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Species differentiation of *Sepia officinalis* and *Sepia hierredda* (Cephalopoda: Sepiidae) based on morphological and allozyme analyses

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Two taxa of commercially exploited cuttlefish, *Sepia officinalis* and *S. hierredda*, are compared for the first time on the bases of quantitative morphology and allozyme polymorphisms. Morphometric measurements and meristic counts of selected soft and hard (cuttlebone) body characters, with allozyme electrophoretic analysis are used. Samples were obtained from north-west Iberian Peninsula and Senegalese waters (West Africa). Significant differences in mantle width, arm and hectocotylus length, numbers of rows of reduced suckers on the hectocotylus and in most cuttlebone measurements were found. Canonical discriminant functions of cuttlebone measurements for males and females were calculated. Allozyme electrophoresis for 33 presumptive loci showed low levels of genetic variability and 13 diagnostic loci between the two *Sepia* taxa. The genetic identities (*I*) in pairwise comparisons of populations of both taxa were $I=0.582–0.596$, which are typical values for congeneric species. These congruent morphological and genetic results strongly suggest that *S. officinalis* and *S. hierredda* are different species.

INTRODUCTION

Cuttlefish are a relatively important marine resource. World total catches of cuttlefish were about 225,000 mt in 1995 of which approximately 45,000 mt were *Sepia officinalis* Linnaeus (1758) or *S. hierredda* Rang (1837) from the Sahara Bank (Eastern Central Atlantic) (FAO, 1998). In the Sahara Bank and off the peninsula of Cape Verde, where the fishing grounds for cuttlefish are located in this Atlantic region, both taxonomic status and geographic distribution patterns of these taxa are still not sufficiently clear. However, the geographical distribution of *S. officinalis* and *S. hierredda* off the north-western coast of Africa shows that both species are sympatric. The southern boundary of *S. officinalis* coincides approximately with the border between Mauritania and Senegal (16°N) and the northern limit of *S. hierredda* is at the latitude of Cape Blanc (21°N) (Hatanaka, 1979).

Sepia officinalis and *S. hierredda* have long been confined within an arbitrary subspecies–species range depending on the author preference. Four subspecies of *S. officinalis* were considered to inhabit the western coast of Africa: *S. officinalis officinalis* L. (1758), *S. officinalis fillouxi* Lafont (1868), *S. officinalis hierredda* Rang (1837) and *S. officinalis vermiculata* Quoy & Gaimard (1832) (Adam, 1941, 1952; Adam & Rees, 1966). These taxa were differentiated on morphological bases, such as body proportions. Although *S. officinalis fillouxi* was considered indistinct from *S. officinalis officinalis* by Mangold (1966), the remaining three have been long considered as subspecies of *S. officinalis* (Hatanaka, 1979; Bakhayokho, 1983, 1991). These three subspecies recently have been considered as species (Khromov et al., 1998), but the separation was based on marked morphological differences, which have not been tested statistically. Therefore, more detailed studies are needed to confirm this separation and to test objectively the species status of these taxa.

Refined morphological analysis combined with genetic studies may be needed as a multidisciplinary approach, which has advantages not conferred by the use of either technique individually. However, until now few cephalopod studies have attempted to compare taxa using both methodologies together (e.g. Augustyn & Grant, 1988). Allozyme electrophoresis has several advantages over morphological studies because allozymes are primary products of the genome (Ayala, 1983; Avise, 1994). Thus, allozyme polymorphisms have proven to be effective for detecting cryptic species and studying phylogenetic relationships in commercially exploited cephalopod resources (e.g. Sanjuan et al., 1996; Carvalho & Nigmatullin, 1998; Pérez-Losada, 1998).

The main aim of the present study was to combine morphological and genetic analyses to discriminate and to clarify the taxonomic status of *S. officinalis* and *S. hierredda*.

MATERIALS AND METHODS

Sample collection

Samples of 71 specimens (33 males and 38 females) of *Sepia officinalis* (SfNW) were obtained from Galician waters (north-west Iberian Peninsula) (Figure 1) during 1992–994. All these specimens were taken from commercial catches on the day of capture, frozen on board at −20°C and transported to the laboratory where they were stored at −72°C until further processing.

Two samples of *Sepia* species were taken from Senegalese waters (West Africa) (Figure 1): sample 1, comprised by 24 specimens of *S. hierredda* (ShSe1) and five of *S. officinalis* (SfSe1) collected on 25 May 1996 (15°50′N 16°52′W, 47-m depth). Sample 2, comprised 85 specimens of *S. hierredda* (ShSe2) collected on 14 August 1997 (15°47′N 16°58′W, 38-m depth). Sample 1 and 21 specimens of sample 2 were used for allozyme analysis. The remaining 64 specimens (32 males and 32 females) of sample 2 were employed for morphological studies. Both samples were taken off the northern boundary of Senegal where the distribution zones of the two species overlap.

Morphological analysis

After thawing at room temperature, individual sexes were determined and the following measurements and counts (variables) from soft-body parts were recorded: dorsal mantle length (ML), body weight (BW), mantle width (MW), length of right arms (AL I, AL II, AL III and AL IV), and for males hectocotylized arm length (HcL) and number of rows of reduced suckers on the hectocotylus (NRSH). The width of the protective membranes of arms and the size and pattern of the suckers on tentacular clubs were other soft-body features previously used to distinguish between these taxa (Adam, 1952). These characters were checked and they exhibited such high intrinsic variation (data not shown) independent of the species membership that they cannot be considered accurate characters with systematic value. Therefore, they were not used in our morphological studies. Adam (1952) also indicated differences between species in the dentition of the chitinous rings of suckers from the arms and tentacular clubs. This character has not been tested in the present study, mainly because sucker-ring tooth counts are too tedious and complicated for routine population analysis. The cuttlebone was extracted from each specimen and the following measurements of this hard structure were recorded (Figure 2): cuttlebone length (CL), cuttlebone weight (CW), striated zone length (SZ), non-striated zone or last loculus length (NSZ), cuttlebone width (CWi), phragmocone length (FL), phragmocone width (FW), inner cone width (ICW), outer cone width (OCW) and cuttlebone thickness (CT). ICW and OCW are actually measured perpendicular to the length axis at the level of the posterior tip of the phragmocone. The spine of the cuttlebone was checked in all specimens and then compared between species. Weights and measurements were recorded to the nearest g or mm. Body and cuttlebone measurements were expressed as percentage of ML and CL, respectively (Appendix 1).

