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Phylogeny of Selected Sepiidae (Mollusca, Cephalopoda) Based on 12S, 16S, and COI Sequences, with Comments on the Taxonomic Reliability of Several Morphological Characters

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Phylogenetic relationships among 11 species of sepiids from Japanese waters and *Sepia officinalis* from Mediterranean were studied using partial sequences of the mitochondrial 12S rRNA, 16S rRNA, and cytochrome *c* oxidase subunit I genes. These three genes had been analyzed in an Atlantic species *S. elagans* and was obtained from database. In the two-gene set analysis (16S+COI), sequence data of another 4 species were added from database. We also studied morphological characters of radulae, tentacular clubs, and cuttlebones. The molecular phylogeny was not congruent with relationships detected by the number of rows in radulae and the arrangement of suckers on the tentacular club. As to the cuttlebone shape, the molecular phylogeny suggests the separation of two groups, *Doratosepion* species with a lanceolate cuttlebone and the others with a broad cuttlebone. Our molecular phylogenetic study revealed these sepiids are separated into four clades. The first clade includes *Sepia officinalis, S. hierrendda, S. bertheloti, S. pharaonis* and *Sepiella japonica*. The second clade consists of *S. latimanus* and *Metasepia tullbergi* from sub-tropical waters. The third clade includes *Sepia esculenta, S. madokai, S. aculeata* and *S. lycidas,* which have a cuttlebone with a prominent spine. The fourth clade consists of *Doratosepion* species complex, *S. kobiensis, S. lorigera, S. pardex, S. peterseni,* and *S.* sp., which are characterized by a narrow cuttlebone with a distinct outer cone at the posterior end. The lack of membranous structures in the cuttlebone is a synapomorphy for this clade. *S. elegans* did not clearly belong to any of these clades and might represent the fifth clade.

Key words: cephalopods, cuttlebone, sepiids, mitochondrial genes, molecular phylogeny

INTRODUCTION

The family Sepiidae is a major group of coleoid cephalopods with a distinct chambered cuttlebone. The cuttlebone is composed of calcium carbonate and serves as a buoyancy device. Sepiids are distributed in Indo-Pacific, Australian, Mediterranean, and African coastal waters. Currently, three genera, *Sepia*, *Sepiella*, and *Metasepia,* are recognized in the Sepiidae (Voss, 1977; Khromov *et al*., 1998; Young *et al*., 1998). On the basis of morphological characters, *Sepiella* and *Metasepia* are considered as valid genera (Adam and Rees, 1966; Khromov *et al*., 1998; Lu, 1998). The Genus *Sepia* comprises a large number of species and exhibits morphological diversity. In an early classification, the Sepiidae were separated into eleven genera based on cuttlebone shape (Rochebrune, 1884). Naef (1921–1923) subsequently divided them into three genera with seven sub-genera. Adam and Rees (1966) recognized only two genera in the Sepiidae. Several authors have also attempted to classify the species at the sub-generic level (Orbigny, 1845–1847; Gray, 1849; Steenstrup, 1875; Hoyle, 1885; Sasaki, 1929; Iredale, 1954; Taki, 1981). Khromov *et al*. (1998) recently divided *Sepia* into six species complexes, namely, *Sepia, Acanthosepion, Rhombosepion, Anomalosepion, Doratosepion*, and *Hemisepius*, based on morphological characters. Thus, in their system, the genus *Sepia* basically exhibits a typological "open system" of species complexes, and a species may be shifted to a different complex when new characters become known or when known characters are reassessed. At present the classification of the genus *Sepia* is in a state of confusion.

In the past decade, molecular data have been utilized for phylogenetic analyses of cephalopod groups, including mitochondrial 16S rRNA (Bonnaud *et al*., 1994, 1996, 1997) and COI gene sequences (Carlini and Graves, 1999; Carlini *et al*., 2001). These studies consistently supported monophyly of the Sepiidae. However, some major problems concerning sepiid relationships remain to be solved, for instance relationships within the Sepiidae and within the *Sepia* species complex. In this study, we examined the relationships of 11 Japanese and one Mediterranean sepiid species, based on phylogenetic analyses of combined DNA sequences from the mitochondrial 16S rRNA, 12S rRNA, and cytochrome *c* oxidase subunit I genes. In addition, we studied whether or not three sets of morphological charac-

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Table 1. Sepiid species and *Loligo bleekeri* for molecular analyses

Species	M.L.[3] (cm)	Sex	Locality[4]	Collection date	Accession no. (12S, 16S, COI)
Sepia esculenta	6.4	male	1	Jan.30.2002	AB192327, AB192319, AB192335
Sepia madokai	7.8	–	2	Feb.14.2002	AB192328, AB192320, AB192336
Sepia lycidas	24.9	male	1	Jan.30.2002	AB192329, AB192321, AB192337
Sepia latimanus	21.0	male	3	Nov.17.2003	AB192330, AB192322, AB192338
Sepia officinalis	25.7	male	4	Sep. 9.2004	AB193808, AB193804, AB193812
Sepia kobiensis	3.5	–	2	Feb.14.2002	AB192331, AB192323, AB193813
Sepia peterseni	9.5	male	2	Feb.14.2002	AB192332, AB192324, AB192339
Sepia pardex	19.5	male	5	Nov.12.2003	AB193805, AB193801, AB193809
Sepia lorigera	20.5	male	6	Jun.14.2004	AB193806, AB193802, AB193810
Sepia sp.[1]	12.3	female	2	Jun.29.2004	AB193807, AB193803, AB193811
Metasepia tullbergi	4.7	male	2	Feb.14.2003	AB192333, AB192325, AB192340
Sepiella japonica	5.7	male	7	Feb.20.2003	AB192334, AB192326, AB192341
Sepia elegans[2]	–	–	8	–	AY293633, AY293657, AY293707
Sepia aculeata[2]	–	–	9	–	– , AF369113, AF350494
Sepia bertheloti[2]	–	–	10	–	– , AY368677, AJ583487
Sepia hierrendda[2]	–	–	10	–	– , AY368675, AJ583492
Sepia pharaonis[2]	–	–	9	–	– , AF369117, AF359555
Loligo bleekeri[2]	–	–	11	–	AB191153, AB191142, AB191293

[1] This species belongs to the *Doratosepion* species and has distinct biserial suckers in the distal portion of the arms I-III in female individuals. This species resembles *Sepia tenuipes*, but is distinguishable from *S. tenuipes* in the possession of unequal suckers on the tentacular club.
[2] These sequences were obtained from databases.
[3] Mantle Length
[4] 1 Minabe, Wakayama, Japan; 2 Irino, Kochi, Japan; 3 Naha, Okinawa, Japan; 4 Morocco; 5 Sakaiminato, Tottori, Japan; 6 Owase, Mie, Japan; 7 Osaka, Japan; 8 Banyuls-sur-mer, France; 9 China; 10 Mauritania; 11 Uozu, Toyama, Japan

ters, the cuttlebone, the tentacular club, and the radula, are reliable characters to indicate phylogenetical groupings.

