Allozyme Electrophoretic Technique and Phylogenetic Relationships in Three Species of Sepia (Cephalopoda: Sepiidae)

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ABSTRACT. Samples of *Sepia officinalis*, *S. orbignyana*, and *S. elegans* from Galician waters (NW Iberian Peninsula) were obtained from commercial catches in three fishing ports. A preliminary screening for 47 and 33 enzymes in mantle muscle and digestive gland, respectively, was carried out using seven buffer systems. Thirty-seven enzyme loci were resolved from these tissues using only two buffer systems. Most enzymes showed equal or higher activity for the digestive gland than for the mantle muscle in freshly-caught samples of *S. officinalis* and *S. orbignyana*. The activity of a large number of enzymes decreased faster in the digestive gland than in the mantle muscle after 6-12 h at room temperature. Consequently, we suggest that mantle muscle rather than digestive gland be used for routine electrophoretic studies in *Sepia* species obtained from commercial catches. A phylogenetic reconstruction analysis, applying the Wagner parsimony method and using the ommastrephid species *Illex coindetii* as outgroup, showed only one most parsimonious tree. *S. orbignyana* and *S. elegans* were found to be a sister group. The topology agreed with that recently obtained from mitochondrial rDNA sequences, and both molecular data are in line with previous morphological results. They confirm the view that *S. orbignyana* and *S. elegans* belong to a different subgenus (*Rhombosipion*) from that of *S. officinalis* (*Sepia* "sensu stricto").

KEY WORDS. Cephalopoda, *Sepia officinalis*, *S. orbignyana*, *S. elegans*, *Rhombosipion*, enzyme activity, phylogeny, North-Eastern Atlantic Ocean

INTRODUCTION

The Genus *Sepia* Linnaeus 1758, order Sepioidea (Mollusca: Cephalopoda), has some systematic and phylogenetic problems. *S. officinalis* (Linnaeus, 1758), *S. orbignyana* (Férussac, 1826) and *S. elegans* (Blainville, 1827), have been catalogued by Khromov (31) into two different subgenera on the basis of the internal cone structure and the shape of the cuttlebone. *S. officinalis* was considered to belong to the subgenus *Sepia* "sensu stricto," whereas *S. orbignyana* and *S. elegans* were classified in the subgenus *Rhombosipion* Rochebrune, 1884. The validity of these, and another four *Sepia* subgenera, has been discussed by Khromov et al. (32). Recently, phylogenetic mitochondrial rDNA studies for 27 decapod cephalopods have indicated that *S. orbignyana* and *S. elegans* are a monophyletic group, separated from *S. officinalis* (5).

Data on genetic variability obtained from allozyme polymorphisms have proved to be effective for detection of genetic differences at the inter and intraspecific level in commercially exploited fish and cephalopod species (13,15,28,49). There have been 21 allozyme studies of cephalopods, which have included ten Loliginidae, ten Ommastrephidae, one Gonatidae, two Octopodidae and one Nautilidae species (12,15,30,50). However, as yet, allozyme polymorphism analyses have not been applied to species of the order Sepioidea. This paper examines three species which represent an important commercial resource throughout their range; combined catches being around 75,000 metric tons (20). The limited dispersive capacity of these species, in contrast with oceanic squids (26), may result in some degree of regional intraspecific genetic differentiation.

The first aim of this paper was to develop horizontal starch gel electrophoresis technique for *Sepia officinalis*, *S. orbignyana*, and *S. elegans* collected from commercial catches, testing enzyme activity in mantle muscle and digestive gland, and after various storage times at room temperature. This will allow studies of the inter and intraspecific genetic variability in these species in the future. The second aim was to determine the phylogenetic relationships among the three *Sepia* species.

