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# PHYLOGENETIC RELATIONSHIPS AMONG THE DECABRACHIA CEPHALOPODS INFERRED FROM MITOCHONDRIAL DNA SEQUENCES

**XIAODONG ZHENG,[1,*] JIANMIN YANG,[1,2] XIANGZHI LIN[1,3] AND RUCAI WANG[1]**

[1]*Mariculture Research Laboratory, Fishery College, Ocean University of China, Qingdao, China 266003;* [2]*Marine Fisheries Research Institute of Shandong Province, Yantai, China 264000;* [3]*College of Oceanography and Environmental Sciences, Xiamen University, China 361005*

***ABSTRACT*** To clarify phylogenetic relationships among the decabrachia cephalopods, especially the family Sepiidae and Sepiolidae, mitochondrial cytochrome *c* oxidase subunit I (COI) gene and partial 16S rRNA gene were sequenced for 13 species. Phylogenetic analyses were performed by the distance and parsimony method. Coleoids were divided into 2 main lineages, Octobrachia and Decabrachia (including Sepiida, Sepiolida, and Teuthida). In all phylogenetic trees, the monophyly of the Sepiolidae and Sepiidae was supported well, but their rank and position within the Decabrachia were not clear. Based on partial COI rDNA and its amino acid sequences, Parsimony analyses showed Sepiolidae, Sepiidae, and the (*Loligo chinensis, Chtenoteryx sicula*) clade from Teuthida were in the same level. Compared with Sepiidae, Sepiolidae was more closely related to Teuthids using 16S rDNA sequences. We are inclined to support the current classification: Sepiolidae and Sepiidae belong to different Orders. According to the phylogenetic analysis, the 2 genera (*Sepiella* and *Sepia*) from the Sepiidae can be distinguished (78% neighbor-joining (NJ); 64% maximum parsimony (MP) in 16S rRNA gene), but do not have visible boundline using COI gene and its amino acid data. This suggests that COI gene may be much fitter to analyze cuttlefish phylogeny at a high taxonomic level (i.e., family), and 16S rRNA gene could be used as a precious tool to analyze taxonomic relationships at the genus level.

***KEY WORDS:*** phylogeny, cephalopods, Decabrachia, COI, 16S rRNA gene

## INTRODUCTION

According to Voss (1977), the Sepioidea includes Spirulidae, Sepiidae, Sepiolidae, Idiosepiidae, and Sepiadariidae. To date, the taxon system is still used in China. The position of the Sepiolidae as a sister group of the Sepiidae and the monophyly of the squids are, however, questioned by some scholars (e.g., Berthold & Engeser 1987, Clarke 1988, Boletzky 1999).

Molecular information from DNA data has been used to clarify the relationships among Cephalopods since the 1990s. The nucleotide divergence between sequences of one gene or a portion of gene from the mitochondrial or nuclear genome can be analyzed phylogenetically. Previous DNA sequence diversity and phylogenetic relationships of octopods have been investigated using the mitochondrial cytochrome *c* oxidase subunit I (mtCO I), mtCO II, mtCO III, 16S rRNA gene (Carlini & Graves1999, Carlini et al. 2001, Bonnaud et al. 1996, Bonnaud et al.1997, Söller et al. 2000, Allcock & Piertney 2002, Piertney et al. 2003). Anderson (2000) sequenced 2 mitochondrial genes (16S rRNA and CO I) to clarify loliginid phylogeny. Sequence analyses from the 3′ end of the mt l-rRNA (16S) gene of decapod cephalopods have shown that this portion of gene was a useful tool for taxonomic relationships at the infrafamilial level (Bonnaud et al. 1994). The COI gene for phylogenetic analysis of the coleoid cephalopods exhibited a high degree of nucleotide sequence variability, with one half of the sites varying in at least one taxon; COI amino acid sequences were highly conserved, but were useful in determining basal-level relationships among the Coleoidea (Carlini et al. 2001).

