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Phylogenetic analysis of cytochrome c oxidase I sequences to determine higher-level relationships within the coleoid cephalopods

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PHYLOGENETIC ANALYSIS OF CYTOCHROME *C* OXIDASE I SEQUENCES TO DETERMINE HIGHER-LEVEL RELATIONSHIPS WITHIN THE COLEOID CEPHALOPODS

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ABSTRACT

Until recently, the higher-level phylogenetic relationships of coleoid cephalopods have remained unclear. A thorough knowledge of the higher-level phylogeny of the group has been limited by the paucity of paleontological data for this poorly-fossilized group and the lack of cladistic analyses of developmental, morphological, and molecular data applied to the coleoids. In this study we analyzed a 657 base pair portion of the mitochondrial cytochrome *c* oxidase I (COI) gene from 48 cephalopod species representing a broad spectrum of coleoid diversity to examine higher-level phylogenetic relationships within the group. The COI gene 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. The evolutionary rate of COI amino acid sequences differed significantly between the two main lineages. The average amino acid sequence divergence within the Octopodiformes was over twice that of the average divergence within the decapods. In addition to analysis of the unweighted data set, phylogenetic analysis was conducted on the data subjected to a single round of rescaled consistency index (RCI) weighting to reduce the effect of homoplasious substitutions in determining phylogenetic structure. To further reduce the influence of homoplastic change and to facilitate bootstrap analysis of the data, a nested analysis of the data was employed, beginning with an analysis of the entire data set to determine proper outgroups to be used in the more restrictive analysis of the decapods and octopods separately. We draw the following conclusions from our analysis of cephalopod COI sequences: (1) the Coleoidea, Octopoda, Vampyromorpha, and Decapoda are monophyletic groups; (2) the Vampyromorpha and Octopoda are sister groups; (3) the Sepioidea, as including the five families Spirulidae, Sepiolidae, Sepiidae, Sepiadariidae, and Idiosepiidae, is polyphyletic; (4) *Spirula* is more closely related to the Teuthoidea than it is to the remaining members of the Sepioidea; and (5) the Oegopsida, as currently defined, is polyphyletic.

Our understanding of higher-level phylogenetic relationships of extant cephalopods, a morphologically diverse class in the phylum Mollusca, is surprisingly rudimentary. With the exception of *Nautilus*, all extant cephalopods are members of the subclass Coleoidea, which are distinct from the Nautiloidea and the extinct subclasses (Orthoceatoidea, Actinoceratoidea, Endoceratoidea, and Ammonoidea) in many ways, most notably in the reduction and internalization or complete loss of shell (Teichert, 1988). Although the fossil record of early cephalopods is rich and demonstrates the success of the group in Paleozoic and Mesozoic times, the mainly soft-bodied coleoid cephalopods are not well-represented with the exception of the extinct belemnoids, whose internal shell was readily preserved. Consequently, the paleontological contribution to the evolutionary history of coleoids is scanty, and the largely contradictory current classifications of the group are based primarily on the morphology of living representatives. Furthermore, many coleoid cephalopods live in relatively inaccessible habitats, such as the bathypelagic waters of the open ocean or abyssal benthic habitats, and are therefore difficult to capture in sufficient

quantity for phylogenetic studies using morphological characters. Although interest in cephalopods has increased significantly over the past few years, the difficulty in obtaining adequate material for morphological studies has restricted the number of attempts to ascertain higher-level phylogenetic relationships within the group. In fact, cladistic analyses of higher-level coleoid cephalopod relationships had not been conducted until the morphological studies by Young and Vecchione (1996) and the molecular studies by Bonnaud et al. (1994, 1997).

The cladistic analysis of morphological characters conducted by Young and Vecchione (1996) has been helpful in elucidating some of the relationships within the Coleoidea, particularly in regard to the Vampyromorpha and Octopoda. However, morphological analysis at such a broad scale remains a practically and technically challenging task, as it requires access to large amounts of study material collected over decades and familiarity with the morphology of the entire subclass. In addition, Young and Vecchione found weak support for the monophyly of the decapods (a single unambiguous synapomorphy: modification of arm pair IV into tentacles) and they did not attempt to determine relationships within the decapods. Analysis of relationships within the coleoids using molecular sequence data is a promising approach to the problem for several reasons. Molecular analyses do not require numerous whole animal specimens. Only small amounts of tissue which are readily fixed in ethanol or various tissue storage buffers are required. Tissue samples may be obtained from around the world and sent by mail conveniently and inexpensively. Finally, the difficult and time consuming task of finding the few (~16 in Young and Vecchione) morphological characters that can be clearly polarized is avoided by using long stretches of molecular characters.

The initial examinations of coleoid relationships using molecular data (Bonnaud et al., 1994; 1997) focused primarily on members of the Sepioidea and Myopsida, and representation was limited with respect to the suborder Oegopsida and order Octopoda. The first study examined approximately 500 base pairs of the mitochondrial 16S rRNA gene from six of the 25 oegopsid families and one (the outgroup) of the eleven families of the Octopoda (Bonnaud et al., 1994). The most recent study examined approximately 500 base pairs of the mitochondrial cytochrome oxidase III gene and included two oegopsid and two octopod families and *Vampyroteuthis infernalis* (Bonnaud et al., 1997). These studies were unable to establish relationships at the interfamilial level and concluded that perhaps a more conserved gene was necessary to determine relationships within the various major groups. The two studies conducted by Bonnaud et al. illustrate the major drawback of using molecular sequence data to construct a phylogeny for coleoid cephalopods: in lieu of an accurate, fossil-based estimate of divergence times within the Coleoidea, it is difficult to choose a gene that will be most suitable for inferring relationships within the group.

In this study we report the results of a phylogenetic analysis of the coleoid cephalopods based on molecular sequence data from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. The COI gene has been shown to be among the most conserved protein-coding genes in the mitochondrial genome of metazoans (Brown, 1985). COI nucleotide and amino acid sequences have been used in several studies focused on resolving relationships between taxa that have diverged over 100 mya. (Folmer et al., 1994; Cummings et al., 1995; Zardoya and Meyer, 1996). At the same time, third codon position nucleotides of the COI gene are highly variable and have proved informative in resolving taxa that have diverged more recently (Van Syoc, 1994; Spicer, 1995; Palumbi, 1996). As we

were interested in determining relationships spanning a wide range of cephalopod diversity, we opted to use the COI gene for phylogenetic analysis of the coleoid cephalopods.

Materials and Methods

Taxonomic Representation.—A portion of the cytochrome *c* oxidase I (COI) gene was sequenced for 48 cephalopod taxa representing a broad spectrum of diversity in the class. The classification, following Voss (1977), and source of the specimens used in this study are given in Table 1. When possible, we attempted to include at least two members of each family. We included taxonomic representatives from each of the five "families" of the order Sepioidea, two genera from the suborder Myopsida, 20 families of the suborder Oegopsida, and six families from the order Octopoda. As the order Vampyromorpha is monotypic, we sequenced two individuals of *Vampyroteuthis infernalis* to account for intraspecific variation in the group. We also included a nautiloid, *Nautilus pompilius*, in our study. Tissue samples from specimens were stored in either 70% ethanol (-20°C) or tissue storage buffer (0.25 M ethylenediamine tetraacetate [EDTA], 20% dimethyl sulfoxide [DMSO], saturated NaCl, pH 8.0) (Seutin et al., 1991) until DNA extractions were performed.

