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Phylogeny of the Sepia officinalis species complex in the east Atlantic extends the known distribution of Sepia vermiculata across the Benguela upwelling region

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1 **Phylogeny of the *Sepia officinalis* species complex in the east**
2 **Atlantic extends the known distribution of *Sepia vermiculata* across**
3 **the Benguela upwelling region**

4
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11
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13
14 In order to manage expanding cephalopod fisheries appropriately, accurate species
15 identification and biogeographic characterisation are fundamental. This study addressed
16 such topics within the *Sepia officinalis* species complex (*Sepia officinalis*, *Sepia hierredda*
17 and *Sepia vermiculata*) with emphasis on African waters. Samples from the currently
18 presumed distributions of *S. vermiculata* and *S. hierredda* (South Africa and Ghana/Angola
19 respectively) were sequenced for the cytochrome *c* oxidase subunit I (COI) and the
20 cytochrome *b* (cytb) genes of the mitochondrial genome, and compared to existing *S.*
21 *officinalis* sequences. Three highly divergent and reciprocally monophyletic clades
22 corresponding to *S. officinalis*, *S. hierredda* and *S. vermiculata* were resolved, representing
23 the first molecular confirmation of the distinct species status of *S. hierredda* and *S.*
24 *vermiculata*. Sequences also revealed that, contrary to expectations based on presently
25 published information, all samples from southern Angola were *S. vermiculata*. This indicates
26 that the species range extends beyond the currently described northern limit and that *S.*
27 *hierredda* and *S. vermiculata* may be indiscriminately harvested in Angolan waters. Finer
28 scale patterns within *S. vermiculata* phylogeography also indicate that the Benguela Current
29 System and/or other environmental factors serve to isolate northern and southern stocks.

30
31 **Keywords:** biogeography, cephalopod, cuttlefish, dispersal, ecosystem compatible
32 exploitation, fisheries management, indiscriminate harvesting

33
34 **Introduction**

36 As many traditionally exploited fin-fish stocks continue to decline there is growing interest in
37 the expansion of cephalopod fisheries (Boyle 2000; Young et al. 2006; Anderson et al. 2011;
38 Jereb et al. 2015). The typical short life cycle of cephalopods renders them vulnerable to
39 overfishing (Rodhouse et al. 2014) and as they fulfil important roles in marine ecosystems,
40 improved assessment and management of stocks will be vital to ensure ecosystem-
41 compatible exploitation (Pierce et al. 1998; Young et al. 2006). Fundamental to this is both
42 accurate species identification and resolution of species ranges (Taylor et al. 2012;
43 McKeown et al. 2015).

44
45 The common cuttlefish *Sepia officinalis* species complex is of importance to both commercial
46 and artisanal fisheries across its range (Reid et al. 2005). Three species are currently
47 described within this complex (Khromov et al. 1998): *Sepia officinalis* (Linnaeus 1758),
48 *Sepia hierredda* (Rang 1837) and *Sepia vermiculata* (Quoy and Gaimard 1832). By far the
49 most extensively studied of these species is *S. officinalis*, an abundant cephalopod within
50 coastal waters of the Mediterranean Sea basin and north-east Atlantic Ocean. The northern
51 distribution of *S. officinalis* extends into the southern North Sea (Gittenberger and Schrieken
52 2004; De Heij and Baayen 2005), and the southern limits are along the north-west coast of
53 Africa, coinciding with the border between Mauritania and Senegal (16° N). Off North-West
54 Africa *S. officinalis* is found in sympatry with *S. hierredda*, the distribution of which extends
55 as far north as Cape Blanc (21° N) (Hatanaka 1979; Guerra et al. 2001). *Sepia hierredda* is
56 found at shallower depths than *S. officinalis* and although it is relatively well characterised in
57 its zone of overlap with *S. officinalis* (e.g. Guerra et al. 2001) there has been limited, if any,
58 research focussed upon *S. hierredda* from its central or southern distribution. Despite this,
59 fisheries data cite the distribution of *S. hierredda* as extending throughout the tropics and
60 subtropics as far south as Tigres Bay in southern Angola (Hatanaka 1979; Roeleveld ~~et al.~~
61 1998[not 'et al.']). A break in the occurrence of this species complex is noted around the
62 Benguela ~~upwelling region~~Current System [In Abstract you refer to 'Benguela Current
63 System'. Best to be consistent.] that occurs off the coast of Namibia, with *S. vermiculata*,
64 the most poorly investigated member of this species complex, thought to be restricted to the
65 coast of southern Africa, occurring from the Western Cape of South Africa into the Indian
66 Ocean as far as central Mozambique (Roeleveld ~~et al.~~1972, 1998[neither are 'et al.'];
67 Khromov 1998). Additionally, trawl data from far farther into the Indian Ocean noted the
68 occurrence of a population of *S. vermiculata* on the Saya-de-Malha Bank of the Mascarene
69 Plateau (Nesis 1993).