The cephalopods, especially decapods, are an important and valuable fishery resource in China, South Korea, and Japan (Nesis & Kir 1982, Okutani 1995). For example, *Sepia esculenta*, *Sepiella maindroni* and *Loligo chinensis* are all high commercial species (Dong 1991). A great deal of fundamental and applied researches on cephalopods, such as fauna, systematics, morphology, embryology, population genetics, and biodiversity have been completed since 1960s (e.g., Lee 1963, Lee 1983, Dong 1993, Lu 1998, Lu 2000, Zheng et al. 2001a, Zheng et al. 2001b, Zheng et al. 2004). There are, however, few documents referring to the molecular evolution and phylogenetics of cephalopods living in the coastal waters of China. In this study 13 cephalopod species are analyzed by the sequence comparison of 16S rRNA and COI gene. The phylogenetic trees are reconstructed. The taxonomic relationships of decabrachia species are discussed.

## MATERIALS AND METHODS

### *Taxon Selection*

Eight decabrachia species and two octopus species were sampled for 16S rRNA gene sequence analysis. Representatives of three other cephalopods (*Euprymna scolopes,* Bonnaud et al. 1994; *Chtenopteryx sicula*, *Grimpoteuthis* sp., Anderson 2000) were added to cladistic analysis.

Eight decabrachia species and one octopus species were sampled for the COI gene sequence analysis. Representatives of 4 other cephalopods (*Nautilius pompilius,* Carlini & Graves1999; *Euprymna scolopes,* Bonnaud et al. 1994; *Chtenopteryx sicula*, *Grimpoteuthis* sp. Anderson 2000) were added to cladistic analysis.

Details on the taxonomic position (following Voss 1977) and origin of the 13 species studied are presented in Table 1.

### *DNA Extraction, Amplification, and Sequencing*

Total genomic DNA was extracted using a CTAB method modified from Winnepenninckx et al. (1993). Regions of the 16S and COI genes were amplified by PCR. The 16S primers sequences were D16SAR 5′-CGC CTG TTT AHY AAA AAC AT-3′, D16SBR 5′-CCG GTC TGA ACT CAG MTC AYG T-3′(Anderson 2000). The primers used for the amplification of

*Corresponding author. E-mail: xdzheng@mail.ouc.edu.cn

**TABLE 1.**

**Classification (Voss 1977) of cephalopod taxa included in this study.**

| Classification | [a]References | Locality | GeneCOI/16S# |
|---|---|---|---|
| Phylum MOLLUSCA | | | |
| Class CEPHALOPDA | | | |
| Subclass NAUTILOIDEA | | | |
| Family Nautilidae | | | |
| *Nautilius pompilius* Linne, 1758 | CA | Waikiki Aquarium | AF000054/— |
| Subclass COLEOIDEA | | | |
| Order SEPIOIDEA | | | |
| Family Sepiidae | | | |
| *Sepia aculeata* Orbigny, 1848 | ZH | East China Sea | AF350494/AF369113 |
| *S. esculenta* Hoyle, 1885 | ZH | Yellow Sea of China | AF359554/AF369114 |
| *S. latimanus* Quoy & Gaimard, 1832 | ZH | South China Sea | AY185506/AF369116 |
| *S. pharaonis* Ehrenberg, 1831 | ZH | South China Sea | AF359555/AF369117 |
| *S. robsoni* (Massy, 1927) | ZH | South China Sea | AF350495/AF369957 |
| *Sepiella maindroni* de Rochebrune, 1884 | ZH | East China Sea | AF340032/AF369118 |
| Family Sepiolidae | | | |
| *Euprymna berryi* Sasaki, 1929 | ZH | South China Sea | AF350493/AF369110 |
| *E. scolopes* Berry, 1913 | AN/BO | Hawaii, USA | AF075417/X79592 |
| Order TEUTHOIDEA | | | |
| Suborder MYOPSIDA | | | |
| Family Loliginidae | | | |
| *Loligo chinensis* Gray, 1849 | ZH | South China Sea | AY185505/AF369955 |
| Suborder OEGOPSIDA | | | |
| Family Chtenopterygidae | | | |
| *Chtenopteryx sicula* (Verany, 1851) | AN | Pacific Ocean | AF075416/AF110097 |
| Order OCTOPODA | | | |
| Suborder CIRRATA | | | |
| Family Cirroteuthidae | | | |
| *Grimpoteuthis* sp. | AN | Monterey Bay, CA USA | AF075419/AF110100 |
| Suborder INCIRRATA | | | |
| Family Octopodidae | | | |
| *Octopus ocellatus* Gray, 1849 | ZH | East China Sea | AF346854/AF369111 |
| *O. variabilis* (Sasaki, 1929) | ZH | East China Sea | —/AF369112 |