MATERIALS AND METHODS

We obtained eleven sepiid species from Japanese waters and *Sepia officinalis* from the Mediterranean (Morocco). The size, sex, and source of specimens are shown in Tables 1 and 2. Tissues for DNA analyses and hard organs for morphological observations were stored in 70% ethanol. Soft organs were stored in 10% formalin. Tissue samples for DNA analysis were obtained from the arm and mantle of fresh or frozen specimens. Genomic DNA was extracted from the tissue using DNeasy® Tissue kit (QIAGEN).

Polymerase chain reaction (PCR) was done in 20 μl containing 7 μl extracted genomic DNA, 2 μl 10× reaction buffer, 1.6 μl 10 mM dNTPs, 1 μl 10 μM each primer, and 0.1 μl TaKaRa *Taq* polymerase. A partial sequence of the mitochondrial 16S rRNA gene was amplified with primers 16sar (5'-cgc ctg ttt (ga)(cat) c aaa aac at-3') and 16sbr (5'-ccg gt (ct) tga act cag atc a (ct) g t-3') (Bonnaud *et al*., 1994), a partial sequence of the 12S rRNA gene with primers 12sd (5'-(ct) aa ac (tc) (ga) gg att aga tac c-3') and 12se (5'-gag (ag) g (tc) gac ggg cg (ga) tgt gt-3'), and a partial sequence of the COI gene with primers LCO1490 (5'-ggt caa caa atc ata aag ata ttg g-3') and HCO2198 (5'-taa act tca ggg tga cca aaa aat ca-3') (Folmer *et al*., 1994). The temperature regimen of 16S and 12S amplification was 1 min at 94°C, 2 min at 45–50°C, 3 min at 72°C for 30 cycles. The temperature regimen of COI gene was 1 min at 94°C, 2 min at 45°C, 2 min at 72°C for 30 cycles. The amplified

Table 2. Sepiid species for morphological analyses

Species	Sex	Cuttlebone Length (cm)	Locality*	Collection date
S. esculenta	female	10.2	1	Oct. 9.2003
S. madokai	–	3.6	2	Feb.14.2002
S. lycidas	male	22.2	3	Jul. 9.2004
S. pharaonis	male	23.2	3	Dec. 8.1989
S. latimanus	–	24.3	4	Apr. 4.2000
S. officinalis	female	25.7	5	Jul. 9.2004
S. kobiensis	female	5.7	6	Jun.29.2004
S. peterseni	male	8.3	6	Jun.29.2004
S. pardex	male	22.4	7	Nov.12.2003
S. lorigera	male	19.6	8	Jun.14.2004
S. sp.	female	11.2	6	Jun.29.2004
M. tullbergi	–	4.2	6	Feb.14.2003
S. japonica	male	13.6	1	Nov.26.2003

* 1 Akashi, Hyogo, Japan; 2 Minabe, Wakayama, Japan; 3 Kyusyu, Japan; 4 Naha, Okinawa, Japan; 5 Morocco; 6 Irino, Kochi, Japan; 7 Owase, Mie, Japan; 8 Sakaiminato, Tottori, Japan

DNA fragment was cloned into pGEM-T Vector (Promega). Plasmid DNA from transformant colonies was purified with QIAprep® Miniprep kit (QIAGEN). Both strands of plasmid DNA were fully sequenced using T7 primer upstream and SP6 primer downstream of insert site by the dideoxy chain-termination method using Applied Biosystems BigDye® Terminators v1.1 (Sanger *et al*., 1977). For each species, we determined 500–518 bp, 390–413 bp, and 652–676 bp from the 16S rRNA, 12S rRNA, and COI genes, respectively. The sequences were deposited in the DDBJ database; their accession numbers are shown in Table 1.

The 12S, 16S, and COI sequence data were combined into one data set together with those of *Loligo bleekeri* (12S, AB191153;