Mean, standard deviation and range of variation for each variable were estimated and compared. Distributions of these measurements were normal. Linear regression

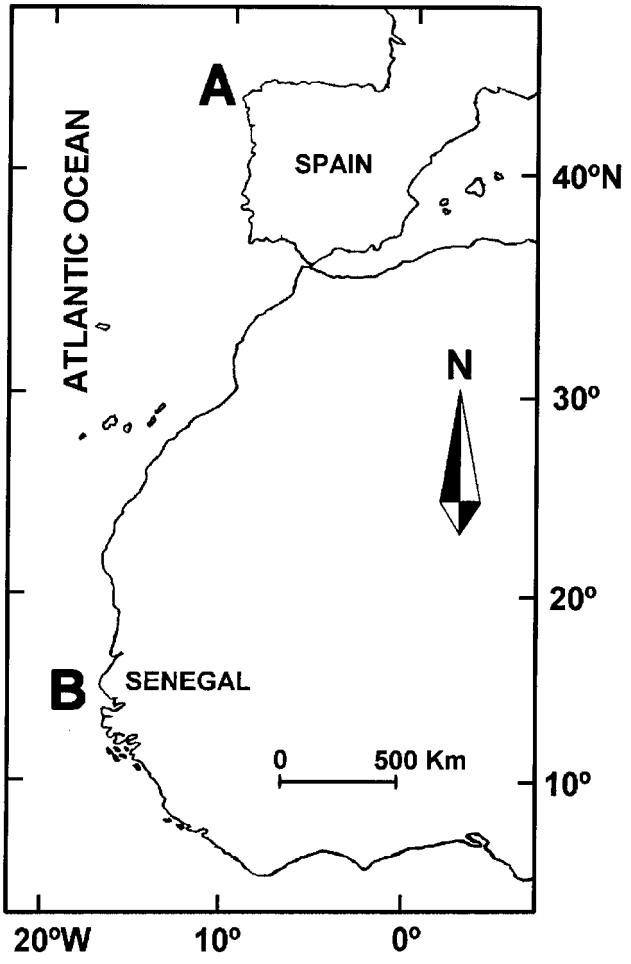

Figure 1. Map showing the origin of the *Sepia officinalis* and *S. hierredda* samples. (A) Galician waters (north-west Iberian Peninsula); (B) Senegalese waters (West Africa).

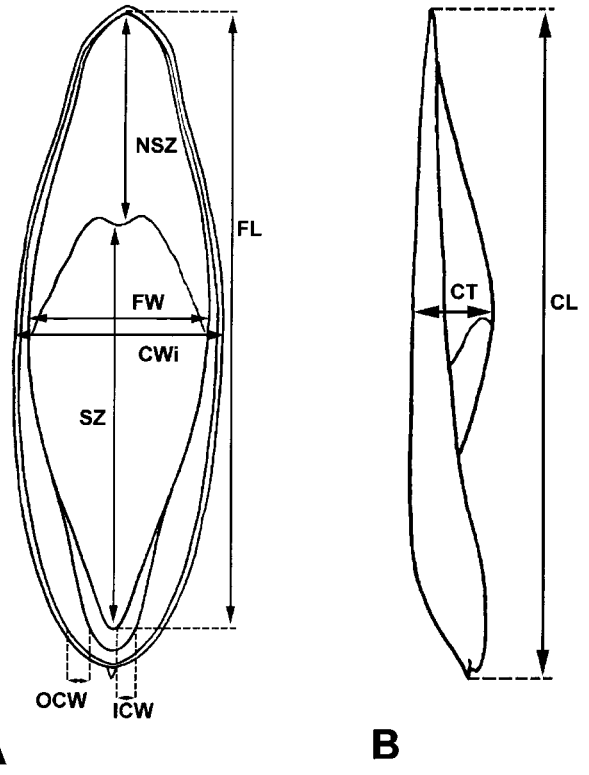

Figure 2. Morphometric measurements carried out on the cuttlebone of *Sepia officinalis* and *S. hierredda*. (A) Ventral view; (B) lateral view. CL, cuttlebone length; CT, cuttlebone thickness; Cwi, cuttlebone width; FL, phragmocone length; FW, phragmocone width; ICW, inner cone width; NSZ, non-striated or last loculus zone length; OCW, outer cone width; SZ, striated zone length.

parameters for each soft-body size variable vs mantle length and cuttlebone size variables vs cuttlebone length were obtained. A Student's *t*-test (Zar, 1984) was carried out to test whether the slopes and intercepts of the regression lines differed significantly between the species.

Two canonical discriminant analyses (CDA) were undertaken using all soft and hard continuous variables and only hard (cuttlebone) continuous variables, respectively. Stepwise analyses were employed comparing sexes separately. For CDA size effect was removed plotting each variable vs ML or CL and then taking the residual for pooled regressions of each variable. CDA were then carried out using residuals as variables (R-variable). For CDA the STATISTICA 5.0 software (StatSoft Inc. 1984–1995) was used. The classification functions were computed for each species.

Although CDA using soft and hard variables was able to separate the two species, several soft-body variables were not available due to damage resulting from collecting method (trawl nets). This caused soft CDA to use too few specimens (e.g. 12 males and 19 females in the case of *S. hierredda*), thus decreasing the effective power of the analysis. For this reason, these results will be not shown on the present paper. However, in spite of this statistical constraint, both soft and hard variables employed to discriminate between species are quite interesting and will be briefly discussed.