MATERIALS AND METHODS

Samples of *Sepia officinalis*, *S. orbignyana*, and *S. elegans* and also of *Illex coindetti* (Vérany, 1839) for comparison, were obtained from Galician waters (NW Iberian Peninsula) by fishing vessels using traps for *S. officinalis*, and bottom trawl for the other species. Specimens were collected from commercial catches in three fishing ports as soon as the fishing vessels tied-up (1-24 h after animals were caught). *S. officinalis* individuals were collected in Vigo, *S. orbignyana* in Ribeira and *S. elegans* and *I. coindetti* in Celeiro (Fig. 1). Fifteen individuals for each species were taken to develop the electrophoretic technique. Subsequently, ten individuals for each species were collected from very freshly commercial catches (1-8 h after animals were caught) for routine electrophoretic analyses. The specimens were sorted and placed into plastic bags, immediately frozen in dry ice and transported to the laboratory, where they were stored at -75°C until required.

Horizontal starch gel electrophoresis based on the method of Harris and Hopkinson (27) and Murphy et al. (35) was carried out. Samples of mantle muscle from the three *Sepia* species were prepared for electrophoresis by first removing the skin and by rinsing off ink residues. About 60 mg of mantle muscle or digestive gland was homogenized with 70 µL of 0.01 M dithiotreitol (DTT) solution for *S. officinalis*, and with 50 µL for *S. orbignyana* and *S. elegans*, because the mantle muscle of the first species was more viscous than those of the two latter cuttlefishes. The homogenate was centrifuged at 12,000 x g for 10 min at 4°C and soaked onto 5 x 11 mm strips of Whatman No 3 paper, except for investigations of ARK and GPI where Whatman No 1 was used to avoid broad smears. Seven buffer systems and different gel buffer solutions were assessed: 1) continuous Tris-Borate-EDTA pH 8.7 (8), with gel buffers at 1 : 4 and 1:9 dilutions of electrode buffer; 2) continuous Tris-Citrate pH 8.0 (48), with gel buffers at 1:9, 1:14, 1:19, and 1:26 dilutions of electrode buffer; 3) continuous Acetate pH 5.6 (44), with gel buffers at 1:9, 1:12, and 1:25 dilutions of electrode buffer; 4) continuous Tris-Citrate pH 7.0 (modified of Ahmad et al. (1), with gel buffers at 1:9, 1:14 and 1:19 dilutions of electrode buffer; 5) continuous Tris-Maleate-EDTA pH 7.4 (9), with gel buffer at 1:9 dilution of electrode buffer; 6) discontinuous Histidine-Citrate (23); and 7) discontinuous Lithium-Hydroxide (42). Starch gels (20 x 18 x 1 cm) of 12% hydrolyzed starch (SIGMA S-4501) were run at 4°C at constant voltage depending of the enzyme system. After electrophoresis gels were sliced into six and stained for

distinct enzymes, except the top slice. Most enzymes were visualized by the histochemical staining recipes of Murphy et al. (35), with the exception of PGM (44), ACP, DDH, MPI and PK (27) and ESTD and PEP (1). Forty-seven and 33 enzymes were screened for the mantle muscle and the digestive gland, respectively, using the seven buffer systems. A total of 26 enzymes showed enough activity and resolution to be genetically interpreted using the Tris-Borate-EDTA pH 8.7 (8; electrode buffer: 180 mM Tris, 100 mM Boric acid, 4 mM EDTA; gel buffer: 1:4 dilution of electrode buffer) and the Tris-Citrate pH 8.0 (48; electrode buffer: 250 mM Tris, 57 mM Citric acid; gel buffer: 1:9 dilution of the electrode buffer) buffer systems (Table 1). These two buffer systems and 26 enzymes were selected for the routine scoring of allozyme genotypes. Terminology and notations for isozymes/allozymes were based on recommendations of Shaklee et al. (43) and IUBMB (29). Arabic numerical suffixes for multiple loci (1, 2, ...) were in order of decreasing anodal mobility. Alleles were identified by relative electrophoretic mobilities, being *100 the most common allele in *S. officinalis*.

