MOLECULAR PHYLOGENY OF COLEOID CEPHALOPODS (MOLLUSCA: CEPHALOPODA) INFERRED FROM THREE MITOCHONDRIAL AND SIX NUCLEAR LOCI: A COMPARISON OF ALIGNMENT, IMPLIED ALIGNMENT AND ANALYSIS METHODS

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ABSTRACT

Recent molecular studies investigating higher-level phylogenetics of coleoid cephalopods (octopuses, squids and cuttlefishes) have produced conflicting results. A wide range of sequence alignment and analysis methods are used in cephalopod phylogenetic studies. The present study investigated the effect of commonly used alignment and analysis methods on higher-level cephalopod phylogenetics. Two sequence homology methods: (1) eye alignment, (2) implied alignment, and three analysis methods: (1) parsimony, (2) maximum likelihood, (3) Bayesian methodologies, were employed on the longest sequence dataset available for the coleoid cephalopods, comprising three mitochondrial and six nuclear loci. The data were also tested for base composition heterogeneity, which was detected in three genes and resolved using RY coding. The Octopoda, Argonautoidea, Oegopsida and Ommastrephidae are monophyletic in the phylogenies resulting from each of the alignment and analysis combinations. Furthermore, the Bathyteuthidae are the sister taxon of the Oegopsida in each case. However many relationships within the Coleoidea differed depending upon the alignment and analysis method used. This study demonstrates how differences in alignment and analysis methods commonly used in cephalopod phylogenetics can lead to different, but often highly supported, relationships.

INTRODUCTION

The class Cephalopoda comprises two extant subclasses, Nautiloidea (*Nautilus* and *Allonautilus*) and the Coleoidea. The Coleoidea contains two subdivisions, the Belemnoidea, which became extinct at the end of the Cretaceous, and the Neocoleoidea, which contains the octopuses, squids and cuttlefishes. Neocoleoid cephalopods are characterized by the reduction and internalization, or complete loss, of the shell and, as a result, they very rarely fossilize well. Therefore, very little information regarding the origins and relationships of extant coleoid cephalopods can be gleaned from the fossil record (Nishiguchi & Mapes, 2007).

Morphological studies have proved to be useful in classifying species within subfamilies and/or genera (e.g. Berthold & Engeser, 1987; Clarke, 1988; Khromov, 1990; Voight, 1993a,b; Young & Vecchione, 1996; Voss, 1988), but less so in determining higher-level relationships. Morphological studies attempting to resolve these relationships have been constrained by the number of characters used with confidence due to "problems primarily involving character independence, apomorphic 'loss', or assessment of homology/homoplasy" (Young & Vecchione, 1996).

Presently, extant coleoids are divided into two superorders, Decapodiformes and Octopodiformes (Berthold & Engeser, 1987). In his website 'The Fossil Coleoidea Page' (http://userpage.fu-berlin.de/~palaeont/fossilcoleoidea/welcome.html), Engeser draws attention to the fact that the term Octopodiformes is in use elsewhere and suggests the use of Vampyropoda (Boletzky, 1992) instead. The Decapodiformes (Decembrachiata Winckworth *sensu* Engeser, loc. cit.) contains the orders Teuthoidea [suborders Myopsida (closed-eye squids) and Oegopsida (open-eye squids) and Sepioidea (families Idiosepiidae (pygmy squid), Sepiidae (cuttlefishes), Spirulidae (ram's horn squid), Sepiolidae (bobtail squids) and Sepiadariidae (bottletail squids)]. Current debate exists on the validity of the ordinal level of classification (Naef, 1921–1923; Voss, 1977; Berthold & Engeser, 1987; Young & Vecchione, 1996). Furthermore, Lindgren, Giribet & Nishiguchi (2004) question whether the suborder Oegopsida is monophyletic.

The Octopodiformes contains the orders Vampyromorpha (vampire 'squid') and Octopoda (pelagic and benthic octopuses), hence the name Vampyropoda (Boletzky, 1992). A sister-taxon relationship between these two orders is accepted primarily based on morphology (Pickford, 1939; Boletzky, 1992; Young & Vecchione, 1996; Engeser, 1997; Young, Vecchione & Donovan, 1998; Carlini, Reece & Graves, 2000), but combined analysis using molecular and morphological data suggests a sister-taxon relationship between the Decapodiformes and Vampyromorpha (Lindgren *et al.*, 2004). The Octopoda comprises the suborders Cirrata (deep-sea finned octopuses) and Incirrata (benthic octopuses and pelagic octopuses, including the argonautoids and blanket octopuses. A sister-taxon relationship between these suborders is also widely accepted (Grimpe, 1921; Naef, 1921–1923; Young & Vecchione, 1996; Voight, 1997). Phylogenetic relationships between the nine Incirrata families remain unresolved and have been debated in the literature (Naef, 1921–1923; Robson, 1929, 1931; Voss, 1977; Young & Vecchione, 1996; Voight, 1997).