Allozyme analysis

Samples of mantle muscle were prepared for electrophoresis using methods previously described for *Sepia*

Table 1. *Regression equations and results of* t*-test analyses to compare slope and intercepts of regressions of morphological characters between males and females of* Sepia officinalis *and* S. hierredda. *Linear equation:* $y=b(x)+a$. *For variable names see Materials and Methods.*

	Males equation				Females equation				Slope comparison		Intercept comparison	
	a	b	r^2	N	a	b	r^2	N	*t*	v	*t*	v
Sepia officinalis												
Character vs ML												
MW	6.016	0.549	0.95	33	11.175	0.531	0.92	38	1.553	67	1.642	68
AL I	−9.963	0.701	0.87	33	−16.639	0.686	0.95	38	−3.571**	67	−3.277*	68
AL II	−9.754	0.698	0.90	32	−15.742	0.685	0.92	38	−3.224*	66	−2.983*	67
AL III	−9.604	0.722	0.91	33	−22.215	0.748	0.94	38	−3.562**	67	−3.444**	68
AL IV	−19.129	0.828	0.90	33	−29.589	0.957	0.96	38	−1.823	67	−1.862	68
HcL	−17.763	0.814	0.91	33								
Character vs CL												
SZ	−5.497	0.464	0.96	33	−4.692	0.445	0.95	38	−1.980	66	−1.717	67
NSZ	5.627	0.468	0.98	33	5.352	0.479	0.96	37	1.561	66	1.346	67
Cwi	0.606	0.367	0.99	33	−2.221	0.407	0.99	38	7.350**	66	4.888**	67
FL	1.432	0.913	0.99	33	0.788	0.919	0.99	37	0.307	66	0.104	67
FW	−0.616	0.325	0.99	33	−3.163	0.358	0.98	38	5.229**	66	3.939**	67
ICW	−0.618	0.049	0.89	33	−1.205	0.052	0.91	38	−0.822	66	−0.959	67
OCW	−1.791	0.108	0.94	32	−2.354	0.116	0.88	38	1.784	65	1.684	66
CT	−2.077	0.148	0.96	33	−3.439	0.162	0.97	38	0.957	65	1.726	67
Sepia hierredda												
Character vs ML												
MW	6.235	0.487	0.77	21	8.898	0.470	0.80	23	−0.093	40	−0.044	41
AL I	−30.679	0.749	0.93	20	−23.669	0.670	0.93	20	0.199	36	0.414	37
AL II	−31.113	0.768	0.96	21	−28.591	0.729	0.91	21	−0.050	38	0.063	39
AL III	−32.665	0.788	0.94	21	−27.984	0.725	0.88	21	−0.114	38	0.050	39
AL IV	−47.793	1.003	0.95	18	−36.519	0.858	0.88	22	−0.123	36	0.181	37
HcL	−50.547	1.035	0.96	14								
Character vs CL												
SZ	−12.581	0.570	0.96	32	−18.851	0.627	0.91	32	1.429	60	1.322	61
NSZ	10.304	0.368	0.90	32	15.172	0.329	0.80	31	−0.608	59	−0.546	60
Cwi	2.375	0.326	0.99	32	1.195	0.347	0.97	32	3.555**	60	2.954*	61
FL	−1.740	0.934	0.99	32	−2.219	0.939	0.99	32	1.316	60	1.292	61
FW	2.495	0.266	0.98	32	0.018	0.297	0.97	32	4.460**	60	3.416*	61
ICW	0.851	0.036	0.83	32	0.314	0.042	0.78	32	1.213	60	0.925	61
OCW	−0.166	0.067	0.88	31	−0.351	0.071	0.88	31	1.390	58	1.308	59
CT	−1.994	0.135	0.98	32	−0.996	0.128	0.97	32	0.885	60	1.269	61

N, number of individuals; r^2, determination coefficient; *, $P<0.05$; **, $P<0.001$.

species (Pérez-Losada et al., 1996; Pérez-Losada, 1998). Standard horizontal starch gel electrophoresis was carried out (Murphy et al., 1996). Twenty-eight enzymes, yielding 33 putative enzyme-coding loci, displayed adequate activity and resolution for consistent interpretation and routine examination (Appendix 2). Different electrophoretic conditions were used to test for homology among loci. Routine electrophoretic conditions and histochemical staining recipes for the enzymes are described in Pérez-Losada (1998) and Pérez-Losada et al. (1999).

Genotype frequencies at polymorphic loci were tested for agreement with Hardy–Weinberg equilibrium expectations by χ^2-tests. Heterogeneity of allele frequencies among *Sepia* samples was tested using a χ^2 homogeneity test. The probability of the null-hypothesis was estimated using Monte Carlo simulation (Roff & Bentzen, 1989). Estimates of genetic variability were calculated for each sample and species. Genetic identity (*I*; Nei, 1972) between *Sepia* samples and their bootstrap confidence estimates were also calculated. The 95% bootstrap confidence limit on *I* was constructed by the percentile method (Felsenstein, 1988).

The computer program BIOSYS-1 (Swofford & Selander, 1981) was used to perform most of the genetic analyses. The probability of the null-hypothesis of the χ^2 statistics was computed using Zaykin & Pudovkin's (1993) computer programs. Nei's (1972) genetic identity (*I*) together with bootstrap confidence estimates were calculated out with the Dbot program (D.V. Zaykin, Tatarenkov & A.I. Pudovkin, personal communication).

Table 2. *Regression equations and results of* t*-test analyses to compare slope and intercepts of regressions of morphological characters between* Sepia officinalis *and* S. hierredda. *Linear equation:* $y=b(x)+a$. *For variable names see Material and Methods and for regression equations see Table 1.*

Sepia officinalis vs *S. hierredda*		Slope		Intercept	
		t	v	*t*	v
Males					
Character vs ML	MW	3.061***	50	2.750**	51
	AL I	5.549****	49	4.436****	50
	AL II	5.243****	49	4.379****	50
	AL III	6.053****	50	4.791****	51
	AL IV	3.353***	47	3.204***	48
	HcL	2.494*	43	2.629*	44
Character vs CL	SZ	−5.392****	61	−4.057****	62
	NSZ	7.843****	61	5.231****	62
	CWi	9.719****	61	5.728****	62
	FL	1.170	61	1.784	62
	FW	13.321****	61	6.227****	62
	ICW	1.898	61	1.146	62
	OCW	13.995****	59	6.268****	60
	CT	6.512****	61	4.725****	62
Females					
Character vs ML	MW	4.607****	57	3.935****	58
	AL I	5.117****	54	4.140****	55
	AL II	3.618****	55	3.395***	56
	AL III	4.168****	55	3.575****	56
	AL IV	3.522****	56	3.016***	57
Character vs CL	SZ	−7.879****	65	−5.212****	66
	NSZ	8.763****	64	5.570****	65
	CWi	11.693****	65	6.221****	66
	FL	0.424	65	0.930	66
	FW	12.097****	65	6.300****	66
	ICW	−0.110	65	−0.687	66
	OCW	13.012****	64	6.450****	65
	CT	8.559****	65	5.283****	66

t, *t*-test value; v, freedom degrees; *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$; ****, $P<0.001$.