[a] References: AN, Anderson F. E. (2000); CA, Carlini, D. B. and Graves, J. E. (1999); BO, Bonnaud, L. Boucher-Rodoni R, Monnerot M. (1994). ZH, the authors. #Genbank accession numbers.

partial COI gene were: HCO2198 (5′-TAA ACT TGA GGG TGA CCA AAA AAT-3′) and LCO1491 (5′-GGT CAA CAA ATC ATA AAG ATA TTG-3′) from Folmer et al (1994). Amplifications were performed under the following conditions: 120 s at 94 °C, then 30 or 35 cycles (COI and 16S, respectively) of 40 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. A total volume of 25 μL reactions consists of 0.5 units of *Taq* (TaKaRa), 0.5 μM each primer, 0.2 μM each dNTP, 2.5–3.5 mM $MgCl_2$, 2.5 μl of 10× buffer supplied with *Taq* and 4 μl (30–50 ng) of template DNA.

The amplified fragments were purified by the PCR fragment recovery Kit (TaKaRa). Purified products were sequenced directly using the ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit and AmplicTaq DNA polymerase with ABI PRISM 377XL DNA sequencer (Applied Biosystem Inc.).

### *Phylogenetic Analyses*

All of the initial sequences were aligned by ClustalX v 1.8 (Thompson et al. 1997). Amino acid sequences of the COI gene were translated using the GENEDOC (Nicholas et al. 1997). The mean nucleotide composition, proportion of transition (ts) by number of the total base substitutions (ts + tv, transitions + transversions) were calculated in MEGA 2 (Kumar et al. 2001). The phylogenetic analyses were performed on both aligned nucleotide sequences and on amino acid sequences, using distance and parsimony methods included in the packages MEGA 2 (Kumar et al. 2001) and PAUP4.0b6 (Swofford 2000). Statistical confidence of a particular cluster of sequences was evaluated by the bootstrap procedure (1,000 resampling replicates).

## RESULTS

### *Genetic Variation of Partial COI Gene Sequence*

The percentage proportion of 4 kinds of base pairs (A, C, G, T) was compared with each other. There was no remarkable difference between these species examined. The average content of base pairs in the nine species examined was 28.12%, 17.51%, 15.71%, and 38.60%, respectively. A + T content was also up to 66%, which is in the same range as in the reference species. High A + T content was an obvious characteristic of cephalopod mtDNA sequences. No gaps were found in all of the sequences we analyzed. A total of 281 nucleotide positions were found to be variable (42.4%). One-hundred and ninety-four variable sites were found at

the third position of codon triplet and the percentage was approximately 69% of all variable nucleotides.

The genetic distance ranged from 0.000 to 0.202 among the Sepiidae. *Sepiella maindroni* could not be separated from other species of the family using distance method, although it belonged to *Sepiella* in the traditional taxonomy. The closest taxon with respect to genetic distance was *Sepia latimanus* (0.000), and the most distant was *Sepia aculeata* (0.202). Compared with the data between Sepiidae and Sepiolidae, the distance was 0.191 ± 0.008 (mean ± SD), slightly closer than between Sepiidae and Teuthida (mainly mentioned Loliginidae and Chtenopterygidae) (0.206 ± 0.007) as well as between Sepiolidae and Teuthida (0.215 ± 0.013). *Nautilus pompilius*, belonging to Nautilida, was the most distant species from the other 12 cephalopods analyzed (0.288 ~0.349).