70
71 Whereas available genetic data support the distinctiveness of *S. officinalis* and *S. hierredda*
72 (Guerra et al. 2001), at present the description of *S. vermiculata* is based solely on

73 divergence from *S. hierredda* and *S. officinalis* in morphological traits (Khromov *et al.* 1998).
74 As such, a primary goal of the present study was to assess the validity of *S. vermiculata* as a
75 species, using mitochondrial DNA (mtDNA) sequencing. A secondary objective was to
76 assess genetic patterns in the context of biogeography, as to date and to the best of our
77 knowledge, there has been no molecular investigation of the *S. officinalis* species complex
78 south of Mauritania. The results, based on mtDNA cytochrome *c* oxidase subunit I (COI) and
79 cytochrome *b* (*cytb*) sequencing, support the species status of *S. vermiculata* but indicate
80 that its range extends further north in the Atlantic Ocean than previously described, and at
81 least as far as southern Angola.

82

83 **Methods**

84

85 **Sampling and mtDNA sequencing**

86 Tissue samples (tentacle clips stored in 95% ethanol) recorded as *S. hierredda* were
87 collected between 2011 and 2016 from artisanal catches in Ghana (Tema fish market) as
88 well as through targeted fishing in southern Angola (Flamingo River) **[Some occurrences in**
89 **the document just 'Flamingo'. Please confirm which is correct.]**, while tissue samples
90 recorded as *S. vermiculata* were obtained from two locations (Bushmans River and Jeffreys
91 Bay) in the Eastern Cape of South Africa (Figure 1).

92

93 Genomic DNA was extracted from all samples using a standard CTAB-
94 chloroform/isoamylalcohol method (Winnepenninckx *et al.* 1993). Partial sequences of the
95 mtDNA COI and *cytb* genes were amplified by polymerase chain reaction (PCR) using
96 species-specific primers developed specifically for this study (COI: *Sepia*COIF 5'-
97 GTAAACCTGGTACACTTTT-3', *Sepia*COIR 5'-TTCTATTTGTAAACCTTCTCATC-3'; *cytb*:
98 *cytb*117F 5'-CCCCCAATCCAAGTTAACAA-3', *cytb*928R 5'-ATGCGGGATGTGAATTATGG-
99 3'). PCRs were performed in a total volume of 20 µl, containing 4 µl template DNA, 2 mM
100 MgCl₂, 0.5 µM forward primer and 0.5 µM of reverse primer, 0.2 mM dNTP mix (20 µM each
101 dATP, dCTP, dGTP, dTTP), 1x reaction buffer [75 mM Tris-HCl, 20 mM (NH₄)₂SO₄] and *Taq*
102 polymerase (BIOTAQ, 5 U/µl). The PCR thermo-profile for COI amplification was: 180 s at
103 95 °C, followed by 40 cycles of 30 s at 95 °C, 45 s annealing at 50 °C and 60 s at 72 °C,
104 followed by a final 5 min extension at 72 °C. For *cytb* amplification, PCR conditions were:
105 180 s at 95 °C, followed by 34 cycles of 30 s denaturing at 95 °C, 30 s annealing at 52 °C
106 and 30 s at 72 °C, again followed by a final 5 min extension at 72 °C. The PCR products
107 were then purified and sequenced using BigDye technology, with sequence identity
108 confirmed using BLAST.

109

110 **Phylogenetic sequence analysis**

111 Phylogenetic relationships among sequences obtained here, as well as other sequences
112 available on GENBANK (Table 1) were inferred using maximum likelihood (ML) trees,
113 constructed for both mtDNA regions in MEGA 6.06 (Tamura et al. 2013) and Bayesian
114 inference performed using MRBayes 3.2 (Ronquist and Huelsenbeck 2003). In both cases
115 HKY+G+I was identified as the best fit substitution model based on the Akaike information
116 criterion (AIC; Akaike 1974) implemented in MODELTEST. For both gene regions *Sepia*
117 *pharaonis* was used as an outgroup as it was the most closely related species for which COI
118 and cytb sequences were available. Maximum likelihood bootstrap values (BS) were
119 calculated using 1 000 bootstrap replicates and Bayesian inference (BI) was calculated
120 assuming unknown model parameters, and run over 5 000 000 generations, sampling the
121 Markov chain every 1 000 generations and using three heated chains and one cold chain. It
122 was considered that convergence had been reached on the basis that the standard deviation
123 of split frequencies was [Word missing. Remove brackets and insert 'was'?] (<0.01), with
124 the first 15% of trees discarded as burn-in. Percentage sequence divergences [plural?]
125 within and between species/clades were calculated using MEGA 6.06.