RESULTS

Morphological analysis

Six soft-body variables (mantle width, four arm lengths and hectocotylized arm length) were significantly different between *Sepia officinalis* and *S. hierredda* (Appendix 1; Tables 1&2). Thus, the mantle of *S. hierredda* was narrower and both the unmodified arms and the hectocotylized arm were shorter than those in *S. officinalis*. Moreover, the number of transverse rows of reduced suckers on the hectocotylus was higher (8–4) in *S. hierredda* than in *S. officinalis* (4–8).

Differences between sexes in each *Sepia* taxon were found for the hard-structure (cuttlebone) variables CW and FW (Table 1). In consequence, subsequent morphometric and CDA analyses were made using sexes separately. Only the phragmocone length (FL) and inner cone width (ICW) did not differ between species in both sexes (Table 2). The significant differences found (Appendix 1) between cuttlebones of the two species were as follows: (i) the striated zone of the cuttlebone of

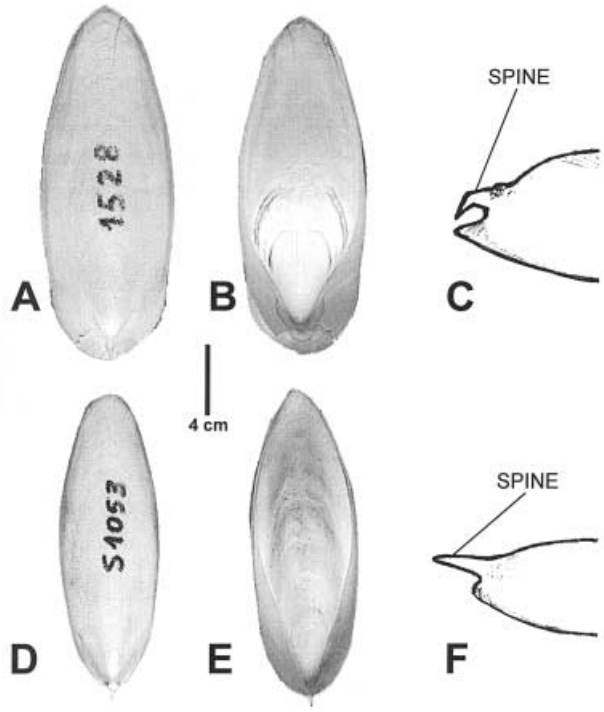

Figure 3. Cuttlebone and spine detail in *Sepia* species. *Sepia officinalis*: dorsal (A) and ventral (B) views of cuttlebone and lateral view of scheme spine (C). *Sepia hierredda*: dorsal (D) and ventral (E) views of cuttlebone and lateral view of scheme spine (F).

Table 3. *Discriminant analysis results made for males and females between* Sepia officinalis *and* S. hierredda *using residuals of each variable (R-variable). (A) Stepwise analysis summary; (B) raw coefficients for canonical variables and classification functions.*

A.	Males			Females		
Step	Variable	*F*	Lambda	Variable	*F*	Lambda
1	R-FW	83.635	0.430	R-OCW	137.309	0.331
2	R-OCW	27.422	0.298	R-FW	25.202	0.241
3	R-NSZ	7.501	0.265	R-ICW	13.279	0.200
4	R-FL	3.642	0.250	R-NSZ	4.692	0.187
5	R-SZ	1.568	0.244	R-FL	1.934	0.181
6	R-ICW	1.814	0.236	R-CT	1.227	0.178
7				R-SZ	1.017	0.175

B.	Males Classification functions			Females Classification functions			
Variable	RC	*Sepia officinalis* (*P*=0.508)	*S. hierredda* (*P*=0.492)	Variable	RC	*Sepia officinalis* (*P*=0.543)	*S. hierredda* (*P*=0.457)
R-FW	−0.405	0.350	−1.085	R-OCW	−0.450	0.986	−0.948
R-OCW	−0.317	0.482	−0.641	R-FW	−0.314	0.865	−0.484
R-NSZ	−0.052	0.092	−0.091	R-ICW	0.826	−2.021	1.528
R-FL	−0.231	0.269	−0.550	R-NSZ	0.013	−0.014	0.443
R-SZ	−0.049	−0.051	0.122	R-FL	−0.224	0.499	−0.464
R-ICW	0.336	−0.130	1.059	R-CT	−0.181	0.345	−0.433
				R-SZ	0.054	−0.083	0.150
Constant	−0.552	−1.437	−3.519	Constant	0.364	−3.369	−2.766
Eigenvalue	3.233			Eigenvalue	4.712		
CR	0.874			CR	0.908		

F, value of F to enter or remove the variable; RC, raw coefficients for canonical variables; CR, canonical value of correlation; *P*, probability proportional to the group size. For variable names see Material and Methods.

S. officinalis was smaller (41.2% as percentage of ML) than in *S. hierredda* (46.9%); (ii) the cuttlebone of *S. officinalis* was wider and thicker (38.1 and 13.2% as percentage of ML, respectively) than the cuttlebone of *S. hierredda* (35.2 and 11.9% respectively); and (iii) the phragmocone and the outer cone of *S. officinalis* were wider than in *S. hierredda*.

On the other hand, the cuttlebone of *S. officinalis* was slightly acuminate at the anterior end while it was very acuminate in *S. hierredda* (Figure 3). Furthermore, the spine of the cuttlebone of *S. officinalis* was usually broken and covered by a chitinous material, especially in adults (Figure 3C), while the spine of the cuttlebone of *S. hierredda* was never broken and never covered (Figure 3F).