In the mid 1990s the first studies using DNA sequence data to estimate phylogenetic relationships within cephalopods were reported (Bonnaud, Boucher-Rodoni & Monnerot, 1994, 1996, 1997; Boucher-Rodoni & Bonnaud, 1996). These studies sequenced portions of 16S rDNA, COII and COIII from 8 to 28 cephalopod taxa. These authors aligned their sequences by

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eye (with the aid of the secondary structure where possible) and analysed the data using neighbour-joining (NJ) and parsimony methods. Although the genes proved useful in helping resolve intrafamilial relationships, little resolution of higher-level relationships was recovered. Subsequently, molecular studies investigating higher-level phylogenetic relationships of cephalopods have sequenced additional mitochondrial genes (Carlini & Graves, 1999; Piertney *et al*., 2003; Nishiguchi, Lopez & Boletzky, 2004; Zheng *et al*., 2004; Guzik *et al*., 2005) including whole mitochondrial genomes (Yokobori *et al*., 2004; Akasaki *et al*., 2006) and also nuclear genes (Carlini *et al*., 2000; Warnke *et al*., 2003; Strugnell *et al*., 2004; Guzik *et al*., 2005; Strugnell *et al*., 2005) often from a greater number of taxa (Carlini & Graves, 1999; Anderson, 2000a, b; Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005) (Table 1).

Furthermore, since these first studies of cephalopod molecular phylogenetics, the range of sequence alignment and analysis methods available to phylogeneticists has increased (Table 1), and debate concerning the best methods to use has flourished (e.g. Wheeler, 1995, Kjer, Gillespie & Ober, 2007). Studies investigating cephalopod phylogenetics have aligned sequences by eye (Carlini & Graves, 1999; Carlini *et al*., 2000; Strugnell *et al*., 2004, 2005) or with the aid of alignment packages (Piertney *et al*., 2003; Yokobori *et al*., 2004; Zheng *et al*., 2004; Guzik *et al*., 2005) and have employed a variety of methods of analysis, including neighbour-joining (Allcock & Piertney, 2002; Warnke *et al*., 2003; Yokobori *et al*., 2004; Zheng *et al*., 2004), parsimony (Carlini & Graves, 1999; Anderson, 2000a,b; Carlini *et al*., 2000; Carlini, Young & Vecchione, 2001; Allcock & Piertney, 2002; Warnke *et al*., 2003; Lindgren *et al*., 2004, 2005; Nishiguchi *et al*., 2004; Zheng *et al*., 2004; Guzik *et al*., 2005), maximum likelihood (ML) (Anderson, 2000a,b; Carlini *et al*., 2000, 2001; Allcock & Piertney, 2002; Warnke *et al*., 2003; Strugnell *et al*., 2004; Yokobori *et al*., 2004; Guzik *et al*., 2005), Bayesian (Strugnell *et al*., 2004, 2005; Guzik *et al*., 2005) and LogDet (Anderson, 2000b; Strugnell *et al*., 2005). Recently, some studies have employed direct optimization where alignment is coupled with tree estimation in a dynamic procedure (Nishiguchi *et al*., 2004; Lindgren *et al*., 2004, 2005) (Table 1).

Although providing some insights [e.g. sister taxon relationships between the suborder Oegospida and family Bathyteuthidae (Strugnell *et al*., 2005)] none of these studies have conclusively resolved all higher-level cephalopod phylogenetic relationships and in many cases the results have been conflicting (see Akasaki *et al*., 2006; Nishiguchi & Mapes, 2007 for review of conflicting decapodiform relationships).

A number of reasons have been suggested for these varying and unresolved relationships. These include the early divergence of taxa, saturated sequence data, insufficient data, insufficient taxa and gene duplication (see Bonnaud *et al*., 1994, 1996; Carlini & Graves, 1999; Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005 for discussion).

The large molecular data sets generated by Lindgren *et al*. (2004) (four genes) and Strugnell *et al*. (2004, 2005) (six genes) contained 18 of the same species (including 6 Octopodiformes and 11 Decapodiformes). Together, these provide the single largest dataset (with regard to sequence length) available for investigating higher-level phylogenetic relationships within

Table 1. Summary of studies of the molecular phylogenetics of coleoid cephalopods.