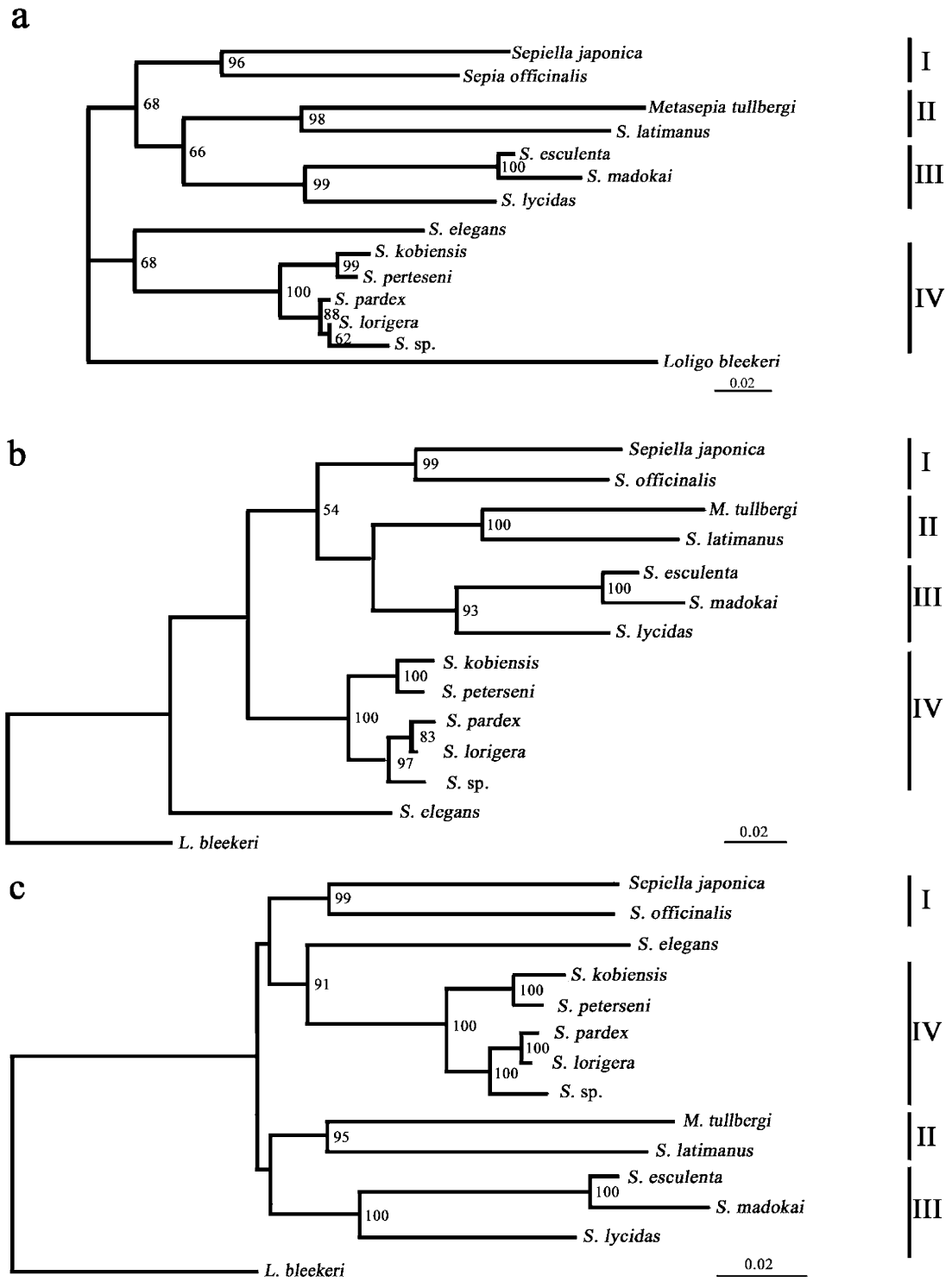

Fig. 1. Phylogenetic trees derived from analyses of sepiid mitochondrial 16S rRNA, 12S rRNA, and COI nucleotide sequences. **a,** Maximum likelihood tree, **b,** Maximum parsimony tree, **c,** Neighbor joining tree. Roman numbers refer to clades, see text for details. Numbers at nodes indicate bootstrap support values >50% (1000 replicates). Bar represents number of substitution per site.

16S, AB191142; COI, AB191293, respectively) as an outgroup. Sequences of the following species were also available from databases and were added to the analysis: *S. aculeata* (16S, AF369113; COI, AF350494), *S. bertheloti* (accession numbers 16S, AY368677; COI, AJ583487), *S. elegans* (12S, AY293633; 16S, AY293657; COI, AY293707), *S. hierrendda* (16S, AY368675; COI, AJ583492), and *S. pharaonis* (16S, AF369117; COI, AF359555). Sequences were aligned using ClustalX ver. 1.83 (Thompson *et al.*, 1997) and SeqPup ver. 0.9 (Gilbert, 1999), and adjusted manually. Indel and non-homologous regions were excluded from the analyses. Aligned sequences 1573bp (16S+12S+COI) and 1161bp (16S+COI) were used for analyses. The aligned 16S+12S+COI

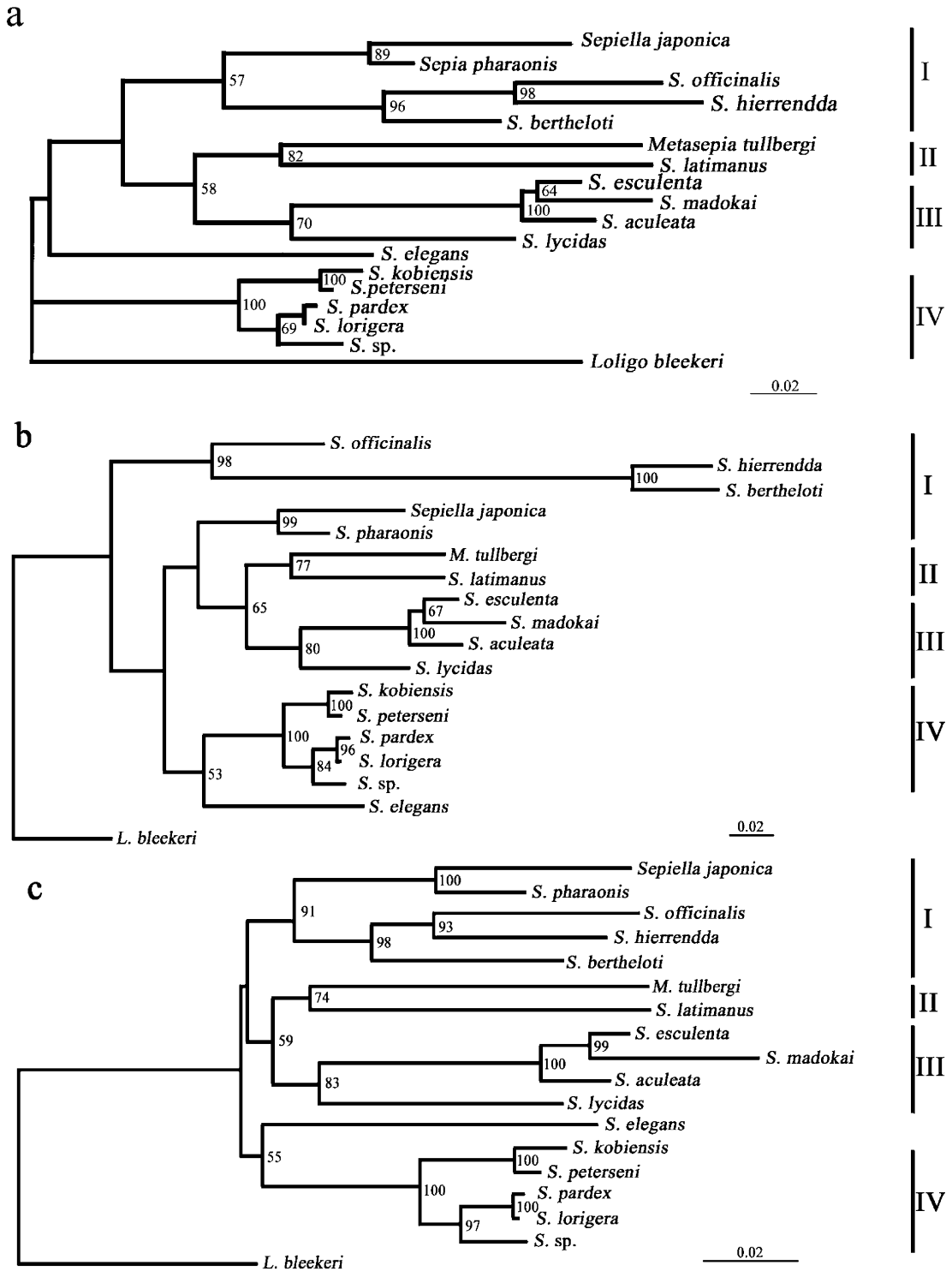

Fig. 2. Phylogenetic trees derived from analyses of sepiid mitochondrial 16S rRNA and COI nucleotide sequences. **a,** Maximum likelihood tree, **b,** Maximum parsimony tree. **c,** Neighbor joining tree. Roman numbers refer to clades, see text for details. Numbers of nodes indicate bootstrap support values >50% (1000 replicates). Bar represents number of substitution per site.

data set included 1019 constant characters and 554 variable characters, of which 335 characters were parsimony informative. The aligned 16S+COI dataset included 754 constant characters and 407 variable characters, of which 266 characters were parsimony informative.

Homogeneity of the data set was tested by the partition-homogeneity test option implemented in PAUP ver. 4.0b10 (Swofford, 2003) with 100 random repartitions. A partition-homogeneity test for the 16S+12S+COI data set showed no significant incongruence (P=0.02). Molecular phylogenetic analyses of the aligned sequences were conducted with PAUP and with PHYLIP ver. 3.6 (Felsenstein, 2004). Maximum-likelihood (ML) analyses used PAUP. Support for ML phylogenetic trees was tested using the maximum likelihood heuristic bootstrap search option (1000 replicates). For the ML analyses, best-fit substitution models were found using Modeltest 3.6 (Posada and Crandall, 1998). The three-gene data set (16S+12S+COI) was analyzed under the GTR+G+I model. The two-gene data set (16S+COI) was analyzed under the GTR+G model. Neighbor-joining (NJ) analyses used two programs, Dnadist and Neighbor in PHYLIP. Evolutionary distances were calculated according to Kimura's two-parameter method (Kimura, 1980). Maximum-parsimony (MP) analyses were done with Dnapars. In the MP analyses, the tree was constructed with one transversion weighted equal to two transitions. Support for MP trees was tested with Seqboot and Consensus in PHYLIP (1000 replicates).