Canonical discriminant analysis (CDA) using only hard (cuttlebone) variables was able to separate both species in six and seven steps for males (Wilks's lambda: 0.236; $F_{(6,\ 58)}$: 31.255; $P<0.000001$) and females (Wilks's lambda: 0.175; $F_{(7,\ 62)}$: 41.739; $P<0.000001$), respectively. The variables employed in the stepwise analysis (Table 3A) were R-FW, R-OCW, R-NSZ, R-FL, R-SZ and R-ICW in males, and R-OCW, R-FW, R-ICW, R-NSZ, R-FL, R-CT and R-SZ in females. In both sexes, residuals of FW (R-FW) and OCW (R-OCW) were the most powerful variables to discriminate between species. The raw coefficients for canonical variables and the values of classification functions for males and females of *S. officinalis* and *S. hierredda* are shown in Table 3B.

Allozyme analysis

Allele frequencies at the 33 enzyme loci are shown in Appendix 2. Thirteen enzyme loci were completely diagnostic between *S. officinalis* from north-west Iberian Peninsula (SfNW) and *Sepia hierredda* (ShSe1 and ShSe2): *AAT-1**, *ACP**, *AK**, *G6PDH**, *GLUDH**, *IDHP**, *MDH-1**, *MEP**, *OPDH-2**, *PEPA**, *PEPB**, *PGDH** and *PGM**. Sample 1 of *S. hierredda* and *S. officinalis* (ShSe1+SfSe1; N=24+5) showed that SfSe1 individuals had the same characteristic alleles as *S. officinalis* from Galician waters (SfNW) for the diagnostic loci, except for *OPDH-2**. The *F*-values for the polymorphic loci in the samples of *S. hierredda* (ShSe1 and ShSe2) and *S. officinalis* (SfNW and SfSe1), as well as in the other samples, did not show significant deviation from Hardy–Weinberg expectations, except for *IDDH** and *MEP** in SfSe1 (F_{SfSe1}, Table 4). However, when sample 1 of *S. hierredda* and *S. officinalis* from Senegalese waters was considered as a whole (ShSe1+SfSe1) because they were not previously identified, the 13 diagnostic loci between *S. hierredda* and *S. officinalis* displayed a highly significant deficit of heterozygotes ($F>0.65$; Table 4). No significant differences in allele frequencies were found between the SfSe1 and

Table 4. *Estimates of* F*-statistics for 18 polymorphic loci in the putative sample of* Sepia hierredda *and* S. officinalis *from Senegal 1 (ShSe1+SfSe1) ($F_{ShSe1+SfSe1}$) and in the subsample of* S. officinalis *from Senegal 1 (F_{SfSe1}), with their significant levels for the χ^2 of goodness-of-fit to Hardy–Weinberg expected proportions. The homogeneity χ^2-values between allele frequencies of samples of* S. hierredda *from Senegal ($\chi^2_{ShSe1-ShSe2}$),* S. hierredda *from Senegal 1 and* S. officinalis *from north-west Iberian Peninsula ($\chi^2_{ShSe1-SfNW}$),* S. hierredda *from Senegal 2 and* S. officinalis *from north-west Iberian Peninsula ($\chi^2_{ShSe2-SfNW}$), and* S. officinalis *from Senegal 1 and north-west Iberian Peninsula ($\chi^2_{SfSe1-SfNW}$), are also shown. Estimated probabilities of homogeneity χ^2 after 1000 runs of Monte Carlo simulations were indicated for each polymorphic locus.*

Locus	$F_{ShSe1+SfSe1}$	F_{SfSe1}	$\chi^2_{ShSe1-ShSe2}$	$\chi^2_{ShSe1-SfNW}$	$\chi^2_{ShSe2-SfNW}$	$\chi^2_{SfSe1-SfNW}$
*AAT-1**	1.000***			168.0***	162.0***	
*ACP**	1.000***		10.0**	168.0***	162.0***	
*AK**	1.000***			168.0***	162.0***	0.2
*ALPDH**				0.8	0.7	
*G6PDH**	1.000***			168.0***	162.0***	
*GLUDH**	1.000***			168.0***	162.0***	
*IDDH**	0.502**	0.655	5.7*	145.8***	162.0***	12.9*
*IDHP**	0.680***	−0.250	0.9	168.0***	162.0***	14.1***
*LAP**			2.3		5.8*	
*MDH-1**	1.000***			168.0***	162.0***	
*MEP**	1.000***	1.000*		168.0***	162.0***	102.3***
*MPI**			1.2		2.9	
*OPDH-1**	−0.018	−0.111	2.3	14.0***	12.3**	1.0
*OPDH-2**	0.868***	−0.111		168.0***	162.0***	12.1
*PEPA**	1.000***			168.0***	162.0***	
*PEPB**	1.000***			168.0***	162.0***	
*PGDH**	0.707***		2.1	166.0***	162.0***	
*PGM**	1.000***			168.0***	162.0***	

Significance levels for χ^2-tests: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Table 5. *Genetic identities (*I*; Nei, 1972) with standard errors (SE), 95% confidence interval (95% CI) and bootstrap estimates (after 1000 runs) for standard Nei's similarities between samples of* Sepia. *Sample codes are as Material and Methods, and Table 4.*

				Bootstrap	
Sample pairs	*I*	SE	95% CI	5%	95%
ShSe1 − ShSe2	0.998	0.001	0.997–1.000	0.997	1.000
SfNW − SfSe1	0.968	0.023	0.924–1.012	0.929	0.999
SfSe1 − ShSe1	0.596	0.086	0.428–0.765	0.465	0.737
SfSe1 − ShSe2	0.593	0.086	0.424–0.762	0.453	0.738
SfNW − ShSe1	0.585	0.087	0.415–0.755	0.448	0.737
SfNW − ShSe2	0.582	0.087	0.412–0.753	0.457	0.721

SfNW samples for the homogeneity χ^2-test, except for *IDDH**, *IDHP** and *MEP** ($\chi^2_{SfSe1-SfNW}$). This last result could be due either to the few individuals of the SfSe1 subsample (N=5) or to the large geographical distance between both samples. However, highly significant heterogeneity was found between SfNW and each of the two *S. hierredda* samples for most loci ($\chi^2_{ShSe1-SfNW}$ and $\chi^2_{ShSe2-SfNW}$). On the contrary, these last two Senegalese samples showed no significant genetic differentiation ($\chi^2_{ShSe1-ShSe2}$) for most loci, except the *ACP** and *IDDH** loci. Estimates of genetic variability for studied samples are shown in the bottom of Appendix 2.