126

127 **Results**

128

129 In total, 52 individuals were sequenced for COI (345 bp) and 32 individuals were sequenced
130 for cytb (500 bp). Phylogenetic analysis of all sequences revealed three strongly supported
131 clades for both mtDNA regions, corresponding to the three described species of *S.*
132 *officinalis*, *S. hierredda* and *S. vermiculata* (Figures 2 and 3). COI and cytb sequences of
133 eight individuals from Ghana yielded two and six haplotypes respectively, which aligned with
134 *S. hierredda* according to BLAST searches. All COI sequences from South Africa ($n = 18$)
135 and Angola ($n = 10$) yielded a single haplotype, and based on phylogenetic placement were
136 concluded to be *S. vermiculata* (Figure 2). For the cytb sequences of 15 individuals from
137 South Africa, two haplotypes were present, with an additional four haplotypes resolved within
138 the cytb dataset for the six individuals sequenced from Angola. Again, all Angolan
139 haplotypes clustered with the South African *S. vermiculata* haplotypes (Figure 3).

140

141 Sequences that fell within the *Sepia officinalis* clade were from locations north of Mauritania,
142 including the English Channel and Mediterranean. As Perez-Losada et al. (2007)
143 demonstrated in their original analysis of the COI sequences used here, high levels of
144 intraspecific phylogenetic structuring was observed within *S. officinalis*, with three well-
145 supported COI clades (BI = 0.82–0.89, BS [Acronym not defined] = 86–99) observed in the
146 subset of COI sequences used in the present analysis. However, within *S. hierredda* and *S.*

Commented [AH1]: As far as I'm aware its always referred to as just sequence divergence with no plural. But I'll leave it to your discretion as to whether you would prefer it to be a plural.

147 *vermiculata* low levels of phylogenetic diversification were observed using COI. The cytb
148 dataset was comparatively more variable than that of COI, with greater levels of intraspecific
149 genetic divergence observed. This was particularly obvious in *S. vermiculata*, where the
150 Angolan sample (a single COI haplotype in Angolan and South African samples) comprised
151 four private haplotypes with moderate support for the divergence of this Angolan sample
152 from the South African sample (BI = 0.80–0.88, BS = 44–51).

153

154 Interspecific genetic distances (percentage sequence divergence) were greatest between *S.*
155 *officinalis* and *S. vermiculata* in both the COI (Table 2) and cytb (Table 3) datasets (COI =
156 13.37%, cytb = 12.20%), followed by *S. officinalis* and *S. hierredda* (COI = 11.37%, cytb =
157 11.71%), with *S. hierredda* and *S. vermiculata* the least genetically different (COI = 5.72%,
158 cytb = 4.83%). Comparatively, intraspecific genetic distances were low for all three species,
159 ranging from 0–1.12% for COI and 0.24–0.53% for cytb.

160

161 Discussion

162

163 Phylogenetic analysis of two mtDNA genes resolved three highly supported and reciprocally
164 monophyletic clades corresponding to *S. officinalis*, *S. hierredda* and *S. vermiculata*.
165 Applying phylogenetic species criteria, this result represents the first molecular genetic
166 confirmation of the distinct species status of *S. vermiculata*. This conclusion was further
167 supported by interspecific genetic distances which were greater than those observed
168 between other closely related but taxonomically distinct cephalopod species (Dai et al. 2012;
169 Amor et al. 2015), as well as ratios of within- to between-species DNA sequence divergence
170 which were in excess of commonly applied species barcoding ratios (Hebert et al. 2004;
171 Meyer and Paulay 2005; Lefebure et al. 2006).