The type of tissue and the sampling time after death can have an effect on the enzyme activity and the banding pattern of isozymes (18,19,27). In order to test the tissue type, the electrophoretically detected enzyme activities of 26 enzymes from freshly-caught samples of the digestive gland and the mantle muscle of *S. officinalis* and *S. orbignyana* were compared. In addition, the influence of time passed at room temperature, which can resemble the sampling time after death, was checked for the 26 enzymes in both tissues. The mantle muscle and the digestive gland from each *S. officinalis* and *S. orbignyana* were divided in three and four pieces, respectively, and kept at room temperature (20°C) for 6 h, 12 h (only for the digestive gland) and 24 h, prior to electrophoresis. Sample strips were loaded on the same gel for each species, and the relative enzyme activities were measured by visual observation of the staining intensity on the gel (see Fig. 2). In this study, *S. elegans* was not included because this species showed similar enzyme activities to *S. orbignyana* in the preliminary screening.

Genotype frequencies at polymorphic loci were tested for conformance to Hardy-Weinberg equilibrium expectations by chi-square tests. Average heterozygosities (H), average number of alleles (Na) and proportion of polymorphic loci using the 0.95 criterion (P) were calculated for each species (36).

A phylogenetic reconstruction analysis using the Wagner parsimony method (21,33) was carried out with the MIX (Mixed Method Parsimony) routine of the Phylip 3.5c computer program (22). Individual alleles were considered as separate characters with their presence and absence as alternative states. One hundred and eighteen characters were considered per species. The ommastrephid species *Illex coindetii* was used as outgroup to root the tree.

RESULTS AND DISCUSSION

Twenty-six enzymes from the mantle muscle and the digestive gland of *Sepia officinalis*, *S. orbignyana*, and *S. elegans* were electrophoretically assayed and gave sufficient activity and good enough resolution for regular interpretation of banding patterns. Twenty-five enzymes were resolved for the mantle muscle of the three *Sepia* species, and 22 and 20 for the digestive gland of *S. officinalis* and *S. orbignyana* respectively (Tables 1 and 2). Isozyme patterns were interpreted on the basis of known quaternary structure in other organisms (27,34,35,39).

Seventeen enzymes gave a single anodic zone of activity for at least one tissue from the three *Sepia* species (Tables 1 and 2). For DDH and FBALD a weak cathodic sub-band, which could not be interpreted, was detected on the gel. ESTD showed a higher enzyme activity in the lowest gel layer than in any other. Each primary band was accompanied by a faint cathodic sub-band in *S. officinalis*, and by an anodic one in *S. orbignyana* and *S. elegans*. Three-banded ESTD heterozygotes were found for *S. elegans*, as typical for a dimeric enzyme. A dimeric esterase, which could correspond with ESTD, has been also reported for *Loligo vulgaris* (EST-1; (2)) and *Beryteuthis roadster* (EST-2; (30)). IDDH showed a cathodic subband which could not be interpreted. Five-banded IDDH heterozygotes were found for *S. officinalis* and *S. elegans*, as typical for a tetrameric enzyme. A monomeric IDDH has been described for the mantle muscle of *L. forbesi* (11); therefore the IDDH structure seems to vary according to the cephalopod species. The LAP stain also highlighted PEPB and consequently a control slice from the same gel was specifically stained for PEPB to distinguish the LAP and PEPB activity bands. LDH exhibited activity only for the digestive gland. This result agrees with those reported by Storey (45) and Dando et al. (17) concerning a higher LDH activity for the digestive gland than for the mantle muscle in *S. officinalis*. Low levels of LDH activity

have been found for the mantle muscle of other cephalopods (41). PEPB and PGM showed two-banded heterozygotes for *S. elegans*, which suggested a monomeric structure, typical for these enzymes. The same structure has been observed for PGM in *L. forbesi* (11), *L. gahi* (14), *L. vulgaris* (2) and *B. magister* (30). SOD showed each primary band accompanied by a faint cathodic sub-band in *S. officinalis*.