Nei's (1972) genetic identity (*I*) between *Sepia* samples and their bootstrap estimates are shown in Table 5. The intraspecific identity values were high ($I>0.968$) and the interspecific identity values were moderate for all the *Sepia* comparisons ($I=0.582$–0.596). Non-overlap in the bootstrap confidence intervals between the intra and interspecific *I*-values for *S. officinalis* and *S. hierredda* indicated significant differences.

DISCUSSION

The present work shows for the first time a rigorous morphological comparison between *Sepia officinalis* and *S. hierredda*, which corroborates some previous studies (Adam, 1941, 1952; Adam & Rees, 1966; Okutani, 1967; Hatanaka, 1979; Khromov, 1998; Khromov et al., 1998). The number of transverse rows of reduced suckers in the hectocotylus is higher (8–4) in *S. hierredda* than in *S. officinalis* (4–8). The length of the striated zone of the cuttlebone is smaller in *S. officinalis* than in *S. hierredda* for individuals of the same ML, and the general form of the cuttlebone is slightly acuminate in *S. officinalis* vs very acuminate in *S. hierredda*. Moreover, for first the time it is shown that both species differ in the length of all arms, including the hectocotilized one, and the width of the mantle.

The cuttlebone is an excellent diagnostic tool for *Sepia* species differentiation. The use of the cuttlebone in the taxonomic study of cuttlefish is very old and has rendered good results (Neige & Boletzky, 1997; Lu, 1998). Khromov (1998) used some descriptive cuttlebone characteristics to separate cuttlefish, including *S. officinalis* and *S. hierredda* in his key to the genera and species of Sepiidae. However, the present study shows, for the first

time, morphometric differences between the cuttlebone of these two species including more different characters than those in Khromov (1998). Besides quantifying the differences, our study also shows differences in some cuttlebone structures not previously reported (e.g. ICW, OCW and CT). One conspicuous feature is the spine of the cuttlebone. This structure is completely different between species for specimens >50-mm ML, although it is not a useful character in juveniles (ML<50 mm). Moreover, the CDA tends to support the view that morphological differentiation is quite strongly developed between *S. officinalis* and *S. hierredda*, consistent with regarding these taxa as species rather than subspecies.

The CDA analysis using both soft and hard-body variables (data not shown; see Material and Methods for reasons) separated the species in nine and seven steps for males (Wilks's lambda: 0.243; $F_{(9, 33)}$: 11.410; $P<0.000001$) and females (Wilks's lambda: 0.170; $F_{(7, 43)}$: 30.018; $P<0.000001$), respectively. The most powerful variable to discriminate between both taxa was the outer cone width (OCW) of the cuttlebone. Although a log-transformation of the measurements beforehand would have increased the reliability of residuals used in the CDA, this was considered unnecessary because the high linearity of each not-transformed variable (character) with respect to ML or CL (Table 1).

Allozyme analysis showed that allopatric populations of *S. hierredda* from Senegalese waters (ShSe1 and ShSe2) and *S. officinalis* from north-west Iberian waters (SfNW) presented 13 diagnostic loci from a total of 33 enzyme loci analysed. A Senegalese sample of five individuals of *S. officinalis* (SfSe1) and sympatric *S. hierredda* (ShSe1), showed 12 diagnostic loci. Moreover, no electrophoretically recognizable hybrids were identified among the species in sympatry, although the number of individuals in the SfSe1 sample is low for a hybridization test. However, considering the great genetic differences found between *S. hierredda* and *S. officinalis*, for sympatric and allopatric populations, these two taxa could be considered as distinct biological species.

Estimates of genetic divergence based on allozyme polymorphisms are generally well-correlated with taxonomic categories based on morphological analyses, although some differences may exist among animal phyla or classes (Thorpe, 1983; Avise, 1994). It has been suggested that where conventional studies leave taxonomic status in doubt, an estimate of genetic divergence from allozyme polymorphisms could provide an objective and useful criterion (Thorpe, 1983) especially at the species level. Focusing on the Cephalopoda, the similarity of congeneric species usually falls within the range I=0.3–0.8, and, with some exceptions, confamilial genera have I-values <0.4 (see Sanjuan et al., 1996). Therefore, the I-values between the two sympatric samples of *S. hierredda* (0.998) or the two allopatric samples of *S. officinalis* (0.968) are typical values for conspecific populations, whereas the I-values between *S. hierredda* and *S. officinalis* (I=0.582–0.596) are characteristic for congeneric species.

In conclusion, both morphological and genetical analyses were concordant and showed clear differences between *S. officinalis* and *S. hierredda*. As described by other authors (Khromov et al., 1998), the morphological differences indicated that these taxa should be considered different species. Additionally, the genetic identity values between sympatric and allopatric populations of the two *Sepia* taxa are typical of different species. Moreover, the genetic identities between *S. hierredda* and *S. officinalis* suggested that *S. hierredda* belongs to the same genus as *S. officinalis*.

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Appendix 1. *Basic statistics for measurements made on body and cuttlebone of* Sepia officinalis *and* S. hierredda.

