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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

25 **1. Introduction**

26 As many traditionally exploited fin fish stocks continue to decline there is growing interest in
27 the expansion of cephalopod fisheries (Boyle 1990; Young et al. 2006). For example, they are
28 becoming an increasingly important fisheries resource in the North East Atlantic (Sacau et al.
29 2005), an area where their exploitation was previously described as relatively low (Caddy &
30 Rodhouse 1998). The typical short life cycle of cephalopods renders them vulnerable to
31 overfishing (Bravo de Laguna 1989) and as they fulfil important roles in marine ecosystems
32 improved assessment and management of stocks will be vital to ensure ecosystem compatible
33 exploitation (Pierce et al. 1998; Young et al. 2006). Accurate, high throughput, species
34 identification of early life history stages would greatly benefit essential studies of life history,
35 stock structure, reproduction, recruitment and abundance; however, for many cephalopods
36 such stages are morphologically indistinguishable. Species specific genetic markers may be
37 applied to many areas of fisheries science (Teletchea 2009) including the identification of
38 early life history stages (Fox et al. 2005).

39 *Loligo forbesi* and *L. vulgaris* are, from a fisheries viewpoint, the most important squid
40 species in the northeast Atlantic (Boyle & Pierce 1994). Within the region their ranges are
41 largely overlapping (Guerra & Rocha 1994), however, *L. forbesi* is more abundant in
42 northern waters while *L.vulgaris* dominates the southern part of its range and is regarded as
43 largely absent from Scottish waters (Pierce et al. 1994a, b). In the English Channel fishery
44 landings data suggest that the life cycles of the two species are out of phase (Robin &
45 Boucard-Camou, 1995), proposed to be a mechanism of for reducing competition.
46 *Alloteuthis media* and its congener *A. subulata* are also of growing commercial interest with
47 both species reported in abundance in the English Channel and Irish Sea (Hastie et al. 2009).
48 The aim of this research was initially to develop a genetic assay permitting species
49 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

55 **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

74 3. Results and Discussion

75 The primers CRESH-F (5'-GAGCAGGCTTAGTTGGTACTTC-3') and CRESH-R (5'-
76 ATGGCTCCAGCTAACACAGG-3') permitted PCR amplification of a 544bp fragment of
77 the COI gene in all individuals tested. PCR's were performed in 25µl total reaction volumes
78 consisting of 1X PCR Buffer, 2mM MgCl₂, 200 µM of each dNTP, 0.2 units of Taq
79 (BioLine), 0.5 µM of each primer, and 2 µl of DNA template. Amplifications involved an
80 initial denaturation step (95 °C for 3 min) followed by 35 cycles of 30 s at 95 °C, 30 s at 52
81 °C and 30 s at 72 °C. Based on sequence comparisons the combination of SfcI and BccI
82 restriction enzymes was predicted to reciprocally differentiate *L. vulgaris*, *L. forbesi* and
83 *Alloteuthis* spp. by a minimum of two site differences (Table 1) with BccI predicted to
84 distinguish *A. media* and *A. subulata* by a single site difference (Table 1). Analysis of control
85 specimens for the three species produced clear restriction patterns with fragment sizes
86 corresponding to expectations from sequences (Fig. 1). The method could not be empirically
87 tested for *A. subulata* due to the unavailability of control samples, however, the absence of
88 any nucleotide differences within the primer sites, and conservation of restriction enzyme
89 cleavage sites among GenBank sequences support the likelihood of the predicted species
90 specific RFLP. Furthermore, the PCR-RFLP assay successfully excluded *A. subulata* from
91 the paralarvae samples (described below). Although dependent on a small number of site
92 differences, analysis of reference individuals and GenBank sequences spanning a wide
93 geographical range did not indicate any ambiguity due to intraspecific polymorphisms.
94 However, as the main focus of this research was the English Channel further analysis of
95 individuals from throughout the respective ranges of each species is recommended,
96 particularly for *A. media* and *A. subulata* as some localised interspecific haplotype sharing
97 has been reported (Lefkaditou et al. 2011).

98

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.

138

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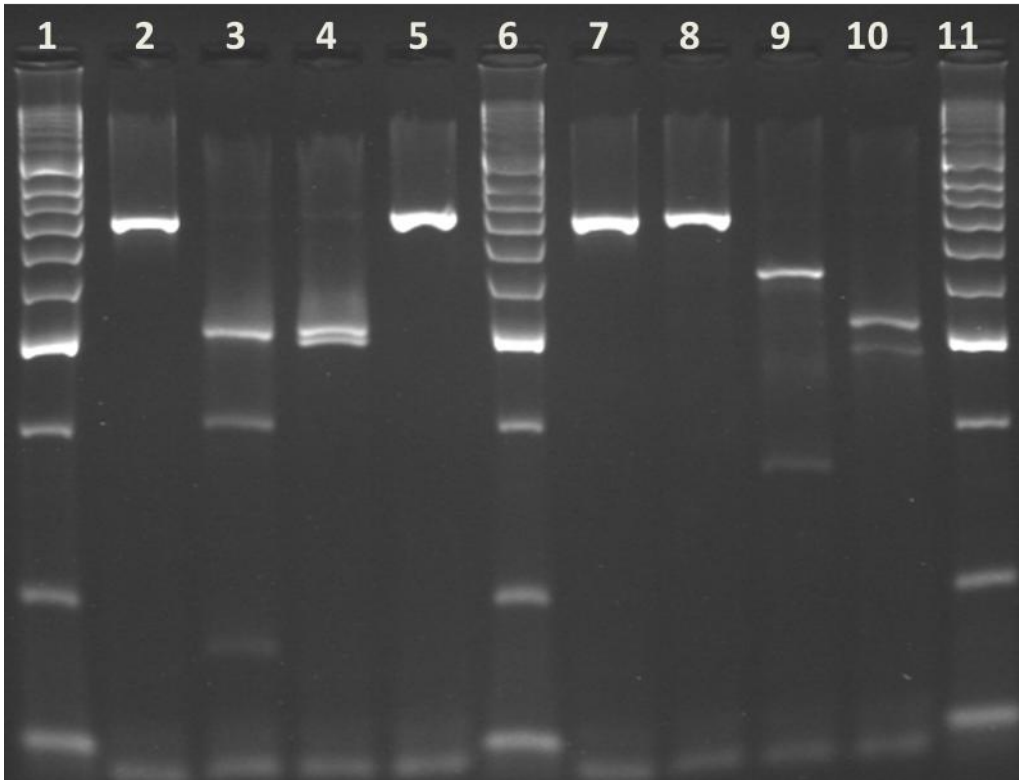
214

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
<i>Loligo vulgaris</i>	277, 352 – 277,192,75	None
<i>Loligo forbesi</i>	277 – 277,267	385 – 385,159
<i>Alloteuthis media</i>	None	254 – 290 254
<i>Alloteuthis subulata</i>	None	254, 436 – 254, 182, 108

218



219

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

223

224