According to the amino acid sequences, the genetic distance was in the range of 0.000 to 0.0056 within the Sepiidae. *Sepiella maindroni* was grouped with the other cuttlefishes, even though they belong to a different genus in the traditional taxonomy. The farthest distance between *Nautilus pompilius* and other cephalopods (0.119 ~0.178) indicated that the relationship between them was the most remote.

### *Genetic Variation of Partial 16S rRNA Gene*

The length of 16S rRNA gene sequences of the 10 species examined ranged from 500 to 514 base pairs (average 507.5 bp). The average of percentage proportion of A, C, G, and T was 34.25%, 9.83%, 17.93%, and 37.99%, respectively. The content of A + T is up to 72%. The result is similar to the above research of the COI gene sequences for cephalopods.

In the Sepiidae, the genetic distance of 5 species of *Sepia* ranged from 0.030 to 0.045, and become a bit higher with *Sepiella* (0.059 ~0.077). The distance between Sepiidae and Sepiolidae (0.085 ± 0.009) was not significant different from that between Sepiidae and Teuthida (0.090 ± 0.013) or Sepiolidae and Teuthida (0.089 ± 0.012). The longest distance (0.173) was observed between Sepiolidae (*Euprymna scolopes*) and Octopoda (*Octopus variabilis*).

### *Phylogeny of Cephalopods Based on COI and 16S rRNA Gene Sequence*

#### Phylogenetic Analysis Based on Partial COI and Amino Acid Sequences

The topology structure of phylogenetic trees was similar using the COI and their amino acid sequences, whatever NJ trees and MP trees. The monophyly of the Sepiolidae (98% NJ, 78%MP in Fig. 1; 90% NJ, 74% MP in Fig. 2) and Sepiidae (50% NJ, 58% MP in Fig. 1; 93% NJ, 87% MP in Fig. 2) was well supported. According to NJ analyses, Coleoids were divided into 2 main lineages, Octobrachia and Decabrachia (including Sepiidae, Sepiolidae, and Teuthida). In the Decabrachia clade, 6 species from the Family Sepiidae grouped with 2 species from the Order Teuthida at first, then grouped with the Family Sepiolidae (NJ trees in Fig. 1 and 2), though low levels of bootstrap support were obtained (see asterisk place in Fig. 1 and 2). Parsimony analyses indicated that the Decabrachia was divided into 3 parallel clades (88% MP in Fig. 1; 96% MP in Fig. 2). Within the Sepiidae clade, *Sepiella* (*S. maindroni*) and *Sepia* could not be divided clearly; and the groups from 5 species in *Sepia* were not consistent with morphologic evidence, which belong to 3 different species complexes (Khromov 1998).

#### Phylogenetic Analysis Based on Partial 16S rRNA Gene Sequence

Both MP and NJ analyses provided strong bootstrap support values for the monophyly of the Coleoid cephalopods (98% MP and 98% NJ), and the monophyly of the Family Sepiidae (64% MP and 78% NJ) as well as Family Sepiolidae (95% MP and 96% NJ) (Fig. 3). Within the Decabrachia clade, Sepiolidae was more closely related with Teuthida (54% MP and 72% NJ). *Sepiella* and *Sepia* were clearly separated (64% MP and 76% NJ). Though the topology structure (NJ tree) in *Sepia* clade was consistent with the classification of species complexes of Sepiidae, bootstrap support values were very low (<50%).

## DISCUSSION

Previous investigations of coleoid systematics have attempted to determine relationships within the Sepiida, Octopoda, and Teuthida through phylogenetic analysis of morphologic and molecular character data. Most recently, Carlini and Graves (1999)