Sex	Variable	N	Mean (mm or g)	SD	Range (mm or g)	%ML	%CL	SD
Sepia officinalis								
Males	ML	33	136.3	43.4	57.6–228.0		103.6	3.1
	BW	33	387.8	300.4	29.7–111.7			
	MW	33	80.8	24.5	32.2–27.5	59.8		4.8
	ALI	33	85.6	32.7	34.5–39.9	61.8		9.0
	ALII	32	85.2	32.5	32.7–45.3	61.6		8.6
	ALIII	33	88.8	32.8	33.8–51.3	64.2		7.4
	ALIV	33	93.8	37.8	35.5–67.0	67.2		9.7
	HcL	33	93.2	36.9	30.7–75.8	66.7		9.5
	NRSH	19	6.2	0.9	4.0–8.0			
	CL	33	131.2	40.3	57.5–205.7	96.6		2.8
	CW	33	17.8	15.7	0.9–56.3			
	SZ	33	55.5	19.2	22.9–94.8		41.8	3.3
	NSZ	33	67.0	19.0	29.2–01.6		51.5	2.5
	CWi	33	48.8	14.9	21.9–78.6		37.2	1.3
	FL	33	121.3	36.8	52.1–90.2		92.5	1.4
	FW	33	42.0	13.1	18.2–68.8		32.0	1.1
	ICW	33	5.8	2.1	2.4–0.7		4.4	0.5
	OCW	32	12.3	4.5	4.7–21.1		9.3	1.0
	CT	33	17.3	6.1	6.3–28.9		13.0	1.1
Females	ML	38	138.1	39.0	70.5–95.5		102.4	2.9
	BW	38	413.0	268.8	57.4–928.6			
	MW	38	84.5	21.6	45.1–22.1	61.8		5.0
	ALI	38	78.1	27.5	27.7–17.7	55.3		6.3
	ALII	38	78.8	27.8	34.8–24.8	56.0		6.4
	ALIII	38	81.2	30.1	34.7–30.3	57.2		7.5
	ALIV	38	89.6	34.4	34.0–44.0	62.8		9.4
	CL	37	133.2	35.9	73.3–88.2	97.8		2.8
	CW	38	20.9	15.3	1.9–48.1			
	SZ	38	55.3	16.8	29.7–85.5		40.7	2.8
	NSZ	37	69.2	17.5	37.9–97.8		52.3	2.6
	CWi	38	52.4	14.7	27.5–73.5		38.9	1.3
	FL	37	123.2	33.0	68.3–76.5		92.5	1.0
	FW	38	45.0	13.0	23.9–64.7		33.3	1.6
	ICW	38	5.8	2.0	2.1–8.8		4.2	0.6
	OCW	38	13.3	4.5	5.9–21.5		9.7	1.2
	CT	38	18.3	5.9	8.6–26.2		13.4	1.1
Both sexes	ML	71	137.3	40.8	57.6–228.0		102.9	3.1
	BW	71	401.3	282.1	26.7–111.7			
	MW	71	82.8	22.9	32.2–27.5	60.9		5.0
	ALI	71	81.6	30.0	27.7–39.9	58.3		8.3
	ALII	70	81.7	30.0	32.7–45.3	58.5		8.0
	ALIII	71	84.7	31.4	33.8–51.3	60.5		8.2
	ALIV	71	91.5	35.8	34.0–67.0	64.8		9.7
	HcL	33	93.2	36.9	30.7–75.8	66.7		9.5
	NRSH	19	6.2	0.9	4.0–8.0			
	CL	70	132.3	37.7	57.5–205.7	97.2		2.8
	CW	71	19.5	15.4	0.9–56.3			
	SZ	71	55.4	17.8	22.9–94.8		41.2	3.1
	NSZ	70	68.2	18.1	29.2–01.6		51.9	2.6
	CWi	71	50.7	14.8	21.9–78.6		38.1	1.5
	FL	70	122.3	34.6	52.1–90.2		92.5	1.2
	FW	71	43.6	13.1	18.2–68.8		32.7	1.5
	ICW	71	5.8	2.0	2.1–0.7		4.3	0.5
	OCW	70	12.8	4.5	4.7–21.5		9.5	1.1
	CT	71	17.8	5.9	6.3–28.9		13.2	1.0
S. hierredda								
Males	ML	32	124.2	34.7	76.0–74.8		99.5	2.5
	BW	32	252.8	178.1	55.1–573.4			

(Continued).

Appendix 1. (*Continued*).

Sex	Variable	N	Mean (mm or g)	SD	Range (mm or g)	%ML	%CL	SD
	MW	21	60.3	17.3	33.6–95.5	54.5		8.3
	ALI	20	50.1	22.5	30.8–01.7	45.0		7.4
	ALII	21	54.0	24.4	32.5–02.5	47.0		7.3
	ALIII	21	54.6	25.4	29.4–10.3	47.4		8.5
	ALIV	18	60.1	30.0	33.9–31.6	53.5		10.6
	HcL	14	63.9	31.9	33.7–24.6	55.3		11.3
	NRSH	17	10.9	2.0	8.0–4.0			
	CL	32	124.4	33.3	76.3–70.5	100.6		2.6
	CW	32	9.9	7.0	2.1–20.8			
	SZ	32	58.3	19.4	32.2–87.4		46.1	4.0
	NSZ	32	56.1	12.9	37.0–82.0		45.7	3.6
	CWi	32	42.9	10.9	28.0–61.6		34.7	1.2
	FL	32	114.4	31.1	69.6–59.5		91.8	0.8
	FW	32	35.6	8.9	23.4–48.9		28.7	1.2
	ICW	32	5.3	1.3	3.2–8.0		4.3	0.5
	OCW	31	8.2	2.4	4.5–1.7		6.6	0.7
	CT	32	14.8	4.5	8.8–21.5		11.8	0.7
Females	ML	32	131.9	31.0	55.2–67.4		99.0	2.3
	BW	32	300.0	160.6	27.1–541.2			
	MW	23	66.6	16.9	26.9–95.6	54.7		7.2
	ALI	20	58.6	21.3	24.7–92.8	46.6		6.5
	ALII	21	58.9	24.6	20.6–95.7	47.3		8.7
	ALIII	21	60.7	24.9	22.0–99.3	48.2		8.8
	ALIV	22	71.6	26.8	34.1–12.7	55.3		9.8
	CL	32	132.8	30.2	60.6–68.2	101.1		2.4
	CW	32	12.1	6.8	1.2–23.4			
	SZ	32	64.5	19.8	27.5–00.4		47.8	5.2
	NSZ	31	58.7	11.1	27.0–74.8		45.1	4.4
	CWi	32	47.3	10.6	23.9–61.4		35.7	1.6
	FL	32	122.5	28.4	53.9–56.8		92.1	1.0
	FW	32	39.5	9.1	20.1–51.4		29.8	1.4
	ICW	32	5.8	1.4	3.4–8.4		4.5	0.6
	OCW	31	9.1	2.3	4.1–3.1		6.8	0.6
	CT	32	16.0	3.9	7.4–20.4		12.0	0.6
Both sexes	ML	64	128.0	32.9	55.2–74.8		99.3	2.4
	BW	64	276.4	169.9	27.1–573.4			
	MW	44	63.6	17.2	26.9–95.6	54.6		7.7
	ALI	40	54.3	22.1	24.7–01.7	45.8		6.9
	ALII	42	56.4	24.3	20.6–02.5	47.2		7.9
	ALIII	42	57.6	25.0	22.0–10.3	47.8		8.6
	ALIV	40	66.4	28.5	33.9–31.6	54.5		10.1
	HcL	14	63.9	31.9	33.7–24.6	55.3		11.3
	NRSH	17	10.9	2.0	8.0–4.0			
	CL	64	128.6	31.8	60.6–70.5	100.8		2.5
	CW	64	11.0	6.9	1.2–23.4			
	SZ	64	61.4	19.7	27.5–00.4		46.9	4.7
	NSZ	63	57.4	12.1	27.0–82.0		45.4	4.0
	CWi	64	45.1	10.9	23.9–61.6		35.2	1.5
	FL	64	118.5	29.8	53.9–59.5		92.0	0.9
	FW	64	37.5	9.2	20.1–51.4		29.3	1.4
	ICW	64	5.6	1.4	3.2–8.4		4.4	0.5
	OCW	62	8.6	2.4	4.1–3.1		6.7	0.7
	CT	64	15.4	4.2	7.4–21.5		11.9	0.7