DNA Extraction, PCR Amplification, Cloning, and Sequencing.—A modification of a protocol designed explicitly for extracting DNA from mollusc tissue (Winnepenninckx et al., 1993) was used for extracting DNA from cephalopod specimens. A small amount (approximately 0.1 g) of muscle tissue from the mantle, fin, arm, or tentacle of preserved specimens was finely diced with a sterile razor blade and placed in a microfuge tube containing 500 µl of isolation buffer (50 mM EDTA, 50 mM Trishydroxymethyl aminomethane [Tris], 150 mM NaCl, pH 8.0), 60 µl of 10% sodium dodecyl sulfate [SDS], 10 µl of 10 mg ml^{-1} ribonuclease A, and 10 µl of 25 mg ml^{-1} proteinase K and was incubated overnight at 37°C. The following morning 10 µl of hexadecyltrimethylammoniumbromide [CTAB] buffer (10% w/v CTAB, 0.7 M NaCl) was added to the samples, which were then incubated for 20 min. at 65°C and allowed to cool to room temperature. Once cool, 350 µl of saturated NaCl was added and the tubes were vortexed at high speed for 15 min. The suspension was extracted once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1), and once with chloroform:isoamylalcohol (24:1) using wide bore pipette tips during transfer of the aqueous phase. High molecular weight DNA was precipitated with 2 volumes of 100% ethanol and collected by either spooling or centrifugation at 4°C. The DNA was washed once with 70% ethanol, dried in a vacuum concentrator, resuspended in 50 µl of sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at 4°C.

Metazoan COI primers, sequences LCO1490 and HCO2198 (Folmer et al., 1994), were ordered from Life Technologies (Gaithersburg, MD). The polymerase chain reaction (PCR) was used to amplify a 710 bp portion of the mitochondrial COI gene using the BRL PCR Reagent System (Life Technologies). A typical 50 µl amplification consisted of the following reagents: 5-10 ng template DNA, 20 mM TrisHCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmoles of each primer, 0.2 mM of each dNTP, and 1.25 units of *Taq* DNA polymerase. An MJ Research PTC-200 (Watertown, MA) thermocycler was used to conduct 40 cycles of the following temperature profile: 94°C for 1 min, 45–47.5°C (depending on the sample) for 1 min., and 72°C for 2 min. A final extension step at 68°C for 7 min followed the 40 cycles of amplification.

COI PCR products were cloned into a plasmid vector using the Original TA Cloning® Kit with pCR™2.1 (Invitrogen Corp., San Diego, CA). Plasmid DNA from transformant colonies was isolated and digested with *Eco*R1 (Life Technologies) to check for presence of the 710 bp COI insert. Both strands of plasmid DNA containing the COI insert were sequenced using the M13 Reverse Primer (New England BioLabs, Beverly, MA) upstream and the T7 Promoter (Life Technologies) downstream of the insert site by Sanger's (1977) dideoxy chain-termination method using the Sequenase® ver. 2.0 Sequencing Kit (United States Biochemical, Cleveland, OH).

After obtaining about 400 base pairs of sequence data for the COI gene fragment of 12 taxa, an internal cephalopod-specific COI sequencing primer was designed with the aid of the computer

Table 1. Classification (Voss, 1977) of cephalopod taxa included in this study.

Classification	[a]Source	[b]Collection number	GenBank accession #
Phylum MOLLUSCA			
Class CEPHALOPODA			
Subclass NAUTILOIDEA			
Family Nautilidae			
Nautilus pompilius Linné, 1758	RY	Waikiki Aquarium	AF000054
Subclass COLEOIDEA			
Order SEPIOIDEA			
Family Idiosepiidae			
Idiosepius pygmaeus Steenstrup, 1881	JS		AF000046
Family Sepiadariidae			
Sepioloidea lineolata Quoy and Gaimard, 1832	AR&MN	ANU5vii95	AF000064
Family Sepiidae			
Sepia officinalis Linné, 1758	MV&RY		AF000062
Sepia opipara Iredale, 1926	AR&MN	ANU4vii95	AF000063
Family Sepiolidae			
Subfamily Heteroteuthinae			
Stoloteuthis leucoptera Verrill, 1878	MV	ALB9402.14.18	AF000068
Heteroteuthis hawaiiensis Berry, 1909	DC	Hokusei Maru, 1996	AF000044
Subfamily Rossiinae			
Rossia palpebrosa Owen, 1834	MV	ALB9402.19.27	AF000061
Family Spirulidae			
Spirula spirula Linné, 1758	MV&RY		AF000066
Order TEUTHOIDEA			
Suborder MYOPSIDA			
Family Loliginidae			
Loligo opalescens Berry, 1911	DC		AF000051
Loligo pealei LeSueur, 1821	SH	NOAA/Chapman957	AF000052
Sepioteuthis australis Quoy and Gaimard, 1832	AR&MN	ANU4vii19	AF000065
Suborder OEGOPSIDA			
Family Ancistrocheiridae			
Ancistrocheirus lesueuri Férussac and d' Orbigny, 1839	RY	Hokusei Maru, 1994	AF000026
Family Architeuthidae			
Architeuthis sp.	TS	F78297	AF000027
Family Bathyteuthidae			
Bathyteuthis abyssicola Hoyle, 1885	DC	Hokusei Maru, 1996	AF000030
Family Brachioteuthidae			
Brachioteuthis beani Verrill, 1881	MV	JSL3749	AF000031
Family Chiroteuthidae			
Chiroteuthis veranyi Férussac, 1835	MV	Hatteras 94 Trawl 13	AF000032
Family Chtenopterygidae			
Chtenopteryx sicula Vérany, 1851	RY	Hokusei Maru, 1994	AF000033
Family Cranchiidae			
Cranchia scabra Leach, 1817	RY	Hokusei Maru, 1994	AF000035
Liocranchia valdiviae Chun, 1906	RY	New Horizon, 1993	AF000050
Family Cycloteuthidae			
Cycloteuthis sirventi Joubin, 1919	RY	Hokusei Maru, 1994	AF000036
Discoteuthis laciniosa Young and Roper, 1969	RY	Hokusei Maru, 1994	AF000037
Family Enoploteuthidae			
Abralia sp.	MV	Hatteras 94 Trawl 20	AF000025
Enoploteuthis reticulata Rancurel, 1970	DC	Hokusei Maru, 1996	AF000039
Family Gonatidae			
Gonatus berryi Naef, 1923	DC	Hokusei Maru, 1996	AF000040
Gonatus onyx Young, 1972	DC	NOAA/DSJ 9606	AF000041

Table 1. Continued.