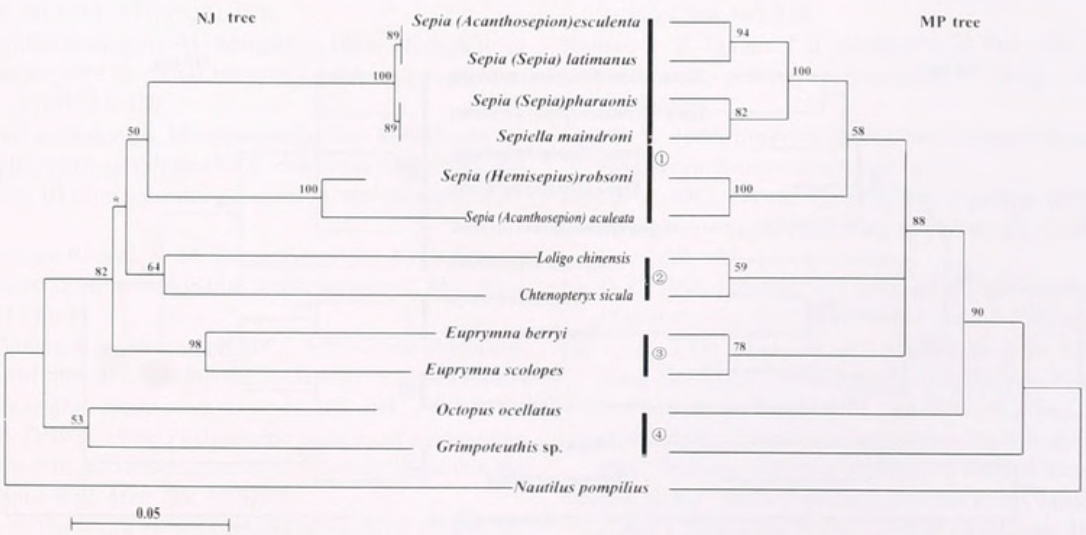


**Figure 1. NJ phylogenetic tree and MP tree based on COI gene data. Boldfaced numbers above branches are bootstrap support values (1,000 replicates). Asterisk indicated bootstrap values less than 50%. *Nautilus pompilius* was used as distant outgroup species. (1) Sepiidae, (2) Teuthida, (3) Sepiolidae, (4) Octopodida.**

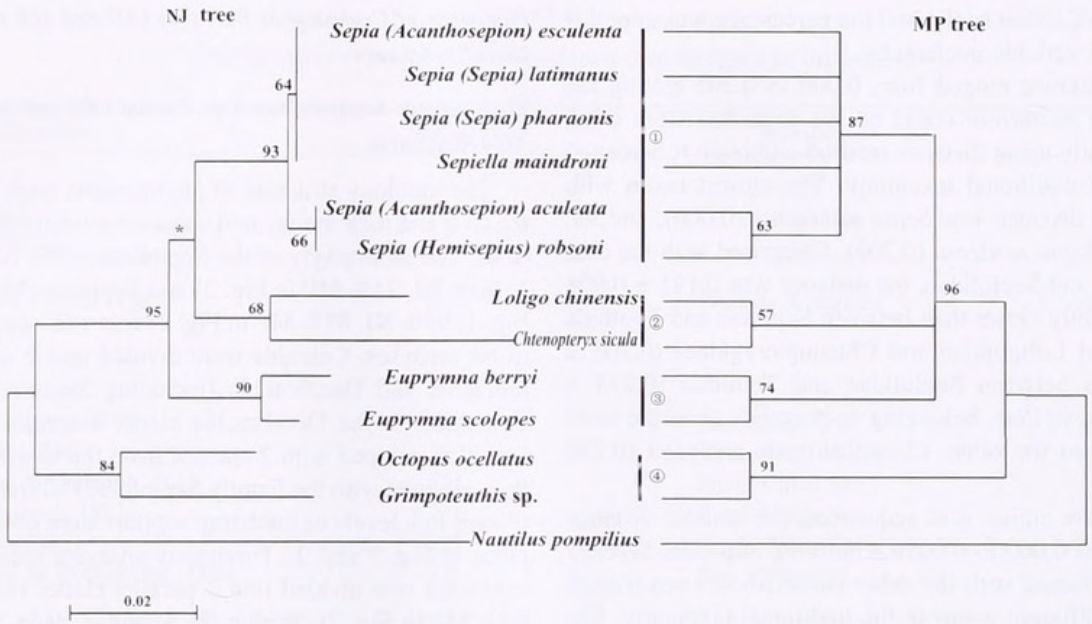


**Figure 2. NJ phylogenetic tree and MP tree based on amino acid of COI gene data. Boldfaced numbers above branches are bootstrap support values (1,000 replicates). Asterisk indicated bootstrap values less than 50%. *Nautilus pompilius* was used as distant outgroup species. (1) Sepiidae, (2) Teuthida, (3) Sepiolidae, (4) Octopodida.**

conducted a higher-level analysis of the coleoid cephalopods. Consistent with them, our results suggest that the coleoids can be divided into 2 main lineages: the Octobrachia and the Decabrachia.