Nine enzymes showed two or three anodic zones of similar or different activity for at least one tissue from the three *Sepia* species (Tables 1 and 2). Two AAT activity zones appeared when the gel was run for 17 h at 5.4 V/cm, AAT-2 appearing very close to the origin (see Fig. 2). The staining solution was modified with respect to the original stain to obtain sufficient enzyme activity (modified amounts: 0.5 g of L-aspartic acid, 0.2 g of α -ketoglutaric acid and 75 mg of fast blue BB salt; (35)). AAT-2 showed three-banded heterozygotes for *S. orbignyana* and *S. elegans*, which suggested a dimeric structure, as usual for this enzyme, and as detected for other cephalopods (*Berryteuthis magister*; (30)). The ACP staining solution was also modified (0.3 g of α -naphthyl phosphate; 27). ACP-3 presented two-banded heterozygotes in *S. orbignyana*, which suggested a monomeric structure, as described for the "red cell" ACP (27). To reveal EST it was necessary to pre-incubate the gel slice in the stain for 30 min, before adding the fast blue BB salt. EST showed two activity zones for the mantle muscle: a stronger anodic EST-2 and a weaker less anodic zone, which was identified as ESTD. The MDH stain also highlighted MEP. To reduce the MEP reaction, 50 mg of pyruvic acid was added to the MDH staining solution. OPDH did not exhibit activity for the digestive gland. OPDH activity for digestive gland decrease rapidly after freezing, probably due to proteolysis (3,46). However, two OPDH activity zones were observed for the mantle muscle of the three *Sepia* spp. OPDH-1 showed two-banded heterozygotes in the three cuttlefishes, which conforms to the usual monomeric structure described for this enzyme in bivalves (*Pecten maximus* (38); and *Tapes decussatus* (40)) and cephalopods (*S. officinalis* (4,45); and *L. vulgaris* (2)). Tissue-specific OPDH isozymes have been found and characterized in cephalopods (*S. officinalis* (17,46,47); and *Loligo vulgaris* (24)) and, exceptionally, in the bivalve *Tapes decussatus* (40). Four possible OPDH isozymes, each for the muscle, heart, brain and digestive gland tissues were described in *S. officinalis* (46). Nevertheless, the results obtained here indicate that more than one "muscle type" OPDH could exist in *Sepia* spp. PK has rarely been employed on

cephalopod studies, being assayed without success in *L. gahi* (14) and *Illex argentinus* (16).

Twenty-nine isozymes were detected for the digestive gland and 31 for the mantle muscle in *S. officinalis* (Table 2). Twenty-four isozymes were observed for both tissues (e.g., AAT-1), five only for the digestive gland (e.g., ACP-1), seven only for the mantle muscle (e.g., FBALD) and one (MEP-2) for neither tissue (Table 2). Of the 24 isozymes noted for both tissues, eight (33.3%) showed the highest activity for the digestive gland (e.g., ACP-3, A3 for the digestive gland versus A2 for the mantle muscle), two (8.3%) for the mantle muscle (e.g., ARK, A3 for the mantle muscle versus A2 for the digestive gland), and 14 (58.3%) showed similar activity for both tissues (e.g., AAT-1, A3 for both tissues) (Table 2). A similar result was found in *S. orbignyana*, where 27 isozymes were detected for the digestive gland and 31 for the mantle muscle (Table 2). Twenty-two isozymes were observed for both tissues (e.g., AAT-1), five only for the digestive gland (e.g., ACP-1), nine only for the mantle muscle (e.g., AK) and one (IDHP-1) for neither tissue (Table 2). Of the 22 isozymes noted for both tissues, nine (40.9%) showed the highest activity for the digestive gland (e.g., ACP-3, A3 for the digestive gland versus A2 for the mantle muscle), seven (31.8%) for the mantle muscle (e.g., AAT-1, A3 for the mantle muscle versus A2 for the digestive gland), and six (27.3%) showed similar activity for both tissues (e.g., AAT-2, A2 for both tissues) (Table 2). Consequently, in freshly-caught samples from both *S. officinalis* and *S. orbignyana* the enzyme activity seems to be higher for the isozymes of the digestive gland (33.3% and 40.9%, respectively) than those of the mantle muscle (8.3% and 31.8%, respectively). This difference in enzyme activity could be due to a greater enzyme concentration for the digestive gland than for the mantle muscle, as usual for a large number of enzymes in marine molluscs.

