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Healey, Amy; McKeown, Niall; Potts, Warren M.; de Beer, Chenelle; Sauer, W. H. H.; Shaw, Paul

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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

AJE Healey[1]*, NJ McKeown[1], WM Potts[2], CL de Beer[2], W Sauer[2] and PW Shaw[1,2]

[1] Edward Llwyd Building, Aberystwyth University, Aberystwyth, Dyfed, Wales

[2] Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown, South Africa

** Corresponding author, e-mail: amh5@aber.ac.uk*

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

Keywords: biogeography, cephalopod, cuttlefish, dispersal, ecosystem compatible exploitation, fisheries management, indiscriminate harvesting

Introduction

As many traditionally exploited fin-fish stocks continue to decline there is growing interest in the expansion of cephalopod fisheries (Boyle 2000; Young et al. 2006; Anderson et al. 2011; Jereb et al. 2015). The typical short life cycle of cephalopods renders them vulnerable to overfishing (Rodhouse et al. 2014) 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). Fundamental to this is both accurate species identification and resolution of species ranges (Taylor et al. 2012; McKeown et al. 2015).

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

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

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

Methods

Sampling and mtDNA sequencing

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

Genomic DNA was extracted from all samples using a standard CTAB-chloroform/isoamylalcohol method (Winnepenninckx et al. 1993). Partial sequences of the mtDNA COI and cytb genes were amplified by polymerase chain reaction (PCR) using species-specific primers developed specifically for this study (COI: SepiaCOIF 5'-GTAAACCTGGTACACTTTT-'3, SepiaCOIR 5'-TTCTATTTGTAAACCTTCTCATC-'3; cytb: cytb117F 5'-CCCCCAATCCAAGTTAACAA-'3, cytb928R 5'-ATGCGGGATGTGAATTATGG-'3). PCRs were performed in a total volume of 20 µl, containing 4 µl template DNA, 2 mM $MgCl_2$, 0.5 µM forward primer and 0.5 µM of reverse primer, 0.2 mM dNTP mix (20 µM each dATP, dCTP, dGTP, dTTP), 1x reaction buffer [75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$] and *Taq* polymerase (BIOTAQ, 5 U/µl). The PCR thermo-profile for COI amplification was: 180 s at 95 °C, followed by 40 cycles of 30 s at 95 °C, 45 s annealing at 50 °C and 60 s at 72 °C, followed by a final 5 min extension at 72 °C. For cytb amplification, PCR conditions were: 180 s at 95 °C, followed by 34 cycles of 30 s denaturing at 95 °C, 30 s annealing at 52 °C and 30 s at 72 °C, again followed by a final 5 min extension at 72 °C. The PCR products were then purified and sequenced using BigDye technology, with sequence identity confirmed using BLAST.

Phylogenetic sequence analysis

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

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.

Results

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

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

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

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

Discussion

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

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

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

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

Conclusion

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

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Figure legends

Figure 1: Sampling sites for *S. vermiculata* and *S. hierredda* across the south-east Atlantic and Indian oceans (GT = Tema, Ghana; AF = Flamingo River, Angola; SB = Bushmans River, South Africa; SJ = Jefferys Bay, South Africa), as well as locations of north-west Atlantic and Mediterranean sequences of *S. officinalis* taken from GENBANK (MA = Mauritania; PF = Faro, Portugal; EC = English Channel; GS = Gulf of Sidra). Coloured areas represent the currently recognised distribution of each species

Figure 2: Bayesian phylogram depicting the relationships between *Sepia officinalis*, *Sepia hierredda* and *Sepia vermiculata* sampled across the east Atlantic Ocean, Mediterranean Sea and Indian Ocean, based upon partial sequences of the mtDNA COI gene. Bayesian inference posterior probabilities are shown above nodes and maximum likelihood bootstrap values are given below. Branch lengths are proportional to the number of nucleotide substitutions and *Sepia pharaonis* is included as an outgroup species. Taxon codes refer to locations in Figure 1

Figure 3: Bayesian phylogram depicting the relationships between *Sepia officinalis*, *Sepia hierredda* and *Sepia vermiculata* sampled across the east Atlantic Ocean, Mediterranean Sea and Indian Ocean, based upon partial sequences of the mtDNA cytb gene. Bayesian inference posterior probabilities are shown above nodes and maximum likelihood bootstrap values are given below. Branch lengths are proportional to the number of nucleotide substitutions and *Sepia pharaonis* is included as an outgroup species. Taxon codes refer to locations in Figure 1

Table 1: Collection locality and GENBANK accession numbers (where applicable) for all samples of the *Sepia officinalis* species complex used in this investigation, * denotes where sequences were obtained from GENBANK

Country	Location	Code	*n* (COI)	*n* (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		*S. officinalis*		2*	AB240155, NC007895
Unknown		*S. pharaonis*	1*	1*	NC02146

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

	S. officinalis	*S. hierredda*	*S. vermiculata*
S. officinalis	1.12 (0.30)	0.11 (0.02)	0.13 (0.02)
S. hierredda	11.37 (1.51)	0.12 (0.12)	0.06 (0.01)
S. vermiculata	13.37 (1.72)	5.72 (1.23)	0.00 (0.00)

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

	S. officinalis	*S. hierredda*	*S. vermiculata*
S. officinalis	0.53 (0.26)	0.12 (0.01)	0.12 (0.01)
S. hierredda	11.71 (1.35)	0.41 (0.18)	0.05 (0.01)
S. vermiculata	12.20 (1.35)	4.83 (0.90)	0.24 (0.13)