The cuttlebone and radula were removed from specimens and fixed further with 70% ethanol for scanning electron microscopy (SEM). The specimens were air-dried overnight. A section of cuttlebone was obtained from the middle of the last loculus. The dried preparations were coated with gold-palladium under reduced pressure in an ion coater (JEOL JFC-1500) and examined with a scanning electron microscope (JEOL JCM-5800). Cuttlebones used for general observation and sketches were stored in 70% ethanol to preserve the form, because chitinous edges warped and broke in the dried condition. The locular index used in this study is that proposed by Choe (1962), the ratio of the length of the last loculus to that of the cuttlebone. The nomenclature of radulae used herein is the standard one for cephalopods (Nixon, 1995).

RESULTS

Molecular phylogenetic relationships among cuttlefishes

Phylogenetic trees (Fig. 1) derived from analyses of 12S rRNA, 16S rRNA, and COI genes consistently suggest that Japanese sepiids are separated into four clades, although some differences were found in the basal branch. The first clade includes *Sepiella japonica* and *Sepia officinalis* from the Mediterranean. The second clade consists of *S. latimanus* and *Metasepia tullbergi*. The third clade includes *Sepia esculenta, S. madokai* and *S. lycidas*. The fourth clade includes the *Doratosepion* species complex, namely,

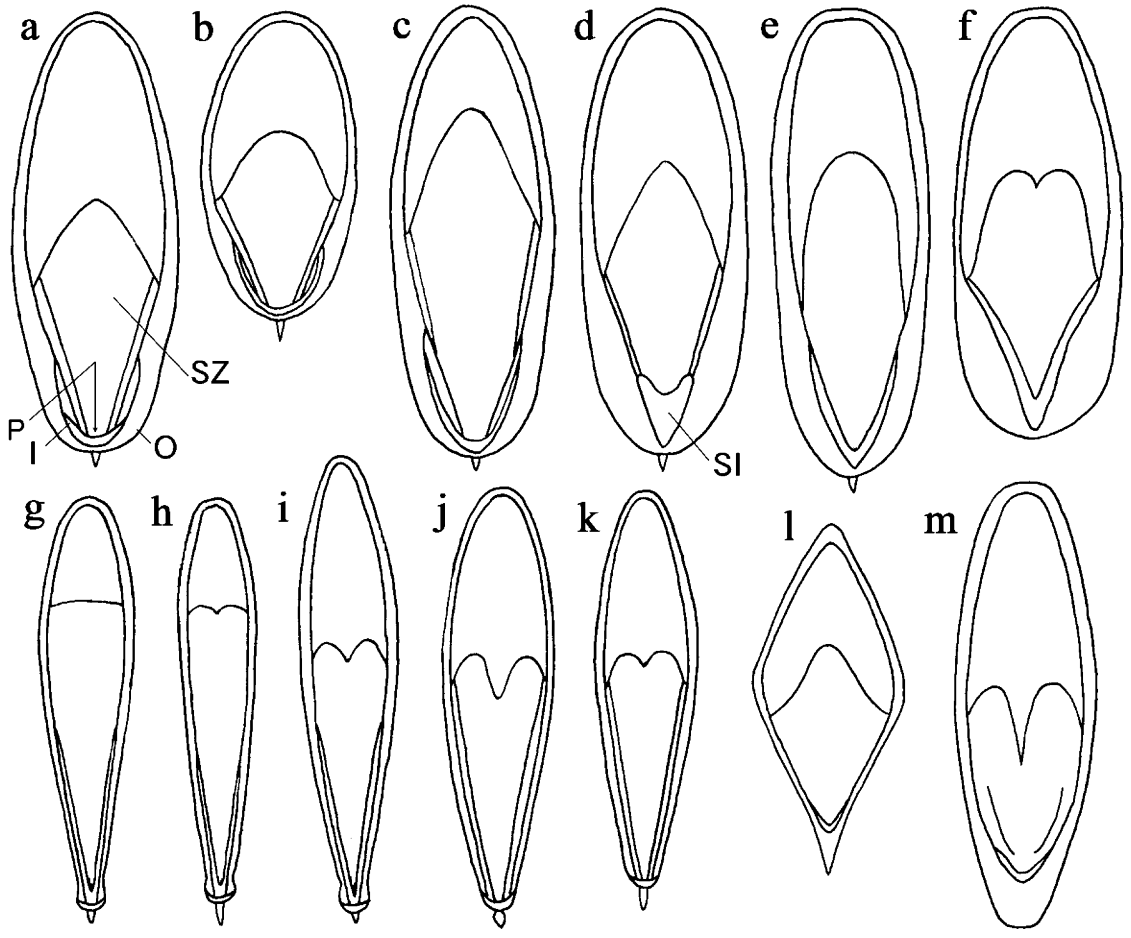

Fig. 3. Drawings of the ventral surface of cuttlebones. **a**, *Sepia esculenta*; **b**, *S. madokai*; **c**, *S. lycidas*; **d**, *S. pharaonis*; **e**, *S. latimanus*; **f**, *S. officinalis*; **g**, *S. kobiensis*; **h**, *S. peterseni*; **i**, *S. pardex*; **j**, *S. lorigera*; **k**, *S.* sp; **l**, *Metasepia tullbergi*; **m**, *Sepiella japonica.* I, inner cone; O, outer cone; P, pocket; SI, secondary inner cone; SZ, striated zone. Cuttlebone lengths are shown in Table 2.