After 24 h 100% of loci (31 isozymes) showed varying enzyme activity for the mantle muscle of both *Sepia* species, and 62% (18 isozymes) and 48% (13 isozymes) for the digestive gland of *S. officinalis* and *S. orbignyana*, respectively (Table 2). Therefore, sufficient enzyme activity in the mantle muscle was maintained for detection in all loci at 24 h, whereas in the digestive gland 38% for *S. officinalis* and 52% for *S. orbignyana* were lost (Table 2). A faster decrease in activity for digestive gland from *S. orbignyana* than relative to *S. officinalis* was observed. The digestive gland is the main organ

involved in the synthesis and secretion of digestive enzymes in *Sepia* spp. (6, 7), and the greater concentration of digestive enzymes presumably have a higher proteolytic degradation in the digestive gland than in the mantle muscle. Consequently, we suggest the use of the mantle muscle rather than the digestive gland for routine electrophoresis of *Sepia* species obtained from commercial catches, because usually the time elapsed since the specimens are caught until the fishing vessels tied up range between 12 h and 24 h.

The allozyme frequencies for 32 enzyme loci from the mantle muscle of *Sepia officinalis*, *S. orbignyana*, and *S. elegans*, and the ommastrephid species *Illex coindetii* are shown in Table 3. Most loci were monomorphic for each *Sepia* species, and three loci were fixed for the three species. Twelve loci were completely diagnostic among the three *Sepia* species, 14 between *S. orbignyana* and *S. elegans*, and 24 between *S. officinalis* and the other two cuttlefishes. No significant deviation from the Hardy-Weinberg expected proportions for any species at any polymorphic locus was detected by the chi-square tests (data not shown). The average heterozygosities (H), the average number of alleles (N_a) and their standard errors (SE), and the proportion of polymorphic loci using the 0.95 criterion (P) are shown in Table 4. No significant differences were detected for the average heterozygosities among the three *Sepia* species using t-test (36). The average heterozygosity estimates fell within the range found for most cephalopods (see for review (10,15,25,30)) and below the average for invertebrate species (0.10; see (37)).

The phylogenetic reconstruction analysis showed only one most parsimonious tree with 123 minimum number of steps (Fig. 3). *Sepia orbignyana* and *S. elegans* made a sister group which shared a common ancestor not shared by *S. officinalis*. The topology of this phylogenetic reconstruction was in agreement with that obtained by Bonnaud et al. (5). According to Khromov (31) and Khromov et al. (32) *S. orbignyana* and *S. elegans* would share a larger number of characters than with *S. officinalis*, and therefore the evolutionary divergence between them would also be smaller than that expected between *S. officinalis* and each of *S. orbignyana* and *S. elegans*. Phylogenetic reconstruction on molecular data, both at allozyme (this paper) and mtDNA levels (5) support this hypothesis, since *S. officinalis* seems to have been reproductively isolated

in a first evolutionary event and the *S. orbignyana* and *S. elegans* monophyletic group in a second.

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FIG. 1. Map of the Galician coast (A), NW Iberian Peninsula, showing the fishing ports (Celeiro, Ribeira and Vigo) where the samples of *Sepia officinalis*, *S. orbignyana*, and *S. elegans* were collected from commercial lots.

FIG. 2. Zymogram corresponding to the AAT.1 * and AAT.2* loci for three specimens of *Sepia orbignyana* (a, b, c) using freshly. caught samples (0 h) and samples kept for 6 h, 12 h, and 24 h at room temperature of the digestive gland (dg) and the mantle muscle (m). O: origin; +: anode.

FIG. 3. The most parsimonius tree found for *Sepia officinalis*, *S. orbignyana*, and *S. elegans* employing the Wagner parsimony method. The ommastrephid species *Illex coindetii* was used as outgroup to root the tree.

