winnepenninckx et al.,

70 (1993). The PCR-RFLP assay was then employed to genotype paralarvae (*n* = 96) sampled

71 from Seine Bay as part of the JUVECEPH 2011 survey. DNA was extracted from individual

72 paralarvae using the Chelex-Proteinase K method in McKeown & Shaw (2008).

73

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 $^{\circ}$C for 3 min) followed by 35 cycles of 30 s at 95 $^{\circ}$C, 30 s at 52 $^{\circ}$C and 30 s at 72 $^{\circ}$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).

99 Unambiguous genotypes were obtained for all paralarvae (i.e. there were no reaction failures

100 or unrecognised genotypes). Of the 96 paralarvae, 95 were classified as *A. media* and one

101 individual was identified as *L. vulgaris*. The validity of species assignments was confirmed

102 by DNA sequencing of the COI amplicon (using the PCR primers) and use of BLASTn for a

103 subset of individuals. Inaccurate species identification of early life history stages has been

104 reported to severely compromise estimates of stock biomass in other commercially important

105 taxa (e.g. Cod, Fox et al. 2005) and the species ratios reported here were surprising as *L.*

106 *vulgaris* was expected to predominate. However, when early development stages are

107 correctly identified they can be used to estimate stock sizes and accurate estimates of

108 sustainable catches. Egg identification in plankton surveys is carried out using molecular

109 markers for stock assessment in cod, whiting and haddock in the southern North Sea (Taylor

110 et al. 2002). Species identification of early life history stages can also be used to construct

111 spatial/temporal spawning maps (Fox et al. 2008; Munk et al. 2009). This is highly relevant

112 to cephalopods as widespread human activities, particularly bottom fishing operations, but

113 also shipping, oil exploration and production have been highlighted as potentially damaging

114 to cephalopod spawning areas (Hastie et al. 2009).

115

116 At present, for *L. vulgaris* and *L. forbesi*, fishery statistics do not distinguish between the two

117 species since they are of similar appearance and equal commercial value (Chen et al. 2006).

118 In the case of *A. media* and *A. subulata* commonly used morphometric characters (e.g.

119 relative fin length) have been shown to provide inaccurate species identification of adults

120 (Anderson et al. 2008). Species misidentification within fishery landings or stock assessments

121 can severely compromise stock sustainability (Garcia-Vazquez et al. 2012). Application of

122 species specific genetic markers in catch estimates and population surveys should therefore

123 be considered for more accurately estimating stock sizes of the two congeners studied here,

124   particularly in light of recent range shifts reported for *Loligo* (Chen et al. 2006) and suggested

125   for *Alloteuthis* (Anderson et al. 2008) that may rapidly alter mixed fisheries.

126

127   DNA based species identification methodologies can be applied to all the different life stages

128   of a marine species and a range of sample types (e.g. whole individuals, biopsies, processed

129   and dried tissue) and thus offer considerable potential to fisheries science. The integration of

130   such methodologies has often been hampered by relatively high per sample costs and by the

131   time and specialist skills needed (Lindeque et al. 2006). By omitting the need for sequencing

132   the PCR-RFLP method described here represents a simple, cost effective means for high

133   throughput screening. Furthermore, although details for two enzymes are reported, digestion

134   with BccI alone is sufficient to discriminate among the four species. The PCR-RFLP method

135   may also be extended to other cephalopod species through judicious selection of cutting

136   enzymes that may be identified from analysis of sequences on GenBank and/or by

137   exploratory sequencing.

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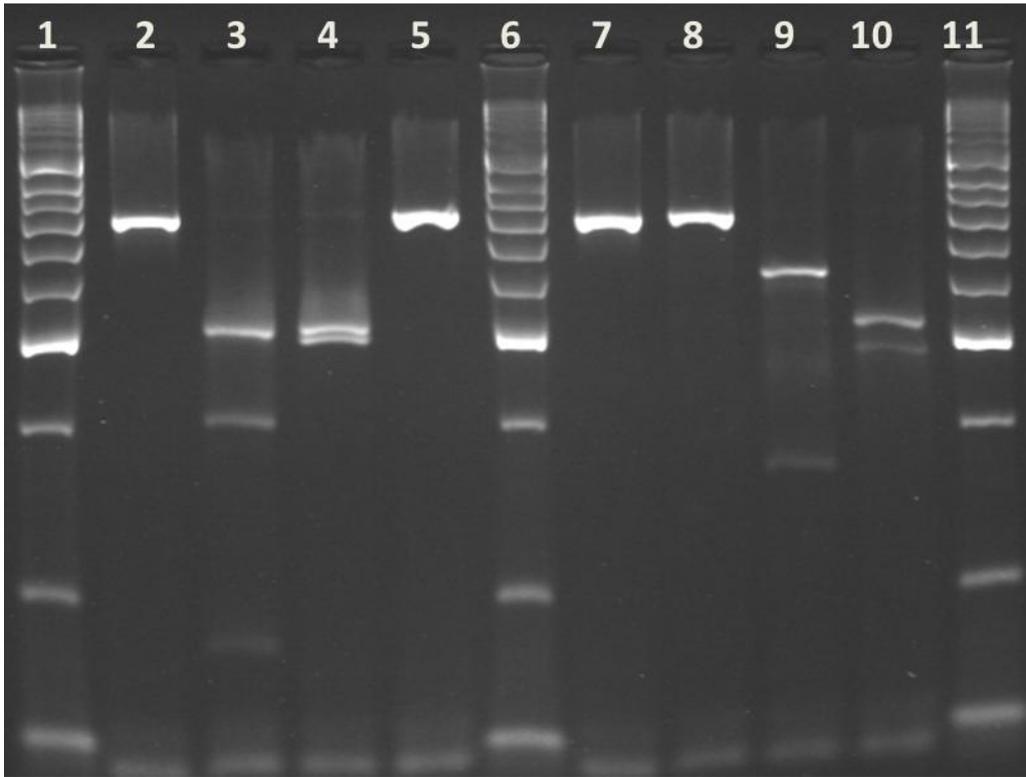
Figure 1. PCR-RFLP patterns for digestion with Sfc I (3 = *L.vulgaris,* 4 = *L. forbesi*, 5 = *A. media*) and Bcc I (8 = *L.vulgaris,* 9 = *L. forbesi*, 10 = *A. media*) and unrestricted PCR products (2 & 7) and molecular weight marker Bioline Hyperladder II (1,6,11).