172

173 Interestingly, and of pertinence to fisheries management of these species, the data
174 presented here show that the distribution of *S. vermiculata* extends further north than
175 previously described, with all samples from southern Angola falling within the *S. vermiculata*
176 clade in both the COI and cytb datasets. Prior to this investigation *S. vermiculata* was
177 considered to be a South African (and Indian Ocean) endemic, the extension of which
178 northward along the west African coast appeared to be limited to southern Namibia by the
179 cold waters of the Benguela upwelling region (Roeleveld 1972, 1998). However the coastal
180 areas of Angola have received comparatively limited prior research, particularly in relation to
181 the abundance and distribution of cephalopods, with the only mention of Angolan cuttlefish
182 coming from the bottom-trawl data of Bianchi (1992), where all *Sepia* caught were broadly
183 classified as belonging to the *S. officinalis* species complex. It may therefore be the case

184 that Angolan cuttlefish have been previously misidentified as *S. hierredda* rather than *S.*
185 *vermiculata*.**[This is a bit confusing. You have just said they were classified as *S.***
186 ***officinalis*, not *S. hierredda*.]** However, as only 10 Angolan samples were included in the
187 COI analysis, the absence of *S. hierredda* could also reflect a greater abundance of *S.*
188 *vermiculata* and/or temporal variance in distribution coinciding with sampling sites. The
189 misidentification of morphologically similar species and over/under representation of species
190 richness and abundance can cause inaccuracies in our understanding of biological,
191 ecological and evolutionary processes (Garcia-Vazquez et al. 2012; Tillett et al. 2012).
192 Consequently a comprehensive genetic analysis of further spatial and/or temporal samples
193 will be needed to accurately assess the extent of overlap or geographical separation
194 between these cuttlefish species.

195
196 Despite an overall lack of genetic diversity and structuring in the COI dataset of *S.*
197 *vermiculata*, analysis of *cytb* sequences revealed some evidence of phylogenetic
198 diversification between individuals from South Africa and Angola, which can be readily
199 aligned with the oceanography of this region. The expanse of coastal habitat between South
200 Africa and southern Angola is dominated by the Benguela ~~Cold Current~~Current System **[In**
201 **Abstract you refer to 'Benguela Current System'. Should be consistency in**
202 **terminology.]** and the associated perennial upwelling system. The Benguela system is an
203 area which, owing to its persistent cool upwelled waters, is generally considered to represent
204 a biogeographic and evolutionary boundary region to many marine species (e.g. Henriques
205 et al. 2014, 2016). More recently, Reid et al. (2016) reported asymmetric gene flow across
206 the Benguela upwelling system from South Africa into Angolan waters, indicating some
207 degree of historical permeability to this system that may help explain the patterns observed
208 here for *S. vermiculata*. This commonly observed restriction to gene flow in association with
209 the Benguela Current ~~S~~system **[Upper case 'S' in Abstract]** is likely enhanced in *S.*
210 *vermiculata* by its life-history characteristics, namely the lack of a highly dispersive pelagic
211 larval stage (Perez-Losada et al. 1999, 2002, 2007; Boyle 2000). In order to
212 comprehensively determine whether there is bi-parentally restricted gene flow across the
213 Benguela upwelling region and indeed between the putative species designations of this
214 study, analysis of nuclear genetic polymorphisms would be required. These findings thus
215 highlight the need for a comprehensive phylogeographic and population genetic evaluation
216 of *Sepia* across the southern African coast in order to fully characterise patterns of genetic
217 connectivity and the drivers behind them.

218
219 **Conclusion**

220

221 Here we not only provide the first molecular confirmation of the species status of *S.*
222 *vermiculata* but also extend this species' known geographical range within the east Atlantic
223 from the west coast of South Africa (Roeleveld 1972, 1998; Reid et al. 2005) to southern
224 Angola, and in doing so highlight the likely incidence of harvesting of misidentified species.
225 This has implications for the management of *Sepia* in southern African waters, which will
226 require a thorough investigation of the abundance and distributional limits of both *S.*
227 *vermiculata* and *S. hierredda* in order to appropriately conserve the biodiversity of this region
228 and negate the detrimental impacts of indiscriminate harvesting. Finally, we reveal subtle
229 patterns of phylogenetic diversification between *S. vermiculata* from South Africa and
230 Angola, indicating that, as for many marine teleosts (Henriques et al. 2014, 2016), the
231 Benguela upwelling region constitutes a biogeographic barrier to dispersal for the Sepiidae.
232 Ultimately this investigation highlights the need for a thorough molecular examination of
233 *Sepia* in west African waters and for this to be integrated into fisheries stock assessment,
234 with the aim of not only determining the stock status of cuttlefish fisheries but also
235 ascertaining the drivers that have promoted both inter- and intraspecific divergence within
236 this species complex.