TABLE 1. Resolved enzymes (abbreviations), E.C. numbers, most effective buffer systems, electrophoretic conditions (V/cm and time (hours) of migration), number of detected loci and quaternary structures for the isozymes of the mantle muscle and the digestive gland in *Sepia officinalis*, *S. orbignyana*, and *S. elegans*

Enzyme	E.C.	Buffer system	V/cm	Time	No Loci	Structure
AAT	2.6.1.1	B	5.4	17	2	-/d
ACP	3.1.3.2	B	4.6	12	3	-/-/m
AK	2.7.4.3	B	5.4	9	1	-
ARK	2.7.3.3	B	5.4	12	1	-
DDH	1.8.1.4	A	10.0	9	1	m
EST	3.1.1.-	B	5.4	9	3	-
ESTD	3.1.1.56	B	5.4	12	1	d
FBALD	4.1.2.13	B	4.6	9	1	-
G3PDH	1.1.1.8	A	12.3	12	1	-
GLUDH	1.4.1.2	A	12.3	9	1	-
GPI	5.3.1.9	A	12.3	15	2	-/d
IDDH	1.1.1.14	B	5.4	12	1	t
IDHP	1.1.1.42	B	5.4	12	2	d/d
LAP	3.4.11.1	B	5.4	12	1	-
LDH	1.1.1.27	B	5.4	15	1	-

MDH	1.1.1.37	B	5.4	12	2	-
MEP	1.1.1.40	A	12.3	12	2	-
MPI	5.3.1.8	A	12.3	12	1	-
OPDH	1.5.1.11	A	12.3	15	2	m/-
PEPA	3.4.13.18	A	12.3	12	1	-
PEPB	3.4.11.4	A	12.3	12	1	m
PEPD	3.4.13.9	A	12.3	12	1	-
PGDH	1.1.1.44	B	5.4	12	1	-
PGM	5.4.2.2	B	5.4	12	1	m
PK	2.7.1.40	B	5.4	15	2	-
SOD	1.15.1.1	B	5.4	9	1	-

PEPA (substrate: gly-leu); PEPB (substrate: leu-gly-gly); PEPD (substrate: phe-pro).

Buffer systems: A, Tris-Borate-EDTA pH 8.7 (gel buffer dilution 1:4); B, Tris-Citrate pH 8.0 (gel buffer dilution 1:9). The quaternary structures are indicated for each isozyme whenever heterozygotes were observed (- = heterozygotes not observed, m = monomeric structure, d = dimeric structure, t = tetrameric structure).

TABLE 2. Relative enzyme activities for 37 isozymes from freshly-caught samples (0 h) and samples kept for 6 h, 12 h (only for the digestive gland) and 24 h at room temperature in the mantle muscle and the digestive gland of *Sepia officinalis* and *S. orbignyana*. The total number of isozymes with no detectable enzyme activity (nd isozymes), and with varying enzyme activity (A_ isozymes), are also given for each species, tissue and time. nd = no detectable, A1 = weak, A2 = moderate, A3 = strong

Isozyme	<i>Sepia officinalis</i>							<i>Sepia orbignyana</i>						
	Digestive gland				Mantle muscle			Digestive gland				Mantle muscle		
	0h	6h	12h	24h	0h	6h	24h	0h	6h	12h	24h	0h	6h	24h
AAT-1	A3	A3	A3	A2	A3	A3	A2	A2	A1	nd	nd	A3	A3	A2
AAT-2	A2	A2	A2	A1	A2	A2	A2	A2	A2	A1	A1	A2	A2	A2
ACP-1	A2	A2	A2	A2	nd	nd	nd	A1	A1	A1	A1	nd	nd	nd
ACP-2	A2	A2	A2	A2	nd	nd	nd	A1	A1	A1	nd	nd	nd	nd
ACP-3	A3	A3	A3	A3	A2	A2	A2	A3	A3	A3	A3	A2	A2	A2
AK	A2	A2	nd	nd	A2	A2	A2	nd	nd	nd	nd	A2	A2	A2
ARK	A2	A2	A1	nd	A3	A3	A3	A1	nd	nd	nd	A3	A3	A2
DDH	A2	A2	A2	A1	A1	A1	A1	A2	A2	A1	A1	A1	A1	A1
EST-1	A2	A2	A1	nd	nd	nd	nd	A2	A2	A2	A2	nd	nd	nd
EST-2	A2	A2	A1	A1	A2	A2	A2	A3	A3	A3	A2	A2	A2	A1
EST-3	A2	A2	A2	A2	nd	nd	nd	A3	A3	A3	A3	nd	nd	nd
ESTD	A3	A3	A3	A2	A2	A2	A2	A3	A3	A3	A3	A2	A2	A2