Classification	[a]Source	[b]Collection number	GenBank accession #
Family Histioteuthidae			
Histioteuthis hoylei Goodrich, 1896	DC	Hokusei Maru, 1996	AF000045
Family Joubiniteuthidae			
Joubiniteuthis portieri Joubin, 1912	DC	Hokusei Maru, 1996	AF000048
Family Lepidoteuthidae			
Lepidoteuthis grimaldii Joubin, 1895	RY	Hokusei Maru, 1994	AF000049
Family Mastigoteuthidae			
Mastigoteuthis magna Joubin, 1913	MV	JSL3750	AF000053
Family Octopoteuthis			
Octopoteuthis nielseni Robson, 1948	RY	Hokusei Maru, 1994	AF000055
Family Ommastrephidae			
Ommastrephes bartramii LeSueur, 1821	DC	Hokusei Maru, 1996	AF000057
Sthenoteuthis oualaniensis Lesson, 1830	RY	Hokusei Maru, 1994	AF000069
Family Onychoteuthidae			
Onychoteuthis compacta Berry, 1913	DC	Hokusei Maru, 1996	AF000058
Family Pholidoteuthidae			
Pholidoteuthis adami Voss, 1956	MV	F/V Contender, 1994	AF000059
Family Pyroteuthidae			
Pyroteuthis addolux Young, 1972	BS	NHI-95-161	AF000060
Family Thysanoteuthidae			
Thysanoteuthis rhombus Troschel, 1857	DC	Hokusei Maru, 1996	AF000070
Order OCTOPODA			
Suborder CIRRATA			
Family Cirroteuthidae			
Cirrothauma murrayi Chun, 1911	BS	NHI-95-291	AF000034
Family Stauroteuthidae			
Stauroteuthis syrtensis Verrill, 1879	MV	F/V Contender, 1995	AF000067
Suborder INCIRRATA			
Family Argonautidae			
Argonauta nodosa Solander, 1786	TS	MOV-F 75026	AF000028
Family Bolitaenidae			
Eledonella pygmaea Verrill, 1884	RY	New Horizon, 1993	AF000038
Japatella diaphana Hoyle, 1885	DC	Hokusei Maru, 1996	AF000047
Family Octopodidae			
Subfamily Bathypolypodinae			
Bathypolypus arcticus Prosch, 1849	MV&RY	ALB9402.1.1	AF000029
Subfamily Graneledoninae			
Graneledone verrucosa Verrill, 1881	MV&RY	F/V Contender, 1994	AF000042
Subfamily Octopodinae			
Octopus tetricus Gould, 1852	TS	MOV-F 78082	AF000056
Hapalochlaena maculosa Hoyle, 1883	TS	MOV-F 78078	AF000043
Family Vitreledonellidae			
Vitreledonella richardi Joubin, 1918	RY	New Horizon, 1996	AF000072
Order VAMPYROMORPHA			
Family Vampyroteuthidae			
Vampyroteuthis infernalis Chun, 1903	RY	Hokusei Maru, 1994	
Vampyroteuthis infernalis Chun, 1903	DC	Hokusei Maru, 1996	AF000071

[a] Source code: AR = Amanda Reid; BS = Brad Seibel; DC = David Carlini; JS = Jayson Semmens; MN = Mark Norman; MV = Michael Vecchione; RY = Richard Young; SH = Scott Herke; TS = Timothy Stranks. The genus and species of each sample was determined by the source indicated.

[b] In cases where collection numbers are not available, the oceanographic cruise from which the sample was obtained is listed instead.

program PC/Gene (Intelligenetics Inc., Geneva, Switzerland). The internal primer, designated LCO1648 following the nomenclature of Folmer et al. (1994), was then used in sequencing reactions in order to obtain sequence data for the internal region of the cloned COI gene fragment. The sequence of the LCO1648 primer, which begins 158 bp downstream of the LCO1490 primer, is as follows: 5'-ta gtt ata cct att ata att gg-3'.

DATA ANALYSIS.—The COI sequence of the outgroup *Katharina* sp. (Mollusca: Polyplacophora) was downloaded from GenBank [KSU56845]. DNA sequences were aligned by eye with the aid of the *Katharina* sp. homologous sequence and compiled in MacClade 3.0 (Maddison and Maddison, 1992). It was not necessary to introduce gaps into the aligned sequences as there were no insertion/deletion events or alignment ambiguities, a finding consistent with the results obtained by Folmer et al. (1994), where no gaps were introduced in the alignment of COI sequences from diverse metazoan phyla. The GenBank accession numbers are provided in Table 1 for all the taxa sequenced in this study. Phylogenetic analysis of the aligned sequences (657 bp, excluding the primer sequences) was conducted using the heuristic tree-search option in PAUP ver. 3.1.1 (Swofford, 1993) on the unweighted data set. The data were then weighted according to the rescaled consistency index (RCI) calculated for each character based on the unweighted tree (base weight = 1000). A single round of RCI-weighting was employed to reduce the effect that homoplasious substitutions have on tree topologies, giving more weight to characters which have greater individual rescaled consistency indices (Kluge and Farris, 1969; Farris, 1989). In this a posteriori method of character weighting, a rescaled consistency index is calculated for each character and is multiplied by the base weight to designate the assigned weight for that character. Perfectly consistent characters (i.e., RCI = 1), those which do not exhibit non homologous distributions on the unweighted tree, are thus assigned the maximal weight of 1000. Those characters which are homoplastic (i.e., RCI < 1) are assigned lesser weights as the product of their individual RCI and the base weight is less than 1000. One cycle of RCI-weighting always produced a single most parsimonious tree. Support for phylogenetic trees was tested using the heuristic bootstrap search command (1000 replicates) in PAUP. MacClade 3.0 was also used to examine character transformations, translate nucleic acid sequences into amino acid sequences, and to generate various assumption sets (weight and character inclusion sets, transition or transversion inclusion sets). Patristic distance matrices were calculated for the unweighted and RCI-weighted data under the various assumption sets (all characters included, first and second codon position characters only, third codon position characters only, transversional or transitional substitutions only) in PAUP, and plotted against percent sequence divergence for all possible pairwise comparisons between 19 randomly chosen taxa to explore the potential impact of positional or transitional saturation on phylogenetic analyses. Protein distance matrices were calculated using the Dayhoff PAM method (Dayhoff, 1978) in the PROTDIST program in PHYLIP 3.57c (Felsenstein, 1995). The protein distance data were transformed using the angular transformation (Sokal and Rohlf, 1981) prior to conducting statistical analyses on the data.

RESULTS

SEQUENCE VARIATION.—The 657 base pair fragment of the COI gene from 50 species comprised 331 variable characters (50.5%), of which 88 (26.6%) characters were first codon position bases (16 of which were parsimony uninformative), 25 (7.5%) were second codon position bases (15 of which were parsimony uninformative), and 218 (65.9%) characters were third codon position bases (4 of which were parsimony uninformative).

Comparisons of sequence divergences within and among the major groups of cephalopod taxa are presented in Table 2. As expected, mean sequence divergences determined from all possible pairwise comparisons within groups were less than mean sequence divergences resultant from pairwise comparisons among groups. For example, the mean sequence divergence (± SD) among the Oegopsida was 17.74 ± 1.61% whereas compari-

Table 2. Comparison of nucleotide sequence divergences within and among the major groups of cephalopod taxa.

	Oegopsida			Myopsida			Sepioidea			Octopoda			Vampyromorpha		
	x[a]	SD[b]	n[c]	x	SD	n	x	SD	n	x	SD	n	x	SD	n
Oegopsida	17.7	1.6	325												
Myopsida	19.7	1.1	78	15.8	1.3	3									
Sepioidea	19.0	1.6	208	19.8	1.6	24	18.5	2.2	24						
Octopoda	20.5	1.7	260	21.4	1.2	30	20.7	1.7	80	17.6	2.4	45			
Vampyromorpha	21.4	1.4	52	21.5	0.4	6	20.4	1.3	16	19.2	1.4	20	1.5	—	1
Nautilus	25.8	1.2	26	26.6	1.2	3	25.6	0.6	8	24.9	0.9	10	26.0	0.2	2

[a] Mean sequence divergence for all possible pairwise comparisons between taxonomic representatives of the two groups in question.
[b] Standard deviation from the mean for all pairwise comparisons between taxa among major groups.
[c] Number of pairwise comparisons between taxa conducted for the major groups examined.

sons between the Oegopsida and other groups ranged from 19% to approximately 26%. However, pairwise comparisons among Coleoid groups did not differ appreciably (e.g., Oegopsida vs Myopsida = 19.72 ± 1.07%, Oegopsida vs Sepioida = 19.04 ± 1.64%). Mean sequence divergences for all comparisons between *Nautilus* and other groups were significantly greater than mean divergences among the various coleoid groups. In contrast to the other within-order sequence comparisons, the low sequence divergence within the Vampyromorpha (1.5%) reflects a comparison within a single species, *Vampyroteuthis infernalis*.