According to the classification of cephalopod taxa (Voss 1977), the Order Sepioidea was comprised of 5 families Spirulidae, Sepiolidae, Sepiidae, Sepiadariidae, and Idiosepiidae (Fig. 4). Although the COI and 16S rDNA data well supported the monophyly of the Decabrachia, we could not confirm the validity of the order Sepioidea. Several studies have questioned this order (Clarke 1988, Bonnaud et al. 1996, Bonnaud et al 1997, Sweeney & Roper1998, Carlini & Graves 1999) (see Fig. 4). Bonnaud et al (1994) analyzed phylogeny of decapod cephalopods based on 3′ end of 16S rDNA nucleotide sequences, and demonstrated that Sepiolids were clearly excluded from the order, and the position of the Spirulidae required further clarification. Referring to paleontology and neontology evidences of cephalopods, Clarke (1988) pointed out that the five families belonged to two orders, that is the Order Sepiida was composed of Sepiidae, Spirulidae and Sepiadariidae; Sepiolidae and Idiosepiidae belonged to the Order Sepiolida. Sweeney and Roper (1998) listed the currently accepted classification of the recent Cephalopoda and addressed that the five families belonged to three orders. Boletzky (1999) indicated that they should be subject to four orders, and the Superorder Decabrachia included 5 orders, Spirulida, Sepiida, Sepiolida, Idiosepiida, and Teuthida (see Fig. 4). Carlini and Graves (1999) pointed out that the Sepioidea were polyphyletic. In all phylogenetic trees, the monophyly of the Sepiolidae and Sepiidae was supported well, but their rank and position within the Decabrachia were not clear. Based on partial COI and its amino acid sequences, Parsimony analyses showed Sepiolidae, Sepiidae as well as the (*Loligo chinensis, Chtenoteryx sicula*) clade from Teuthida were in the same level (88% in Fig. 1 and 96% in Fig. 2). Compared with Sepiidae, Sepiolidae was more closely related to Teuthids (72% NJ and 54% MP) using 16S rDNA. We are inclined to support the current classification: Sepiolidae and Sepiidae belong to different orders. As for the rank and position of other families (e.g., Idi-