Table 3. Morphological characters of sepiid species

Species	Cuttlebone							Number of longitudinal row of radula	Size of suckers on tentacular club
	Overall shape	Spine	Outercone	Innercone	Type[1]	Locular index %±S. D. (n)	Membranous supporting structure		
Sepia esculenta	oval	present	broad	U-shaped	III	33.1±5.2 (10)	present	7	equal
Sepia madokai	oval	present	broad	U-shaped	III	33.3 (1)	present	7	equal
Sepia lycidas	oval	present	broad	U-shaped	III	26.5±2.8 (10)	present	7	equal
Sepia pharaonis	oval	present	broad	U-shaped	I	36.2 (1)	present	7	equal
Sepia latimanus	oval	present	broad	U-shaped	I	33.7 (1)	present	7	unequal
Sepia officinalis	oval	present	broad	U-shaped	I	32.8 (1)	present[2]	7	unequal
Sepia kobiensis	lanceolate	present	cup-shaped	V-shaped	II	31.6 (1)	absent	7	unequal
Sepia peterseni	lanceolate	present	cup-shaped	V-shaped	II	31.3 (1)	absent	7	unequal
Sepia pardex	lanceolate	present	cup-shaped	V-shaped	II	41.4 (1)	absent	7	equal
Sepia lorigera	lanceolate	present	cup-shaped	V-shaped	II	40.4±3.8 (10)	absent	7	unequal
Sepia sp.	lanceolate	present	cup-shaped	V-shaped	II	46.4 (1)	absent	7	unequal
Metasepia tullbergi	rhomboidal	absent	absent	minute	*Metasepia*	38.1 (1)	present	6	unequal
Sepiella japonica	oval	absent	absent	minute	*Sepiella*	46.3 (1)	present	7	equal[3]

[1] **I** indicates a cuttlebone with a prominent spine, a deep broad groove in the striated zone, L-shaped anterior striae, and the pocket-like cavity in the inner cone. **II** indicates a lanceolate cuttlebone with an inverted U-shaped anterior striae, U-shaped inner cone, and a cup-shaped outer cone. **III** indicates a broad and elongated cuttlebone with the U-shaped inner cone, but without the pocket-like cavity in the inner cone.
[2] Budelmann *et al*. (1997)
[3] Minute equal-sized suckers.

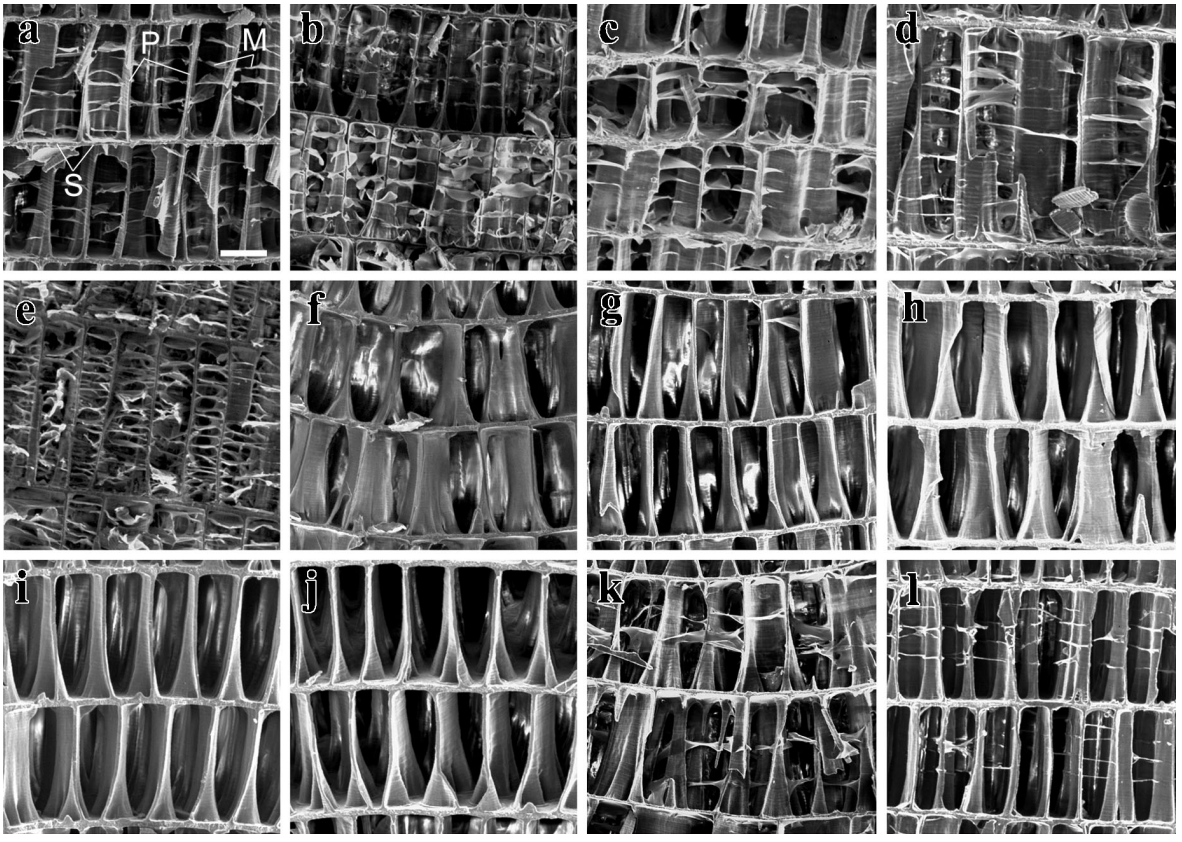

Fig. 4. Scanning electron micrographs of the internal structure of cuttlebones. **a**, *Sepia esculenta*; **b**, *S. madokai*; **c**, *S. lycidas*; **d**, *S. pharaonis*; **e**, *S. latimanus*; **f**, *S. kobiensis*; **g**, *S. peterseni*; **h**, *S. pardex*; **i**, *S. lorigera*; **j**, *S.* sp; **k**, *Metasepia tullbergi*; **l**, *Sepiella japonica*. Membranous structures (M) are found in *Sepia esculenta* (a), *S. madokai* (b), *S. lycidas* (c), *S. pharaonis* (d), *S. latimanus* (e), *Metasepia tullbergi* (k), and *Sepiella japonica* (l). P, pillar; S, septum. Scale bar represents 100 μm.

S. kobiensis, S. lorigera, S. pardex, S. peterseni, and *S.* sp. All trees support monophyly of the *Doratosepion* species complex with high bootstrap values (Fig. 1). *Sepia elegans* forms a sister group with the fourth clade in the ML and NJ trees (Figs. 1a, c).

In phylogenetic trees (Fig. 2) reconstructed from analyses of the two-gene data set (16S+COI), including an additional four sepiid species, four clades were also found in the ML and NJ trees (Fig. 2). The first clade includes three additional species, *Sepia bertheloti* (Mauritania), *S. hierrendda* (Mauritania), and *S. pharaonis* (China). All trees exhibit the close relationship among *S. bertheloti, S. hierrendda* and *S. officinalis*. *Sepiella japonica* is more closely related to *S. pharaonis* than *S. officinalis.* The third clade includes an additional species, *Sepia aculeata* from China. The second and fourth clades show the same topology as the result of analyses using the 3-gene data set. *Sepia elegans* appeared to be close to the forth clade in the MP and NJ trees (Fig. 2b, c).

Morphology of cuttlebone

Cuttlebone shapes are diverse and distinct among sepiid species (Fig. 3; Table 3), with differences reflected in the locular indices. Different species can have nearly identical locular indices, the values of which are not indicative of either genera or species complex (Table 3). Based on the cuttlebone shape, the genus *Sepia* separates into the following three groups.

The first group consists of *S. esculenta*, *S. madokai*, and *S. lycidas*, each with a prominent spine (Figs. 3a–c). The cuttlebones of these species are also similar in the presence of a deep broad groove in the striated zone, L-shaped anterior striae, and the ventral ledge behind the pocket-like cavity in the inner cone. This group contains representatives of two species complexes, *Acanthosepion* (*S. esculenta* and *S. lycidas*) and *Rhombosepion* (*S. madokai*).