The percentage of substitutions occurring at third codon position characters and pooled first and second codon position characters (Fig. 1A) and transitional or transversional substitutions (Fig. 1B) are plotted against percent sequence divergence for sequence comparisons of the unweighted data. As sequence divergence increased, the percentage of substitutions decreased for third codon position characters, and increased for the pooled first and second codon position characters. There did not appear to be a clear relationship between the percentage of transitional or transversional substitutions and sequence divergence for comparisons of the unweighted data. Figures 1C,D show the same comparisons for the RCI-weighted data. When the data were weighted, third codon position characters accounted for the majority of substitutions at low sequence divergences. At greater divergences, the percentage of substitutions at third codon position characters dropped dramatically, and first and second codon position substitutions accounted for the majority of sequence variation. Comparisons between transitional and transversional substitutions demonstrated that transversions accounted for approximately 2/3 of the substitutions for the RCI-weighted data, regardless of sequence divergence. These results support the use of RCI-weighting for this data set, where many of the third codon position substitutions are likely to be a true "signal" rather than "noise"; thus downweighting of all third codon characters would lead to loss of phylogenetic information. Transversional weighting is also unwarranted as there did not appear to be any relationship between the percentage of transversional substitutions and sequence divergence, nor a substantial bias in the total number of transversions (43% of all substitutions in the unweighted analysis) versus transitions (57%). As has been demonstrated by other studies using mitochondrial protein-coding gene sequences (Meyer, 1994), third codon position characters were highly variable; however, exclusion of third codon characters would result in the elimination of approximately two thirds of the phylogenetically informative characters. Nevertheless, there

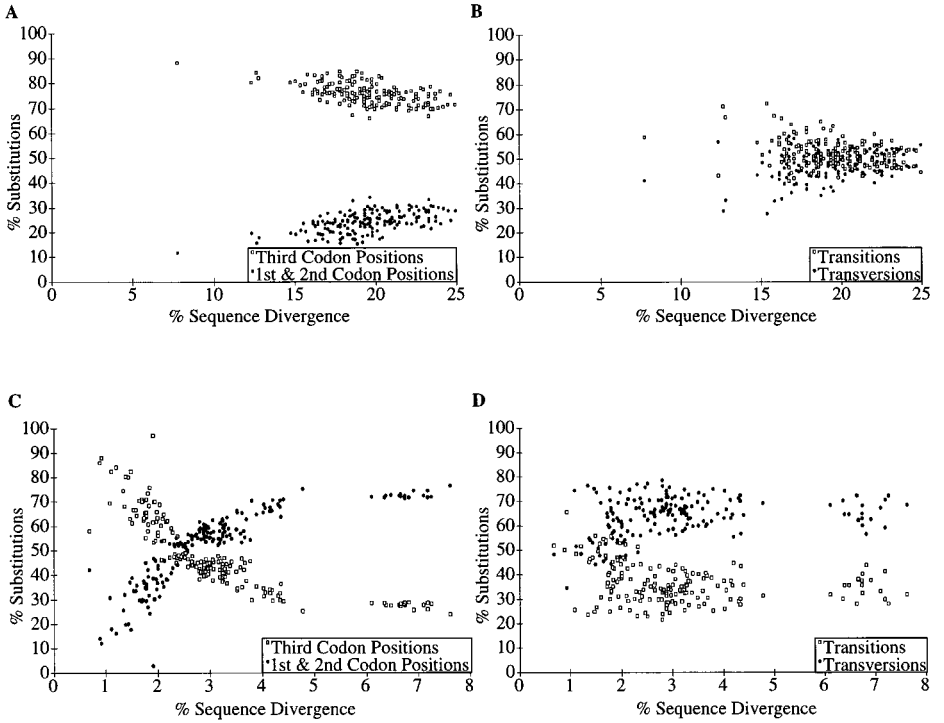


Figure 1. The relationship between sequence divergence and type of substitution plotted for all pairwise comparisons among 20 randomly chosen taxa calculated from patristic distance matrices generated in PAUP v. 3.1.1. A. Unweighted data: percentage of substitutions occurring at third codon position characters (open boxes) and pooled first and second codon position characters (filled circles) plotted as a function of percent sequence divergence . B. Unweighted data: percentage of substitutions involving transitional (open boxes) and transversional (filled circles) changes plotted as a function of sequence divergence. C and D. The same relationships are plotted as in Figures 1A and 1B, respectively, with patristic distance matrices calculated from the RCI-weighted data set.

are clear differences within third codon characters with respect to the degree of variation where 30.6% of third codon characters were twofold variable (exhibited two character states), 27.9% were threefold variable, and 41.5% were fourfold variable. RCI-weighting enabled differential downweighting of all characters, where highly variable characters were severely downweighted (e.g., most fourfold variable third codon characters), and some were not downweighted at all (e.g., most second codon position characters). There was also significant heterogeneity in base composition at third codon position characters (chi-square = 434.0, df = 147, $P < 0.001$) whereas base composition bias at first and second codon characters was insignificant (chi-square = 36.6, df = 147, $P > 0.9$).

COLEOID PHYLOGENETIC RELATIONSHIPS.—A heuristic search of the unweighted data yielded the single most parsimonious tree depicted in Figure 2A. For this tree and all trees discussed below, bootstrap support values are given below nodes supported by over 50% of 1000 heuristic bootstrap replicate searches, and the branch lengths are proportional to the number of unambiguous changes occurring along each branch. The tree generated from unweighted analysis supports the monophyly of the Coleoidea, Decapoda, Cirrata,

Myopsida, and various families represented by more than one taxon (Oegopsida: Enoploteuthidae, Cranchiidae, Gonatidae, Ommastrephidae; Incirrata: Bolitaenidae; Sepioidea: Sepiidae and Sepiolidae). However, the octopods are split into two groups, one of which contains *Vampyroteuthis*. The branch separating the two octopod clades is defined by a single synonymous substitution (C->T: Leu->Leu) occurring at base 219, a first codon position nucleotide. Other taxonomic groupings not supported by maximum parsimony analysis of the unweighted data set include the Cycloteuthidae (as including *Discoteuthis*) and the Pholidoteuthidae (as including *Lepidoteuthis*). The Pyroteuthidae and Ancistrocheiridae are closely aligned but clearly separate from the Enoploteuthidae. The majority of the relationships determined from analysis of the unweighted data set were not supported by bootstrapping.

The single most parsimonious tree generated from a heuristic search of the data weighted to the rescaled consistency index is illustrated in Figure 2B. The tree from the RCI-weighted data supports the monophyly of the Coleoidea, Octopodiformes (Octopoda + Vampyromorpha [Berthold and Engeser, 1987]), Octopoda, Cirrata, Decapoda, Sepiolidae, Teuthoidea, Myopsida, Oegopsida and most families represented by more than one taxon (Oegopsida: Enoploteuthidae, Gonatidae, Ommastrephidae; Sepioidea: Sepiidae; Incirrata: Bolitaenidae). However, some oegopsid families are not supported by parsimony analysis of the RCI-weighted data, including the Cycloteuthidae, Pholidoteuthidae, and Cranchiidae. As with analysis of the unweighted data, the Ancistrocheiridae and Pyroteuthidae are related to each other but remain distinct from the Enoploteuthidae. Sepiolids group outside of the remaining decapods. Bootstrapping the RCI-weighted data resulted in ≥50% support for a greater number of nodes (17 supported) than did bootstrap analysis of the unweighted data set (13 supported).