237

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243

244 **References**

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383 **Figure legends**

384

385 **Figure 1:** Sampling sites for *S. vermiculata* and *S. hierredda* across the south-east Atlantic and Indian
386 oceans (GT = Tema, Ghana; AF = Flamingo [River](#), Angola; SB = Bushmans River, South Africa; SJ =
387 Jefferys Bay, South Africa), as well as locations of north-west Atlantic and Mediterranean sequences
388 of *S. officinalis* taken from GENBANK (MA = Mauritania; PF = Faro, Portugal; EC = English Channel;
389 GS = Gulf of Sidra). Coloured areas represent the currently recognised distribution of each species
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391 **Figure 2:** Bayesian phylogram depicting the relationships between *Sepia officinalis*, *Sepia hierredda*
392 and *Sepia vermiculata* sampled across the east Atlantic Ocean, Mediterranean Sea and Indian
393 Ocean, based upon partial sequences of the mtDNA COI gene. Bayesian inference posterior
394 probabilities are shown above nodes and maximum likelihood bootstrap values are given below.
395 Branch lengths are proportional to the number of nucleotide substitutions and *Sepia pharaonis* is
396 included as an outgroup species. Taxon codes refer to locations in Figure 1
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398 **Figure 3:** Bayesian phylogram depicting the relationships between *Sepia officinalis*, *Sepia hierredda*
399 and *Sepia vermiculata* sampled across the east Atlantic Ocean, Mediterranean Sea and Indian
400 Ocean, based upon partial sequences of the mtDNA cytb gene. Bayesian inference posterior
401 probabilities are shown above nodes and maximum likelihood bootstrap values are given below.
402 Branch lengths are proportional to the number of nucleotide substitutions and *Sepia pharaonis* is
403 included as an outgroup species. Taxon codes refer to locations in Figure 1
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409 **Table 1:** Collection locality and GENBANK accession numbers (where applicable) for all samples of the *Sepia officinalis*
 410 species complex used in this investigation, * denotes where sequences were obtained from GENBANK

| Country | Location | Code | <i>n</i> (COI) | <i>n</i> (cytb) | GenBank accession numbers |
|-----------------|----------------|-----------------------|----------------|-----------------|---------------------------|
| South Africa | Jeffreys Bay | SJ | 8 | 7 | |
| South Africa | Bushmans River | SB | 10 | 8 | |
| Angola | Flamingo River | AF | 10 | 6 | |
| Ghana | Tema | GT | 8 | 8 | |
| Mauritania | | MA | 4* | | EF416525–EF416528 |
| Portugal | Faro | PF | 4* | | EF416384–EF416387 |
| English Channel | | EC | 4* | 1 | EF416306–EF416309 |
| Gulf of Sidra | | GS | 4* | | EF416535–EF416538 |
| Unknown | | <i>S. officinalis</i> | | 2* | AB240155, NC007895 |
| Unknown | | <i>S. pharaonis</i> | 1* | 1* | NC02146 |

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Table 2: Pairwise genetic distances between *Sepia officinalis*, *Sepia hierredda* and *Sepia vermiculata* based on partial sequences of the mtDNA COI gene. Percentage sequence divergence between putative species/clades are given below the diagonal with *P*-distances above the diagonal. Intraspecific percentage sequence divergence is on the diagonal. Standard error for all distance values are given in parentheses

| | <i>S. officinalis</i> | <i>S. hierredda</i> | <i>S. vermiculata</i> |
|-----------------------|-----------------------|---------------------|-----------------------|
| <i>S. officinalis</i> | 1.12 (0.30) | 0.11 (0.02) | 0.13 (0.02) |
| <i>S. hierredda</i> | 11.37 (1.51) | 0.12 (0.12) | 0.06 (0.01) |
| <i>S. vermiculata</i> | 13.37 (1.72) | 5.72 (1.23) | 0.00 (0.00) |

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Table 3: Pairwise genetic distances between *Sepia officinalis*, *Sepia hierredda* and *Sepia vermiculata* based on partial sequences of the mtDNA cytb gene. Percentage sequence divergence between putative species/clades are given below the diagonal with *P*-distances above the diagonal. Intraspecific percentage sequence divergence is on the diagonal. Standard error for all distance values are given in parentheses

| | <i>S. officinalis</i> | <i>S. hierredda</i> | <i>S. vermiculata</i> |
|-----------------------|-----------------------|---------------------|-----------------------|
| <i>S. officinalis</i> | 0.53 (0.26) | 0.12 (0.01) | 0.12 (0.01) |
| <i>S. hierredda</i> | 11.71 (1.35) | 0.41 (0.18) | 0.05 (0.01) |
| <i>S. vermiculata</i> | 12.20 (1.35) | 4.83 (0.90) | 0.24 (0.13) |

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