FBALD	nd	nd	nd	nd	A2	A2	A2	ND	nd	nd	nd	A2	A2	A1
G3PDH	A3	A3	A2	nd	A3	A3	A3	A1	nd	nd	nd	A3	A3	A3
GLUDH	nd	nd	nd	nd	A1	A1	A1	nd	nd	nd	nd	A1	A1	A1
GPI-1	A2	A2	A1	nd	A2	A2	A2	nd	nd	nd	nd	A2	A2	A2
GPI-2	nd	nd	nd	nd	A2	A2	A2	nd	nd	nd	nd	A2	A2	A2
IDDH	A2	A2	A2	A1	A2	A2	A2	A1	nd	nd	nd	A2	A2	A2
IDHP-1	A2	A2	A2	nd	A2	A2	A1	nd	nd	nd	nd	nd	nd	nd
IDHP-2	A2	A2	A1	nd	A3	A3	A3	A1	nd	nd	nd	A3	A3	A3
LAP	A3	A3	A3	A3	A2	A2	A2	A3	A3	A3	A2	A2	A2	A2
LDH	A3	A3	A3	A3	nd	nd	nd	A3	A3	A2	A1	nd	nd	nd
MDH-1	A2	A2	A2	A1	A2	A2	A2	A2	nd	nd	nd	A2	A2	A2
MDH-2	A2	A2	A1	nd	A2	A2	A1	A1	nd	nd	nd	A2	A2	A2
MEP-1	A2	A2	A2	A1	A2	A2	A2	A2	A1	nd	nd	A2	A2	A2
MEP-2	nd	nd	nd	nd	nd	nd	nd	A2	A1	nd	nd	A1	A1	A1
MPI A3	A3	A3	A3	A2	A3	A3	A3	A2	A1	nd	nd	A2	A2	A2
OPHD-1	nd	nd	nd	nd	A2	A2	A2	nd	nd	nd	nd	A1	A1	A1
OPHD-2	nd	nd	nd	nd	A3	A3	A3	nd	nd	nd	nd	A3	A3	A3
PEPA	A3	A2	A1	nd	A2	A2	A2	A1	nd	nd	nd	A2	A2	A2
PEPB	A3	A3	A3	A3	A2	A2	A2	A3	A3	A3	A2	A2	A2	A2
PEPD	A3	A3	A3	A3	A2	A2	A2	A3	A2	A2	A1	A2	A2	A2

PGDH	A2	A2	A1	nd	A2	A2	A2	A2	nd	nd	nd	A2	A2	A2
PGM	A2	A2	A1	nd	A2	A2	A2	A2	A1	nd	nd	A2	A2	A2
PK-1	nd	nd	nd	nd	A2	A2	A2	nd	nd	nd	nd	A2	A2	A1
PK-2	nd	nd	nd	nd	A3	A3	A3	nd	nd	nd	nd	A3	A3	A2
SOD	A2	A2	A2	A1	A1	A1	A1	A2	A2	A2	A2	A1	A1	A1
nd isozymes	8	8	9	19	6	6	6	10	18	23	24	6	6	6
A_ isozymes	29	29	28	18	31	31	31	27	19	14	13	31	31	31

TABLE 3. Allele frequencies for 32 presumptive loci from mantle muscle in *Sepia officinalis* (Sof), *S. orbignyana* (Sor), *S. elegans* (Sel), and *Illex coindetii* (Ico). The number of individuals assayed was ten for each locus and species.