A heuristic search of the inferred COI amino acid sequences yielded many equally parsimonious trees. The search was terminated after 14,900 trees had been generated by the analysis. Although the nucleotide sequences demonstrate significant variability, the amino acid sequences were highly conserved (only 17.4% of the characters were phylogenetically informative). Within major lineages, phylogenetically informative variability in the amino acid sequences was virtually nonexistent, resulting in the generation of many equally parsimonious trees. The strict consensus (not shown) of the 14,900 trees generated from the amino acid data split the coleoids into two major groups, the Decapoda and the Octopodiformes (Octopoda + Vampyromorpha). Bootstrap analysis of the amino acid data was not conducted due to the large amount of time required to complete a single replicate. The amino acid data, although of little use in constructing relations within the octopods and decapods, were useful in strongly confirming placement of *Vampyroteuthis* with the octopods, and in demonstrating the monophyly of the two major groups of coleoid cephalopods.

Analysis of the entire data set permitted proper selection of outgroups to be used for the next level of nested analyses. Use of distant outgroup taxa which are highly diverged from the coleoids (i.e., *Katharina* sp. and *Nautilus pompilius*) made it difficult to determine which character states in the ingroup are pleisiomorphic relative to other states. In order for the outgroup to be of maximal value in the polarization of characters it is necessary to use the most closely related but exclusive sister group as the outgroup (Wheeler, 1990). Unfortunately, for analysis of the Coleoidea as a whole, the most appropriate outgroups are extinct (Berthold and Engeser, 1987). For analysis of relationships within the Octopoda and Decapoda, outgroups were chosen on the basis of the relationships

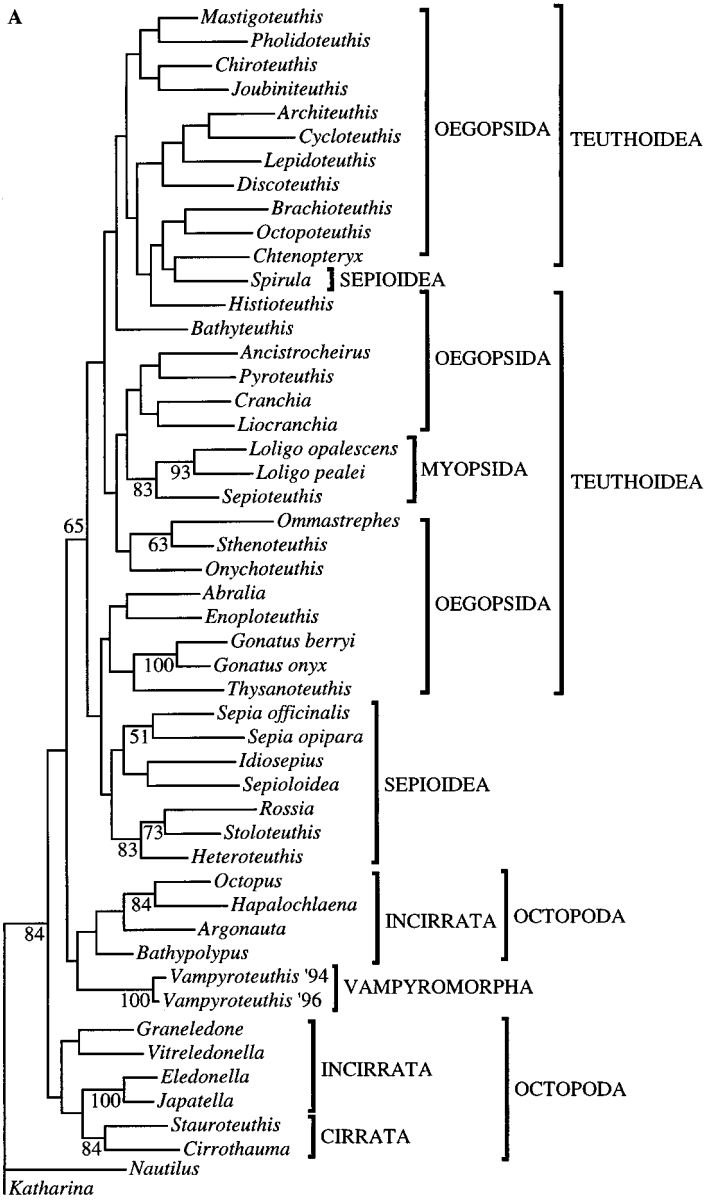
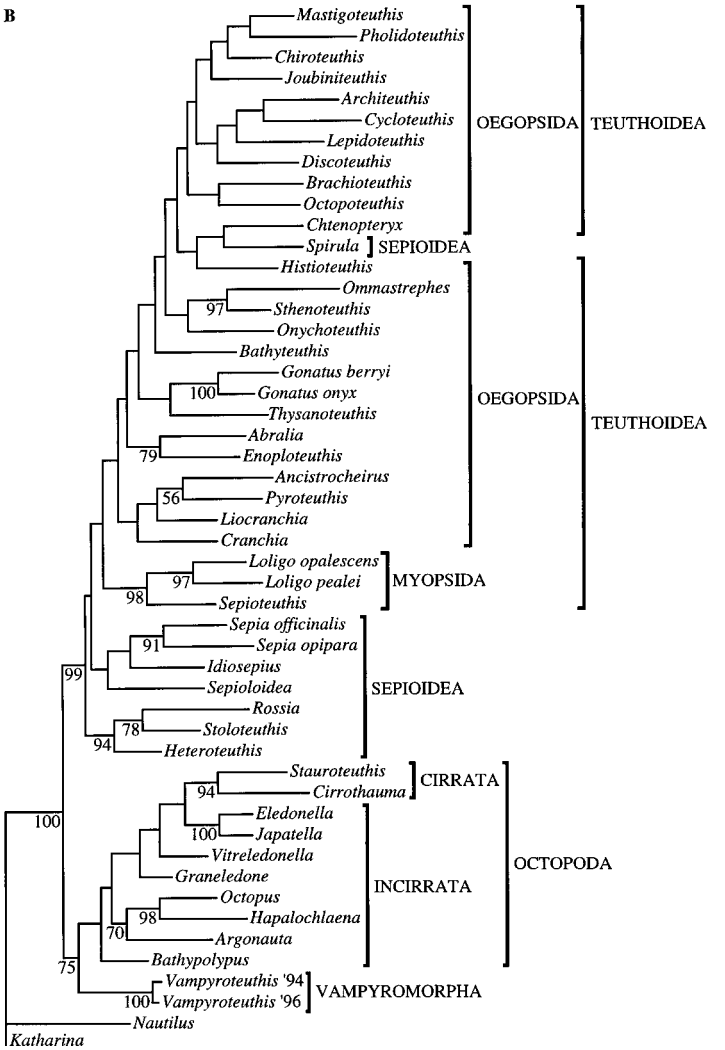


Figure 2. Most parsimonious trees produced from phylogenetic analysis of coleoid cephalopod COI nucleotide sequences. Bootstrap support values > 50% are indicated below nodes. Branches lengths are drawn proportional to the number of unambiguous changes. Indicated on the right are the orders and suborders to which the terminal taxa belong according to the classification of Voss (1977). A. Single most parsimonious tree generated from a heuristic search of the unweighted data (Tree Length = 3305, Consistency Index = 0.182, Retention Index = 0.342). B. *(opposite page)* Most parsimonious tree generated from a heuristic search of the data subjected to a single round of RCI-weighting with a base weight equal to 1000 (TL = 221,902, CI = 0.425, RI = 0.533).