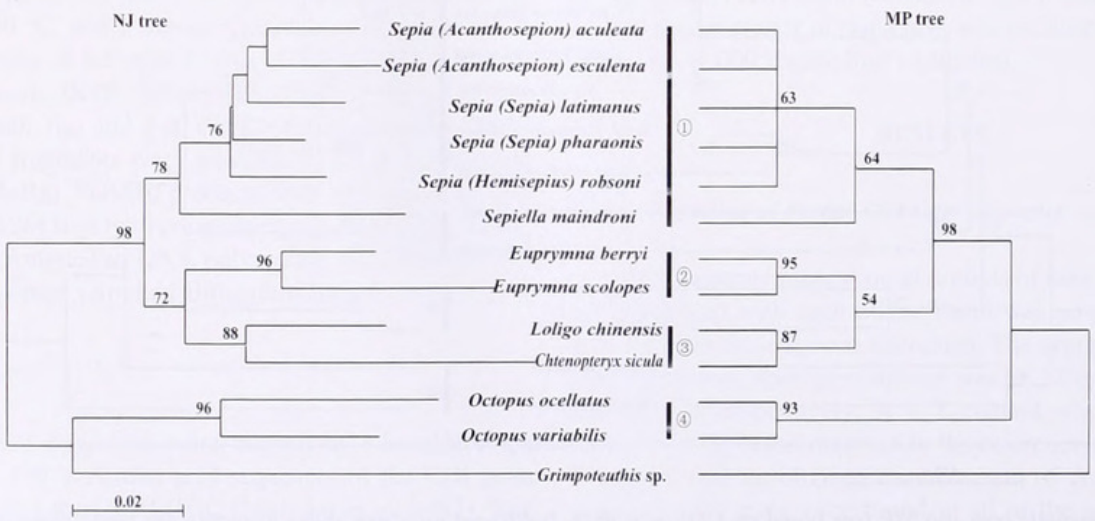


**Figure 3. NJ phylogenetic tree and MP tree based on based on 16S rRNA gene data. Boldfaced numbers above branches are bootstrap support values (1000 replicates). *Grimpoteuthis* sp. was used as distant outgroup species. (1) Sepiidae, (2) Sepiolidae, (3) Teuthida, (4) Octopodidae.**

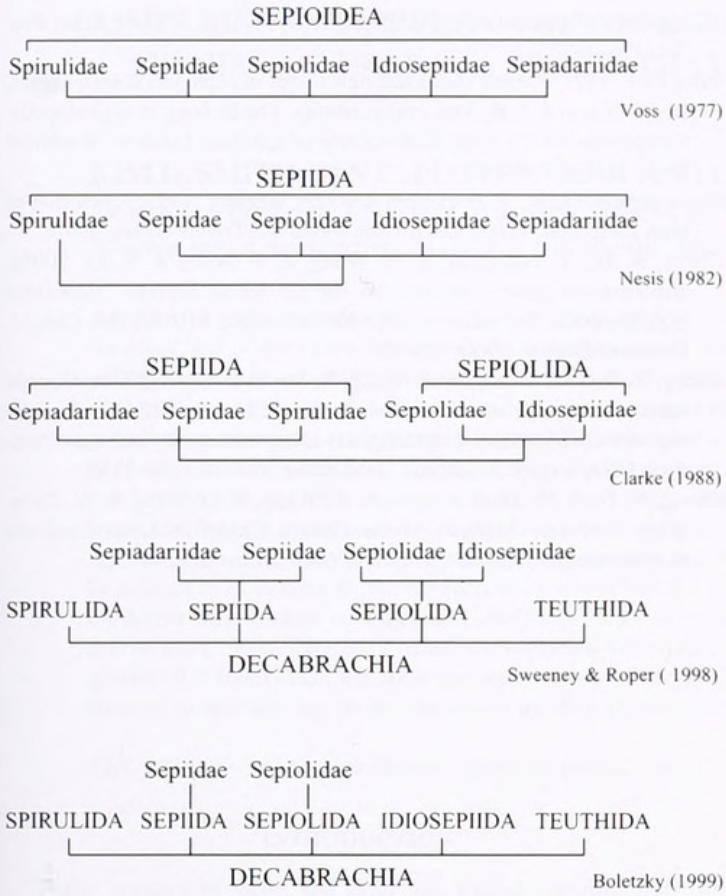


**Figure 4. Sepiid relationships with other taxa according to various authors. Ordinal rank indicated by capital letters.**

osepiidae, Spirulidae, etc), such researches will be carried out further and testify these families' phylogenetic relationship.

According to the COI gene and amino acid sequence data, the family-level relationship was exhibited clearly, but the genus *Sepiella* (*S. maindroni*)was grouped with other species from *Sepia* (see Fig. 1 and 2). *Sepiella* was obviously separated from the *Sepia* groupings based on the 16S rDNA sequences (see Fig. 3), which demonstrated that they belonged to two separate genera. It shows possibility that mtDNA sequence fragments (e.g., 16S rDNA, COI) with different evolutionary rate are fit for different taxa phylogeny. The 16S rDNA of Cephalopods is a precious tool to analyze taxonomic relationships at the genus level, and COI gene is fitter at the family level. Of course, 3 species complexes within the genus *Sepia* were not well supported in all the MP trees. The recognition of subgeneric rank in *Sepia* is not used because more analyses on morphologic, behavioral, biochemical, and molecular data are needed to resolve the question (Lu 2004, pers. comm.). We will examine more extensive sequence data of the species of *Sepia* for more refined information of species complexes or subgeneric rank.

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