Reference	Focal taxa	Genes used	No. of species	Sequence alignment method	Analysis method(s)
Bonnaud *et al.* (1994)	Decapodiformes	16S	28	eye (2° structure)	NJ, P
Bonnaud *et al.* (1996)	Decapodiformes	16S, COIII	8	eye	NJ, P
Boucher-Rodoni & Bonnaud (1996)*	Coleoidea	16S	10		NJ, P
Bonnaud *et al.* (1997)	Coleoidea	COIII, COII	17	eye	NJ, P
Bonnaud *et al.* (1998)	Onychoteuthidae	16	14	eye	NJ, P
Carlini & Graves (1999)	Coleoidea	COI	48	eye	P
Anderson (2000)	Loliginidae	16S, COI	~30	Clustal and eye	P, ML, LogDet
Anderson (2000)*	Loliginidae	16S, COI	53	Clustal and eye	P, ML
Carlini *et al.* (2000)	Coleoidea	actin	44	eye	P, ML
Carlini *et al.* (2001)	Octopoda	COI	29	eye	P, ML
Allcock & Piertney (2002)	Octopodidae	16S	9	Clustal X and eye	NJ, P, ML
Piertney *et al.* (2003)	Cirrata	16S	27	Clustal X and eye	NJ, P, ML
Warnke *et al.* (2003)	Decapodiformes	complete 18S	8	Clustal V, MegAlign, checked by eye	NJ, P, ML
Bonnaud *et al.* (2004)	Nautilus	complete 18S	3	eye	2° structure
Lindgren *et al.* (2004)*	Coleoidea	complete 18S, 28S, hist. COI	60	POY	P
Nishiguchi *et al.* (2004)	Sepiolidae	12S, 16S, COI, 28S	30	POY	P
Strugnell *et al.* (2004)	Octopodiformes	16S, 12S, COI, rhod, pax-6, ODH		eye	ML, Bayesian
Yokobori *et al.* (2004)	Coleoidea	whole mitochondrial genome	3	ClustalX	NJ, ML
Zheng *et al.* (2004)	Decapodiformes	COI, 16S	13	ClustalX v1.8	NJ, P
Guzik *et al.* (2005)	Octopodinae	COIII, cyt b, ef-1α	30	Sequencher 3.1	P, ML, Bayesian
Lindgren *et al.* (2005)	Gonatidae	12S, 16S, COI	39	POY	P
Strugnell *et al.* (2005)	Coleoidea	16S, 12S, COI, rhod, pax-6, ODH	35	eye	Bayesian, LogDet
Takumiya *et al.* (2005)	Coleoidea	12S, 16S, COI	36	SeqPup v. 0.9, ClustalX ver1.83	NJ, P, ML
Akasaki *et al.* (2006)	Coleoidea	whole mitochondrial genome	5	–	ML

*note these studies also used further information in some analyses in addition to gene sequences, e.g. morphology, allozymes, immunology etc.
Abbreviations: cyt b, *cytochrome b apoenzyme*, COI, *cytochrome c oxidase subunit I*; 16S, 16S rDNA; 12S, 12S rDNA; 28S, 28S rDNA; 18S, 18S rDNA; ODH, *octopine dehydrogenase*; rhod, *rhodopsin*; hist, *histone* H3; ef-1α, elongation factor-1α; All sequences were of partial fragments unless otherwise stated.
NJ, neighbour-joining; P, parsimony; ML, maximum likelihood.

the subclass Coleoidea. In the present study we used two methods to align these data: by eye and implied alignment using POY; and also three methods of analysis: parsimony, ML and Bayesian, to investigate the effect of these analyses on the resulting phylogeny. The effect of base composition heterogeneity upon coleoid phylogenetic relationships was also investigated.

MATERIAL AND METHODS

Eighteen species were used in the present study, including representatives from each higher-level taxon within the subclass Coleoidea (Table 2). Portions of nine genes were included, three mitochondrial genes (12S rDNA, 16S, rDNA, COI) and six nuclear genes (28S rDNA, 18S rDNA, *histone*, *octopine*

Table 2. Accession numbers of each of the genes used in this study.