determined from phylogenetic analysis of the entire data set. In the analysis of the Octopoda, *Vampyroteuthis* was selected as the outgroup because the RCI-weighted data placed vampire squids outside of the remaining octopods. In addition, several morphological characters place vampire squids outside of the remaining Octopodiformes: arms II, sucker stalks, arm protective membranes, visceropericardial coelom, dorsal mantle cavity, funnel valve, nuchal cartilage, superior buccal lobes of the brain, and inferior frontal system of the brain (Young and Vecchione, 1996). We also elected to use *Vampyroteuthis* as the outgroup for analysis of the decapods. While the results from phylogenetic analysis of the RCI-weighted data support use of the sepiolids as outgroup to the remaining decapods, the results from the unweighted analysis of the entire data set do not. Furthermore, the morphological evidence does not support separating the sepiolids from the decapods. The

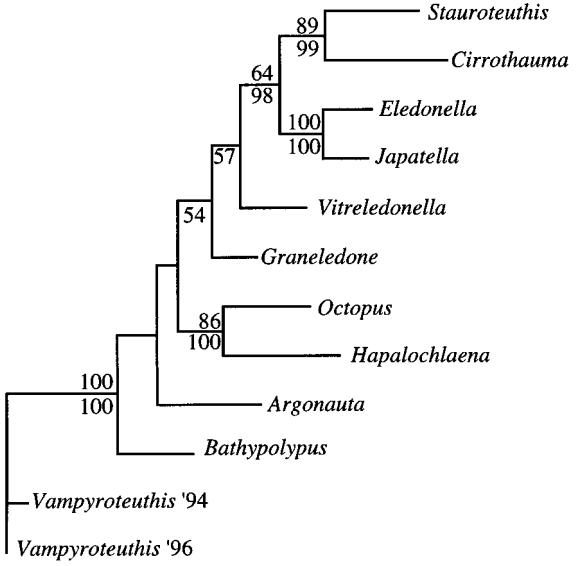


Figure 3. Most parsimonious tree produced from phylogenetic analysis of octopod COI nucleotide sequences using the Vampyromorpha as outgroup. Analysis of the unweighted data (TL = 692 , CI = 0.529 , RI = 0.468, bootstrap indices > 50% above nodes) and the RCI-weighted data (TL = 190, 139) , CI = 0.751 , RI = 0.710, bootstrap indices >50% below nodes) resulted in an identical tree topology.

deliberately conservative selection of *Vampyroteuthis* as outgroup for analysis of the decapods ensures that the assumption of ingroup monophyly would not be violated and avoids the incorrect polarization of characters and confusing cladogram that would result from the selection of an improper outgroup.

OCTOPOD PHYLOGENETIC RELATIONSHIPS.—The most parsimonious trees generated from the unweighted and RCI-weighted data for the octopods are identical (Fig. 3). The octopods are clearly distinct from *Vampyroteuthis*. This division is supported in all bootstrap replicates of both the unweighted and RCI-weighted data. The division of the octopods into the suborders Cirrata and Incirrata is not supported as the bolitaenids appear to be more closely related to the cirrates than to the incirrates, such that the Incirrata are paraphyletic. Strong bootstrap support (98%) for the monophyly of the (bolitaenid cirrate) clade was obtained from analysis of the RCI-weighted data, and some support (64%) was obtained from analysis of the unweighted data. The monophyly of the (bolitaenid cirrate) clade was also supported when the data were transversionally weighted (TV:TI = 2:1 or 3:1) or when third codon position substitutions were downweighted (by 1/2 or 1/3) relative to substitutions at first and second codon position characters (not shown). Members of the family Octopodidae did not cluster. Somewhat surprising is the placement of *Argonauta*, which groups more closely to the octopodids than does *Bathypolypus*.

DECAPOD PHYLOGENETIC RELATIONSHIPS.—The results from a heuristic search of the unweighted and RCI-weighted decapod data resulted in the single most parsimonious trees presented in Figure 4A,B, respectively. These trees are identical with the exception of the placement of the (*Architeuthis Cycloteuthis*) clade, which groups with the (*Mastigoteuthis Pholidoteuthis*) clade in the unweighted analysis and groups outside of

the ((*Mastigoteuthis Pholidoteuthis*) (*Lepidoteuthis Octopoteuthis*)) clade in the RCI-weighed analysis. Analysis of the decapods using *Vampyroteuthis* as the outgroup supports the monophyly of the Decapoda, Teuthoidea (*Spirula* is included), Myopsida, and various families. Results from analysis of the more restricted data set also differed in some of the relationships indicated by the unweighted and RCI-weighted data when all the taxa were included in the analysis. Some interesting differences include the close alignment of *Chtenopteryx* and *Spirula* with the myopsids, the placement of the enoploteuthids outside of the remaining teuthoids, and the division of the Sepioidea (exclusive of *Spirula*) into three distinct clades. As in the analyses of the entire data set, bootstrap levels were elevated in the RCI-weighted analysis where several oegopsid clades were supported by ≥50% of the replicates. Moderate bootstrap support was found for a close relationship between *Chiroteuthis* and *Joubiniteuthis* and also for *Thysanoteuthis* and *Gonatus*. Low levels of bootstrap support were obtained for close relationships between *Mastigoteuthis* and *Pholidoteuthis*, *Lepidoteuthis* and *Octopoteuthis*, and the (*Ancistrocheirus Pyroteuthis*) clade with the cranchiid clade. Bootstrap analysis of the RCI-weighted data resulted in strong support of all clades consisting of a single family with the exception of the cranchiids which exhibited a low level (54%) of support and the cycloteuthids which did not group together.

Evolution of the COI Protein.—The rate of evolution of the COI protein in the two major lineages, the Decapoda and the Octopodiformes, was examined by conducting pairwise comparisons of COI amino acid sequences within the two groups. Pairwise comparisons within decapods resulted in a mean of 2.28% amino acid sequence divergence as calculated by the Dayhoff PAM model of protein evolution, in which the probabilities of change from one amino acid to another are scaled in terms of a common unit which equals a 1% change between two amino acid sequences (Felsenstein, 1995). Pairwise comparisons within the Octopodiformes resulted in an average amino acid sequence divergence of 5.93%. Statistical analyses of the arcsine-transformed distance data indicated a significant difference between the two groups in the rates of evolution of the COI fragment used in this study (single-factor ANOVA, $F = 151.39^{***}$, $F_{0.001[1,694]} = 10.30$). Statistical analysis of the unweighted amino acid data, which assumes equal probabilities for all amino acid transitions, also resulted in highly significant differences between the two groups (single-factor ANOVA, $F = 204.24^{***}$), where the mean amino acid sequence divergences for decapods and Octopodiformes were 2.35% and 5.53%, respectively.

Discussion

Previous investigations of coleoid systematics have attempted to determine relationships within the Octopoda, Sepioidea, and Myopsida through phylogenetic analysis of morphological and molecular character data. Most recently, Young and Vecchione (1996) conducted a higher-level analysis of the coleoid cephalopods. Consistent with Young and Vecchione (1996), and as was independently determined in an earlier study by Berthold and Engeser (1987), our results suggest that the coleoids can be divided into two main lineages, the Octopodiformes (Octopoda + Vampyromorpha) and the Decapoda (Sepioidea + Teuthoidea).