The second group contains *S. kobiensis, S. peterseni, S. pardex, S. lorigera*, and *S.* sp., which have a lanceolate cuttlebone (Figs. 3g–k). Inverted U-shaped anterior striae are found in the striated zone. The inner cone is U-shaped with narrow limbs. These features are characteristics of the *Doratosepion* species complex.

The third group includes *S. officinalis, S. pharaonis* and *S. latimanus* (Figs. 3d–f). They have broad and elongated cuttlebones with the U-shaped inner cone, but lack the pocket-like cavity in the inner cone, in contrast to the first group. This group is heterogeneous with respect to cuttlebone characters, although all three species belong to the *Sepia* species complex. *Sepia officinalis* is characterized by a minute spine (Fig. 3f, not seen in this ventral view). *Sepia*

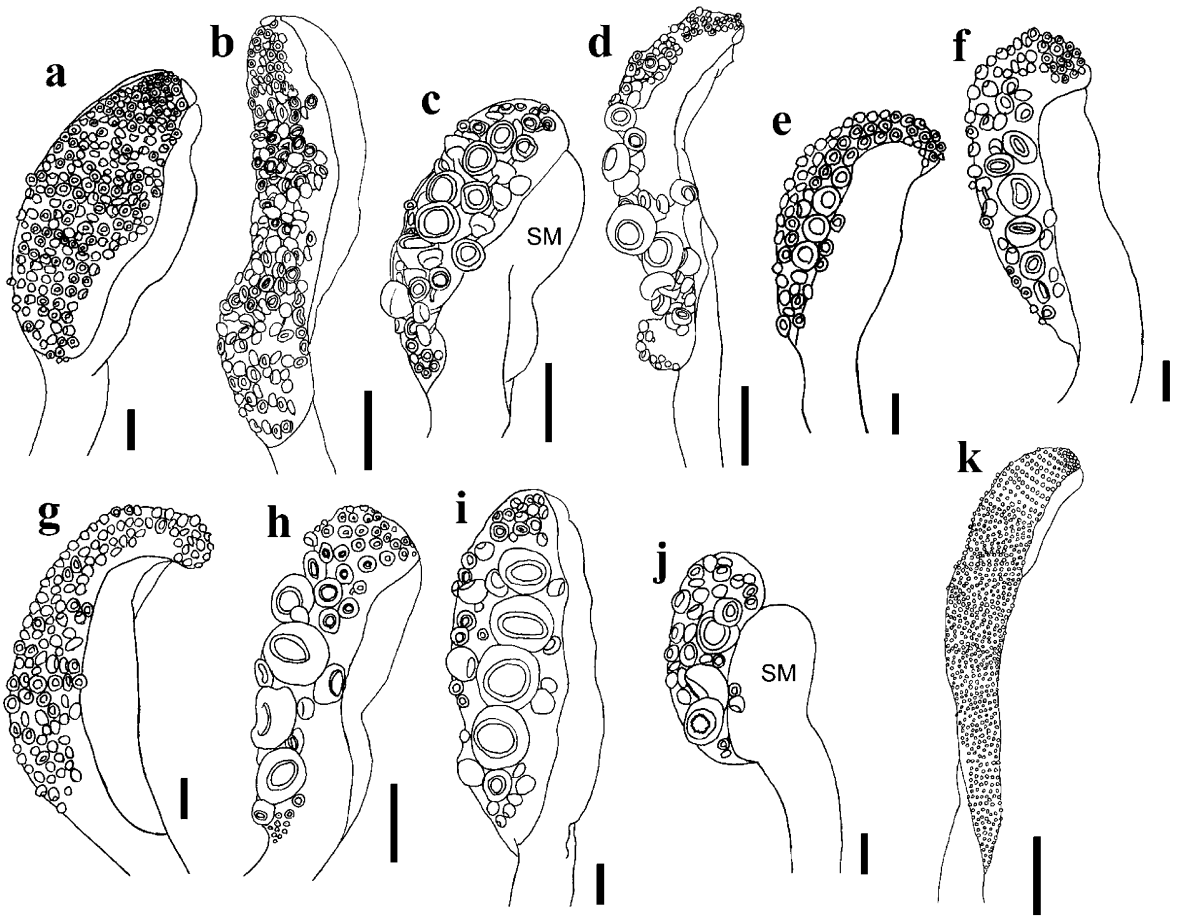

Fig. 5. Drawings of the tentacular club. **a**, *S. esculenta*; **b**, *S. lycidas*, **c**, *S. latimanus*; **d**, *S. officinalis*; **e**, *S. kobiensis*; **f**, *S. peterseni*; **g**, *S. pardex*; **h**, *S. lorigera*; **i**, *S.* sp.; **j**, *Metasepia tullbergi*; **k**, *Sepiella japonica.* SM, swimming membrane. Scale bars represent 2mm for a, g, and i, 1 mm for e, f, and j, 10 mm for b–d, h, and k.

pharaonis has a distinct, broadly U-shaped secondary inner cone (Fig. 3d). *Sepia latimanus* possesses a strong, robust spine (Fig. 3e).

The cuttlebone of *Metasepia tullbergi* is rhomboidal and acute anteriorly (Fig. 3l). The inner cone is V-shaped. The outer cone is absent. The striated zone is shallow and furrowed in the median area. The dorsal surface is chitinous.

The cuttlebone of *Sepiella japonica* is elliptical (Fig. 3m). The inner cone is broadly V-shaped with short limbs. The outer cone is broad. A high protuberance occurs in the last loculus.

The air chambers of the cuttlebone are shown in Fig. 4. The closed air chambers consist of horizontal septa with transverse pillars. The distance between pillars is constant, but the distance between septa becomes smaller from the dorsal side to the ventral side of the cuttlebone. A difference was found in the presence or absence of a membranous structure suspended between pillars (Table 3). Membranous structures were not found in the *Doratosepion* species complex, that includes *Sepia kobiensis, S. peterseni, S. pardex, S. lorigera* and *S.* sp. (Figs. 4f–j). The other cuttlefish species have membranous structures, although these structures are absent in the dorsal two or three septa.

Tentacular club

Tentacular clubs of sepiids examined can be separated into following three types (Table 3). (1) Four or five remarkable, large-sized suckers are found in *Sepia latimanus*, *S. officinalis*, *S. kobiensis*, *S. peterseni*, *S. lorigera*, *S.* sp., and *Metasepia tullbergi* (Figs. 5c–f, h–j). This type includes several species of the *Doratosepion* complex, *S. kobiensis*, *S. peterseni*, *S. lorigera*, and *S.* sp. (Figs. 5e, f, h, i). *Sepia pharaonis* also has this type (Khromov *et al*., 1998). *Sepia latimanus* and *M. tullbergi* are characterized by a large swimming membrane. (2) Small, equal-sized suckers are found in the tentacular clubs of *S. esculenta, S. lycidas, S. madokai* (not shown), and *S. pardex* (Figs. 5a, b, g). The tentacular club of *S. madokai* is almost identical to that of *S. esculenta* (see also Adam and Rees, 1966). (3) *Sepiella japonica* represents the third type, with minute, equal-sized suckers on the club (Fig. 5k).