Locus	Allele	Sof	Sor	Sel	Ico
AAT-1*	*90	0	0	0	1
	*100	1	0	0	0
	*110	0	1	0	0
	*120	0	0	1	0
AAT-2*	*20	0	0.75	0	0
	*80	0	0.25	0.85	0
	*100	1	0	0	0
	*110	0	0	0.15	0
	*120	0	0	0	1
ACP-3*	*50	0	0	1	0
	*90	0	0.15	0	0
	*100	1	0	0	0
	*120	0	0	0	1
	*140	0	0.85	0	0
AK*	*100	1	0	0	0
	*120	0	1	1	0
	*300	0	0	0	1
ARK*	*80	0	0	0	1
	*90	0	1	1	0
	*100	1	0	0	0
DDH*	*100	1	0	0	0
	*110	0	0	0	1
	*120	0	0.95	1	0
	*130	0	0.05	0	0
EST-2*	*100	1	1	1	0
	*120	0	0	0	1
ESTD*	*50	0	0	0.05	0
	*60	0	1	0.15	0

	*70	0	0	0	1
	*80	0	0	0.80	0
	*100	1	0	0	0
FBALD	*100	1	0	0	0
	*105	0	1	0	1
	*120	0	0	1	0
G3PDH*	*60	0	0	0	1
	*100	1	0	0	0
	*120	0	0	1	0
GLUDH*	*100	1	0	0	0
	*120	0	1	1	0
	*200	0	0	0	1
GPI-1*	*50	0	0	1	0
	*90	0	1	0	0
	*100	1	0	0	0
	*110	0	0	0	1
GPI-2*	*50	0	0	0.95	0
	*70	0	0	0.05	0
	*90	0	1	0	0
	*100	0	0	0	1
	*110	0	0	0	1
IDDH*	*50	0	0	0	1
	*70	0	0	0.35	0
	*80	0.30	0	0	0
	*100	0.70	1	0	0
	*110	0	0	0.065	0
IDHP-1*	*80	0.05	-	-	-
	*100	0.95	-	-	-
IDHP-2*	*100	1	0	0	0
	*130	0	0.95	0	0
	*140	0	0	1	0
	*150	0	0.05	0	0
	*250	0	0	0	1

LAP*	*50	0	0	1	0
	*80	0	0	0	1
	*90	0	1	0	0
	*100	1	0	0	0
MDH-1*	*90	0	0	1	0
	*100	1	0	0	0
	*110	0	0	0	1
	*120	0	1	0	0
MDH-2*	*100	1	1	1	0
	*120	0	0	0	1
MEP-1*	*100	1	0	0	0
	*110	0	1	1	0
	*120	0	0	0	1
MEP-2*	*100	-	1	1	-
MPI*	*70	0	0	0	1
	*90	0	1	1	0
	*100	1	0	0	0
OPDH-1*	*50	0	0	0.75	0
	*60	0	0.90	0	0
	*80	0	0.10	0.25	0
	*100	0.80	0	0	0
	*120	0.20	0	0	0
	*140	0	0	0	1
OPDH-2*	*-50	0	0	0	1
	*40	0	0	1	0
	*50	0	1	0	0
	*100	1	0	0	0
PEPA*	*100	1	1	1	0
	*130	0	0	0	1
PEPB*	*80	0	0	0	1
	*100	1	0	0.10	0
	*120	0	1	0.90	0
PEPD*	*40	0	0	0	1

PGDH*	*80	0	1	0	0
	*100	1	0	1	0
	*30	0	0	1	0
	*60	0	1	0	0
	*100	1	0	0	0
PGM*	*200	0	0	0	1
	*60	0	0	0	0.95
	*100	1	0	0	0.05
	*200	0	1	0	0
	*220	0	0	0.95	0
PK-1*	*240	0	0	0.05	0
	*50	0	0	0	1
	*100	1	0	0	0
	*110	0	1	1	0
	*70	0	0	1	1
PK-2*	*90	0	1	0	0
	*100	1	0	0	0
	*90	0	1	1	0
	*100	1	0	0	0
	*120	0	0	0	1

TABLE 4. Average heterozygosities (H), average number of alleles (Na) with their standard errors (SE), and proportion of polymorphic loci using the 0.95 criterion (P) are shown for each species

	<i>S. officinalis</i>	<i>S. orbignyana</i>	<i>S. elegans</i>	<i>I. coindetii</i>
H (SE)	0.028 (0.017)	0.034 (0.015)	0.061 (0.022)	0.003 (0.001)
Na (SE)	1.10 (0.05)	1.16 (0.07)	1.26 (0.09)	1.03 (0.03)
P	0.097	0.161	0.226	0.033