Within the Octopodiformes we found the Vampyromorpha to be sister taxon to the Octopoda, a result which again was consistent with those obtained by Young and Vecchione (1996). Our results within the Octopoda differed from those obtained by Young and

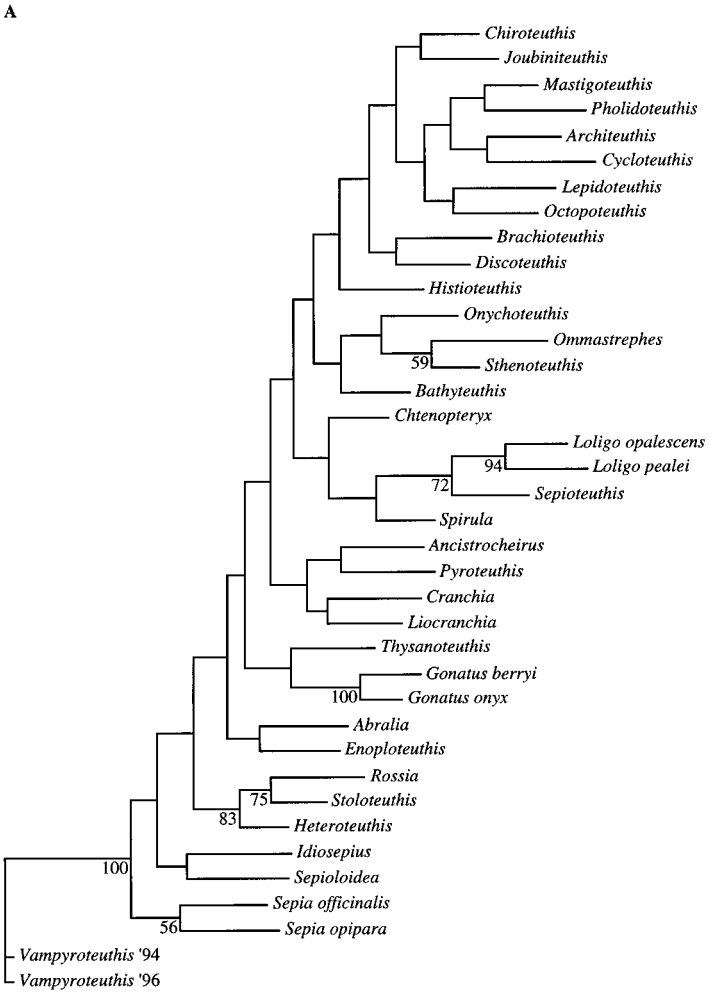
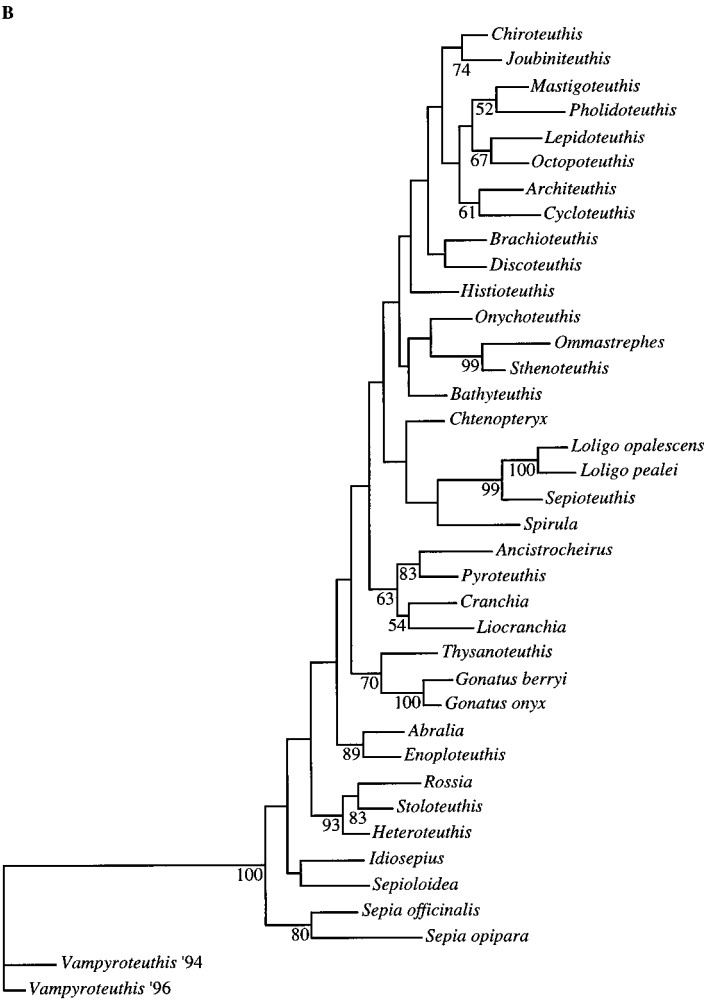


Figure 4. Results from phylogenetic analysis of decapod (Sepioidea + Teuthoidea) COI sequences using *Vampyroteuthis* as outgroup. Bootstrap support values >50% are indicated below nodes. Branches lengths are drawn proportional to the number of unambiguous changes. A. Most parsimonious tree generated from maximum parsimony analysis of the unweighted data (TL = 2466, CI = 0.214, RI = 0.323). B. *(opposite page)* Most parsimonious tree produced from analysis of the RCI-weighted data (TL = 169,389, CI = 0.397, RI = 0.463).

Vecchione and the scenario proposed by other researchers (Naef, 1921–1923; Robson, 1932; Berthold and Engeser, 1987; Voss, 1988; Voight, 1993) wherein the Cirrata are sister taxon to the Incirrata, and both suborders are considered monophyletic. We found the Incirrata to be paraphyletic, as the bolitaenids grouped more closely with the cirrates than they did with the remaining incirrates. At least one morphological character may suggest a bolitaenid-cirrate affinity: the presence of horizontal arm septa in both groups, although Young and Vecchione (1996) interpreted these as separate character states since the arrangement is not identical in the two groups. However, taxonomic sampling of the bolitaenids and cirrates in this study is limited, and the inclusion of additional bolitaenid



(or bolitaenoids such as the Idioctopodidae and Amphitretidae-both monotypic) and cirrate taxa (especially a highly benthic species, such as an opisthoteuthid) is needed to confirm a possible relationship between the two groups. The COI data are also not consistent with the sister group status of the cirrates and incirrates. The cirrates have been considered primitive, having diverged from the incirrates early on in the evolution of the Octopoda. The placement of the cirrates using the COI data is puzzling unless the derived status of the cirrate taxa is an artifact of the longer period for which the COI gene evolved in the older cirrate lineage. The positions of *Bathypolypus* and *Argonauta* are unexpected as *Bathypolypus* is an octopodid and would be expected to group more closely with the other octopodids (*Octopus*, *Hapalochlaena*, *Graneledone*) than *Argonauta* (Argonautidae). The lack of other argonautoid families (e.g., Alloposidae, Ocythoidae, Tremoctopodidae) in our representation of octopod diversity may have contributed to this anomalous result which was not supported by bootstrap analyses.