Radulae

The radula of sepiids is simpler than that of the other cephalopod taxa. The sepiid species examined, excepting *Metasepia tullbergi,* have the homodont-type radula, with six rows of lateral unicuspid teeth and a single row of central rhachidian teeth (Fig. 6; Table 3). In *M. tullbergi,* the radula consists of six rows of lateral unicuspid teeth (5 individuals examined) (Fig. 6h); the central rhachidian teeth were not present in this species. *Sepia latimanus* has distinct, wide-based rhachidian and first lateral teeth (Fig. 6d).

Fig. 7 shows relationships among sepiid species with the distribution of morphological characters indicated. Japanese sepiids are separated into four clades. The molecular phylogeny was not congruent with relationships detected by the number of rows in radulae and the arrangement of suckers on the tentacular club. In the cuttlebone shape, the molecular phylogeny suggests the separation of two groups,

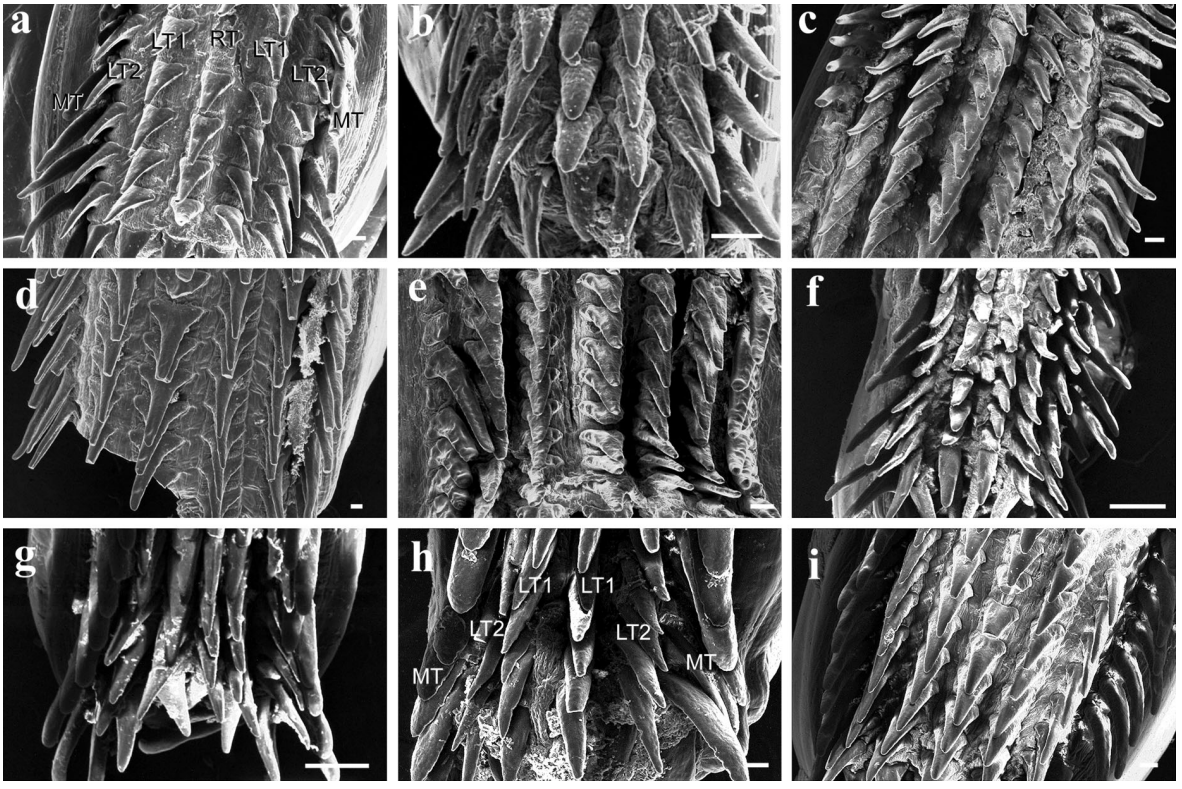

Fig. 6. Scanning electron micrographs of radulae. **a**, *Sepia esculenta*; **b**, *S. madokai;* **c**, *S. lycidas*; **d**, *S. latimanus*; **e**, *S. officinalis*; **f**, *S. lorigera*; **g**, *Sepia* sp. ; **h**, *Metasepia tullbergi* **i**, *Sepiella japonica.* RT, rhachidian tooth; LT1, lateral tooth 1; LT2, lateral tooth 2; MT, marginal tooth. Bar represents 100 μm.

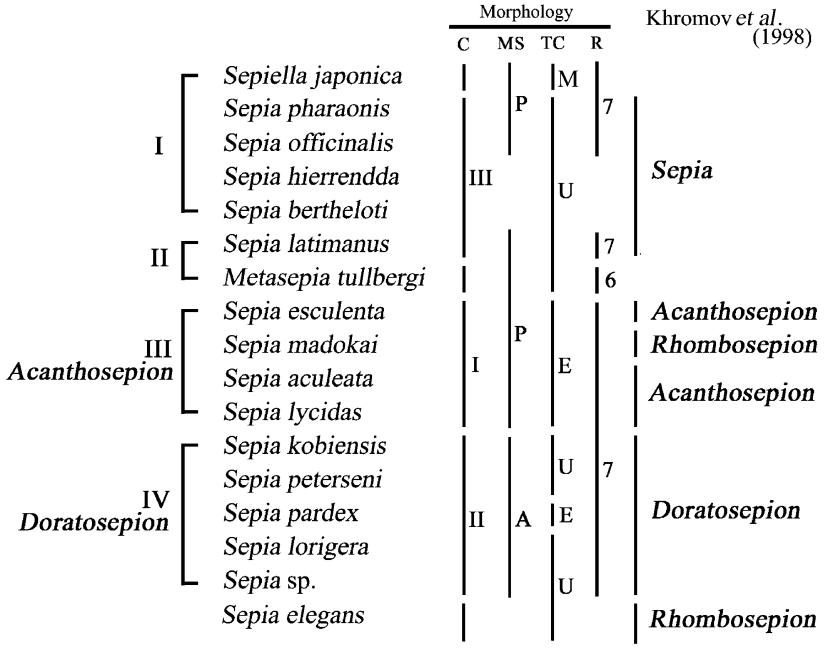

Fig. 7. Four clades (I–IV, left) sepiids detected by molecular phylogenetic analyses (refer to Fig. 2), with the distribution of states of several morphological characters (cuttlebone shape [C], membranous structures suspended between pillars [MS], the distribution of suckers on the tentacular club [TC], and number of tooth row in the radulae [R]) indicated by bars. See Table 3 for details of cuttlebone shape (I–III). *Sepia aculeata* has a long-oval cuttlebone with pocket-like cavity, rounded anterior striae (Khromov *et al*., 1998). This belongs to the type I cuttlebone. *Sepia bertheloti* has a long-oval cuttlebone with a spine. *Sepia hierrendda* has a robust cuttlebone with a minute spine and concave lateral outline in the anterior one-third (Khromov *et al*., 1998). Their cuttlebones lack a pocket-like cavity. Thus they are assigned to the type III cuttlebone. For membranous structures, P indicates membranous structure is present, and A indicates it is absent. For tentacular clubs, M indicates minute suckers. U indicates unequal-sized suckers found, and E indicates equal-sized suckers found. For the radula, the numbers indicates number of tooth rows in the radula. The farthest right column of bars indicates membership in species complexes by Khromov *et al*. (1998).