ML, dorsal mantle length; BW, body weight; MW, mantle width; AL I, AL II, AL III and AL IV, length of right arms; HcL, hectocotylized arm length; NRSH, number of rows of suckers on hectotylus; CL, cuttlebone length; CW, cuttlebone weight; SZ, striated zone length; NSZ, non-striated or last loculus zone length; Cwi, cuttlebone width; FL, phragmocone length; FW, phragmocone width; ICW, inner cone width; OCW, outer cone width; CT, cuttlebone thickness. %ML, mean of ((Variable/ML)*100); %CL, mean of ((Variable/CL)*100); SD, standard deviation.

Appendix 2. *Allele frequencies for 33 loci in the samples of* Sepia hierredda *(ShSe1 and ShSe2) and* S. officinalis *(SfSe1) from Senegalese waters and* S. officinalis *from north-west Iberian waters (SfNW). Sample 1 of Senegalese waters included ShSe1 and SfSe1, and sample 2 is ShSe2. Mean observed heterozygosity (Ho) and expected heterozygosity (He) (unbiased estimated), mean number of alleles (Na) and percentage of polymorphic loci based on the 95% (P_{95}) criterion for the analysed loci in each species are shown at the end of table. The loci AAT-2*, ARK*, DDH*, EST*, ESTD*, FBALD, G3PDH*, GAPDH*, GPI-1*, GPI-2*, MDH-2*, PEPD*, PK-1*, PK-2* and SOD* were monomorphic for all samples.*

	Samples					Samples			
Locus	ShSe1	ShSe2	SfSe1	SfNW	Locus	ShSe1	ShSe2	SfSe1	SfNW
AAT-1*					IDDH*				
N	24	21	5	60	N	24	21	5	60
*100	0	0	1	1	*75	0	0	0.100	0
*110	1	1	0	0	*85	0.104	0	0.500	0.400
					*90	0	0.024	0	0
ACP*					*100	0	0	0.400	0.600
N	24	21	5	60	*105	0.896	0.976	0	0
*50	0	0.095	0	0					
*85	1	0.810	0	0	IDHP*				
*100	0	0	1	1	N	24	21	5	60
*115	0	0.095	0	0	*60	0.021	0	0	0
					*80	0.979	1	0	0
AK*					*85	0	0	0.800	0.242
N	24	21	5	60	*100	0	0	0.200	0.758
*100	0	0	1	1					
*160	1	1	0	0	LAP*				
					N	24	21	5	60
ALPDH*					*100	1	0.952	1	1
N	24	21	5	60	*105	0	0.048	0	0
*90	0	0	0	0.017					
*100	1	1	1	0.983	MDH-1*				
					N	24	21	5	60
G6PDH*					*95	1	1	0	0
N	24	21	5	60	*100	0	0	1	1
*100	0	0	1	1					
*110	1	1	0	0	MEP*				
					N	24	21	5	60
GLUDH*					*75	0	0	0.800	0
N	24	21	5	60	*85	1	1	0	0
*100	0	0	1	1	*100	0	0	0.200	1
*125	1	1	0	0					
MPI*					PEPB*				
N	24	21	5	60	N	24	21	5	60
*90	0	0.024	0	0	*100	0	0	1	1
*100	1	0.976	1	1	*105	1	1	0	0
OPDH-1*					PGDH*				
N	24	21	5	60	N	23	21	5	60
*90	0	0.024	0	0	*100	0	0	1	1
*100	1	0.952	0.900	0.758	*110	0.826	0.929	0	0
*110	0	0.024	0.100	0.242	*130	0.174	0.071	0	0
OPDH-2*					PGM*				
N	24	21	5	60	N	24	21	5	60
*80	1	1	0.100	0	*100	0	0	1	1
*100	0	0	0.900	1	*120	1	1	0	0
PEPA*					Ho	0.013	0.025		0.039
N	24	21	5	60	(SE)	(0.008)	(0.013)		(0.022)
*100	0	0	1	1	He	0.016	0.023		0.038
*120	1	1	0	0	(SE)	(0.010)	(0.011)		(0.021)
					Na	1.1	1.2		1.1
					(SE)	(0.1)	(0.1)		(0.1)
					P95	0.061	0.061		0.091

N, sample size; SE, standard error.