Although the monophyly of the Decapoda is well supported by our COI data, we could not confirm the validity of the order Sepioidea as defined by Voss (1977). Several studies

have reached this conclusion, although the way they have divided the Sepioidea has differed substantially (Fioroni, 1981; Berthold and Engeser, 1987; Clarke, 1988; Khromov, 1990; Bonnaud et al., 1994). *Spirula* did not group with any of the sepioids but consistently grouped with the teuthoids, a result concordant with the results of Bonnaud et al. (1994). Our data support an affinity between the Sepiadariidae and Idiosepiidae and do not support placement of the Idiosepiidae within the Oegopsida, as suggested by Bonnaud et al. (1997). It is important to recognize that many of the differences between our study and previous molecular studies may be the result of very different taxonomic sampling schemes. We included a greater proportion of oegopsid respresentatives in this study whereas Bonnaud et al. (1994, 1997) included a greater proportion of sepioid taxa in their work. The monophyly of the Sepiolidae was well supported in all of our analyses, however, their rank and position within the Decapoda is not clear. Clarke (1988) and Bonnaud et al. (1994, 1997) recommend raising the sepiolids to ordinal rank. While results from analysis of the entire COI data set appear to support this contention, results from analysis of the decapods alone using *Vampyroteuthis* as outgroup do not. As the status of the Sepioidea is likely to remain a matter of debate for some time to come, we refrain from making premature recommendations regarding the taxonomy of the five sepioid families. We feel that such taxonomic revisions should await the results of additional molecular and morphological phylogenetic studies.

The monophyly of the Myopsida was strongly supported in all the analyses. A relationship between *Chtenopteryx* and the myopsids obtained in the analysis of the decapods is consistent with morphological evidence (J. Z. Young, 1991) and recent biochemical evidence (Brierley et al., 1996) which suggests that *Chtenopteryx* is a bathypelagic myopsid squid. In contrast to the parsimony results of Bonnaud et al. (1994, 1997), the myopsids grouped within rather than outside of the Oegopsida. This result, and also the placement of *Spirula* within the oegopsids as sister taxon to *Chtenopteryx* (in the analysis of the entire data set) or as sister taxon to the myopsids (in the analysis of the decapods alone) indicates that the suborder Oegopsida, as defined by Voss (1977), is polyphyletic.

Only two studies have attempted to resolve the Oegopsida, both of which used morphological character data (Toll, 1982; Clarke, 1988). Clarke's analysis of relationships within the group did not use an explicit method (such as maximum parsimony) and is based on subjective interpretation of morphological similarity. Toll used an array of morphological characters all related to the same structure, the gladius, to determine relationships among the Oegopsida. The morphology of the gladius in oegopsid squids, however, is not sufficiently variable to conclusively determine relationships among all the families within the group. The two studies do not agree in their placement of most families within the Oegopsida. Clarke's study divided the oegopsid squids into two major clades, with the cranchiids grouping outside of one clade and the gonatids grouping outside of the second clade. Toll placed the Thysanoteuthidae and Ommastrephidae outside of all the remaining oegopsid squid families. Results from our analysis of the decapods place the Enoploteuthidae outside of the remaining teuthoids, with the Thysanoteuthidae and Gonatidae also placing near the base of the teuthoid tree. Interestingly, unlike either morphological study the COI data support a close relationship between the Thysanoteuthidae and Gonatidae. Clarke considered the Ancistrocheiridae to be distinct from the Enoploteuthidae and Pyroteuthidae, which he regarded as closely related. Toll found all three families to be closely related but relationships among them were left unresolved. We found the pyroteuthids and ancistrocheirids to be closely related, but clearly distinct

and somewhat distant from the enoploteuthids. Like those of Clarke, our results support a close relationship between the Octopoteuthidae and Lepidoteuthidae. Also consistent with Clarke's results, the Chiroteuthidae, Joubiniteuthidae, and Mastigoteuthidae were found to be related; the close relationship between these families has also been suggested by R. E. Young (1991). Unlike either study, we found that the Onychoteuthidae and Ommastrephidae consistently grouped together. Another difference between the studies of Clarke and Toll is in the placement of the Histioteuthidae. The COI data place *Histioteuthis* outside of a large clade consisting of primarily bathypelagic squids, a result somewhat more similar to the results of Clarke in that *Histioteuthis* is not very closely related to the enoploteuthids, ancistrocheirids, or pyroteuthids as suggested by Toll. As many of the relationships within the oegopsid squids were not entirely consistent between the two taxonomic inclusion/exlusion treatments nor between the different weighting schemes, and because bootstrap analyses of the COI data did not lend support to most of the oegopsid nodes, most relationships within the Oegopsida remain obscure.

Our failure to conclusively determine phylogenetic relationships among oegopsid families may be due to the use of an inappropriate gene for constructing family-level relations in the group. The COI sequence is highly conserved at the amino acid level and at first and second codon position characters at the nucleotide level. However, third codon position characters which may be informative in determining relationships between taxa which have diverged more recently, were highly variable and homoplastic change may have masked phylogenetic signals at the family level. Alternately, the lack of resolution within the Oegopsida may be the result of non-dichotomous branching events (Hoelzer and Melnick, 1994). Perhaps the ancestral oegopsid taxon gave rise to several new families in a very short period of time or simultaneously, yielding a polytomous branching pattern. Several life-history traits of oegopsid squids are characteristic of species which are likely to produce polytomous branching patterns in inferred phylogenies, including cryptic speciation (Smith et al., 1981; Brierley et al., 1993; Yeatman and Benzie, 1993), rapid evolutionary rate (Bonnaud et al., 1994; O'Dor, 1995) and cosmopolitan distributions (O'Dor, 1988).

A knowledge of the potential selective forces operating on the evolution of the COI amino acid sequences in cephalopods would be helpful in determining whether the inferred phylogenetic patterns are the result of shared ancestry or of convergence. It is entirely possible that the COI protein may be more conserved for some phylogenetic groups than for others. For example, we found significantly greater variation in the amino acid sequences within the Octopodiformes (mean divergence = 5.9%) than we did within the decapods (mean divergence = 2.3%). These differences may be due to more restrictive requirements in the function of the protein in the decapods, which are generally more mobile than members of the Octopodiformes, in which the evolution of the COI protein may be somewhat less constrained. If this is true, then our lack of resolution for the Oegopsida, which represent the most mobile and metabolically-active cephalopods, may be due to greater constraints on the evolution of the COI protein in the oegopsid squids. In support of this hypothesis, we found a low level of amino acid sequence variability within the Oegopsida (mean divergence = 0.98%) in comparison to the Sepioidea (mean divergence = 4.02%). Unfortunately, comparisons in the evolutionary rate of the COI protein between oegopsids and myopsids are not meaningful, due to the low number (3 species) and close relationship (all are members of the family Loliginidae) of myopsid taxa used in this study.

Our results, particularly in reference to the oegopsid squids, must be interpreted with caution as bootstrap analyses failed to lend substantial support for many clades. Although bootstrap support does not necessarily confirm that a certain relationship is "true," lack of bootstrap support does indicate instability in the data set. Use of reweighting techniques to lend stability to highly homoplastic data sets is controversial, but in the absence of appropriate *a priori* weighting methods, reweighting techniques are the most commonly accepted alternative. Nevertheless, limited confidence can be placed in relationships determined from analysis of the RCI-weighted data when they differ from those obtained in unweighted analysis of the data set, thus, further analysis of oegopsid relations is clearly warranted. We are confident, however, that the following conclusions can be drawn from our COI data: (1) the Coleoidea, Octopoda, Vampyromorpha, and Decapoda are monophyletic groups; (2) the Vampyromorpha and Octopoda are sister taxa; (3) the Sepioidea, as including the 5 families Spirulidae, Sepiolidae, Sepiidae, Sepiadariidae, and Idiosepiidae, is polyphyletic; (4) *Spirula* is more closely related to the Teuthoidea than it is to the Sepioidea; and (5) the Oegopsida, as currently defined, is polyphyletic.

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