Doratosepion species with a lanceolate cuttlebone and the others with a broad cuttlebone.

DISCUSSION

The molecular phylogenetic analyses separated Japanese sepiids into four groups. The first group includes *Sepiella japonica* and the Mediterranean species *Sepia officinalis*. Analyses of the two-gene data set that includes four sepiid species from outside Japanese waters showed that *Sepia officinalis* is rather close to *S. hierrendda* and *S. bertheloti* from the eastern Atlantic Ocean. *Sepiella japonica* shows a close relationship to *S. pharaonis* from China. *Sepiella* is a unique sepiid characterized by a tentacular club with a large number of minute, equal-sized suckers and a spineless cuttlebone. This feature has also been reported in previous review literature (Khromov *et al*., 1998; Lu, 1998) and supports the separation of *Sepiella* from the other sepiid species. *Sepiella* has been recognized as valid genera based on such distinct morphological characters (Adam and Rees, 1966; Khromov *et al*., 1998; Lu, 1998). In the molecular phylogenetic analyses, however, *Sepiella* forms a clade with *Sepia* species. Species with spineless cuttlebones, such as *Sepiella japonica*, *Metasepia tullbergi*, and *Sepia elegans*, are a minority among sepiid species. The molecular data revealed no relationship among these spineless species, suggesting that the spines were lost in each lineage. *S. officinalis* has a minute spine for its large-sized cuttlebone. It appears to be secondarily reduced rather than rudimentary. Therefore, the presence or absence of the spine may be uninformative for relationships among sepiid species. Our results indicate that *Sepiella* is a member of the *Sepia* species complex.

The second group contains *S. latimanus* and *M. tullbergi* that inhabit sub-tropical waters. Although in body size *Metasepia tullbergi* is small and *S. latimanus* is very large, their tentacular clubs are similar in the shape of the swimming membrane and the arrangement of suckers. These similarities are consistent with the molecular data. Between these two species, however, distinct differences are found in the cuttlebone and radulae. In *M. tullbergi*, the radula is unique among sepiid species.

In cephalopods the radula consists of chitinous teeth, 7 to 9 rows in coleoids and 13 rows in nautiloids (Nixon, 1968, 1988, 1995; Aldred, *et al*., 1983; Mangold and Bidder, 1989). Coleoid cephalopods generally have simpler radulae than the other molluscan taxa. In squids, the tricuspid type of rhachidian teeth occurs (Aldrich *et al*., 1971). Many octopus species have a peculiar type of heterodont radulae (Solem and Roper, 1975). Among the cephalopod taxa, cuttlefishes exhibit one of the simplest radulae with unicuspid teeth only. Thus, the gross structure in sepiid radulae, such as the numbers of rows, is characteristic of cuttlefishes, but it may not be informative to clarify the relationships among sepiid species.

It is plausible that the morphology of *Metasepia* changed considerably after this group diverged from the common

ancestor with *S. latimanus*. *S. latimanus* has a broad elliptical cuttlebone without a pocket-like cavity in the inner cone. It is characteristic of *Sepia* complex species, to which Khromov *et al*. (1998) assigned *S. latimanus*. The molecular data show *M. tullbergi* and *S. latimanus* to be a sister taxa. Both lack a pocket-like cavity and together comprise the sister group to the *Acanthosepion* complex, members of which have a pocket-like cavity. This suggests the pocket-like cavity appeared in the clade of the *Acanthosepion* complex species after the branch of the clade of *M. tullbergi* + *S. latimanus*.

The third group includes *Sepia esculenta*, *S. lycidas*, *a*nd *S. madokai*, which have in common several morphological features, such as the shape of the inner cone. Khromov *et al*. (1998) treated *S. madokai* as a member of the *Rhombosepion* species complex, rather than the *Acanthosepion s*pecies complex containing *S. esculenta* and *S. lycidas*. However, the present study showed that *S. madokai* is closely related to *S. esculenta*, on the basis of both morphological and molecular data. The cuttlebone of *S. madokai* is very similar to that of juveniles of *S. esculenta* (Okutani *et al*., 1987), which was once treated as belonging to the subgenus *Platysepia* (Taki, 1981). Our molecular data support a close relationship between these two species. *S. madokai* should be assigned to *Acanthosepion*. In phylogenetic trees reconstructed from analyses of the 2-gene data set (16S+COI), *Sepia aculeata* from China is included in the third clade. This sepiid species also has previously been placed in the *Acanthosepion* species complex (Khromov *et al*., 1998).

The fourth group consists of *Doratosepion* species complex, including *S. kobiensis*, *S. perterseni, S. pardex, S. lorigera* and *S.* sp.. The *Doratosepion* species complex is the most speciose group of cuttlefishes, containing 41 species among a total of 112 nominal sepiid species (Reid, 2000). About 20 species of sepiids have been described from Japanese waters (Okutani *et al*., 1987; Kubodera, 1997, 2000, 2001) and among them *Doratosepion* species represent 70% (14/20). The *Doratosepion* species are characterized by a narrow cuttlebone, the distinct shape of the outer cone, and the absence of membranous structures suspended between pillars. These features of the cuttlebone support monophyly of the *Doratosepion* species complex. With the exception of *S. pardex*, the *Doratosepion* species included in our study have unequal-sized suckers on the tentacular club, but some *Doratosepion* species, for instance *S. tenuipes* and *S. erostrata*, are reported to possess equal-sized suckers (Okutani *et al*., 1987). This variation in sucker size is not congruent with the *Doratosepion* concept. This molecularly well-defined group is also characterized by sexual dimorphism in arm length, except in small-sized species (Okutani *et al*., 1987). In the *Doratosepion* species complex, *S. kobiensis* and *S. peterseni* were closely related. Their cuttlebones are narrower and more weakly calcified on the dorsal surface than for the other *Doratosepion* species examined. The bodies of *S. kobiensis* and *S. peterseni* are smaller than those of the other *Doratosepion* species. These two species probably have evolved through progenesis.

Sepia elegans from the eastern Atlantic Ocean has been placed in the *Rhombosepion* species complex (Khromov *et al*., 1998), because it possesses a lanceolate cuttlebone. This sepiid species forms a sister group with the *Doratosepion* species complex in some trees, although bootstrap support was low. *S. elegans* possibly represents the fifth species complex, rather than belonging to the *Doratosepion* species complex.

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