	Mitochondrial genes			Nuclear genes					
	12S rDNA	16S rDNA	COI	28S rDNA	18S rDNA	hist.	ODH	pax-6	rhod.
Nautiloidea									
Nautilida									
Nautilidae									
Nautilus pompilius	AY616965	AY377628	AY557514	AF311688	AY557452			AY617039	
Coleoidea									
Octopodiformes									
Vampyromorpha									
Vamyroteuthidae									
Vampyroteuthis infernalis	AY545077	AY545101	AF000071	AY557548	AY557459	AY557408	AY545114	AY545139	AY545163
Octopoda									
Allopsidae									
Haliphron atlanticus	AY616942	AY616971	AY557516	AY557549	AY557460	AY557409	AY616910	AY617016	AY617040
Argonautidae									
Argonauta nodosa	AY545080	AY545104	AY557517	AY557551	AY557462	AY557411	AY545117	AY545142	AY545166
Bolitaenidae									
Japetella diaphana	AY545093	A252766	AY545192	AY557552	AY557463		AY545130	AY545155	AY545179
Octopodidae									
Eledone cirrhosa	AY616946	AY616973	AY557520	AY557556	AY557467		AY616992	AY617020	AY617043
Graneledone verrucosa	AY545091	AY545111	AF000042	AY557557	AY557468	AY557413	AY545129	AY545153	AY545177
Decapodiformes									
Sepiolida									
Sepiolidae									
Heteroteuthis hawaiiensis	AY616873	AY616884	AF000044	AY293703	AY557472	AY557416	AY616906	AY616937	AY616922
Sepiida									
Sepiidae									
Sepia officinalis	AY545098	X9570	AJ583491	AY557560	AY557471	AY557415	AY545135	AY545160	AF000947
Idiosepiida									
Idiosepiidae									
Idiosepius pygmaeus	AY545095	AJ001647	AY545193	AY293684	AY557477	AY557421	AY545132	AY5157	AY545181
Spirulida									
Spirulidae									
Spirula spirula	AY545097	AY293659	AY293709	AY557563	AY557476	AY557420	AY545134	AY545159	AY545183
Teuthida Myopsdia									
Loliginidae									
Sepioteuthis lessoniana	AY616869	AJ001649	AY131036	AY557566	AY557480	AY557424	AY616902	AY616933	AY616918
Teuthida Myopsdia									
Bathyteuthidae									
Bathyteuthis abyssicola	AY616958	AJ000104	AF000030	AY557568	AY557483	AY557427	AY617002	AY617032	AY617057
Octopoteuthidae									
Octopoteuthis nielseni	AY616957	AY616983	AF000055	AY557591	AY557507		AY617011–AY617013	AY617031	AY617056
Cranchiidae									
Cranchia scabra	AY616962	DQ280046	AF000035	AY557571	AY557487	AY557430	AY617014 AY617015	AY617036	AY617061
Ommastrephidae									
Illex coindetii	AY616963	AY616985	AY617065	AY557593	AY557509	AY557450	AY617008 AY617015	AY617037	AY617062
Sthenoteuthis oualaniensis	AY545100	X79582	AF000069	AY557595	AY557511	AY557452	AY545137	AY545162	AY545185
Ommastrephes batramii	AY616866	AY616880	AF000057	AY557594	AY557510	AY557451	AY616899	AY616930	AY616915

dehydrogenase [ODH], *pax*-6 and *rhodopsin*). Sample details and methodologies used to obtain DNA sequences from these species are outlined in Lindgren *et al*. (2004) and Strugnell *et al*. (2005). Accession numbers for these sequences are listed in Table 2.

Sequence alignment and homology assessment

Two methods of sequence alignment were used within this study (1) by eye, and (2) using implied alignment using the homology scheme via POY (Wheeler, 2003; Giribet, 2005).

Aligned by eye

DNA sequences were compiled and aligned by eye in Se-Al v2.0a11 Carbon (Rambaut, 2002). Gaps were inserted where necessary to allow sequences to be aligned. Sequence data that were not alignable using this method were removed prior to analyses. Sequence alignment files are available on request. The total concatenated sequence length was 5,651 bp, of which 2,219 bp were variable.

Dynamic homology and implied alignments

Sequence data were analysed by using the direct optimization method described by Wheeler (1996) and implemented in the computer program POY. This method directly assesses the number of DNA sequence transformations (evolutionary events) required by a phylogenetic topology without the use of multiple sequence alignment. This is accomplished by generalization of existing character optimization procedures, including insertion and deletion events (indels) in addition to base substitutions. This method treats indels as processes, as opposed to the patterns implied by multiple sequence alignment (Wheeler, 1995). It is claimed that this method generates more efficient (and therefore simpler) explanations of sequence variation than multiple sequence alignment (Wheeler, 1996). Direct optimization, although computationally intense, is much less demanding than parsimony-based multiple sequence alignments when congruence among partitions is used as a criterion (Wheeler & Hayashi, 1998). The implied alignments produced via POY were used for both ML and Bayesian analyses. These sequences were concatenated for ML and Bayesian analysis (6,377 bp, of which 2,330 bp were variable).

Base composition heterogeneity

PAUP*4.0b10 (Swofford, 1998) was used for χ^2 tests of composition homogeneity of the sequence data aligned by eye. Tests of base homogeneity were based on variable sites only. Where base composition heterogeneity was detected it was RY coded to remove base composition heterogeneity.

The three sequence data sets, (1) implied alignments, (2) aligned by eye, (3) aligned by eye and RY coded were analysed using three methods, (a) parsimony, (b) maximum likelihood, (c) Bayesian analysis. It is important to note that the sequence data aligned by eye were analysed using parsimony analyses in PAUP rather than POY.

Dynamic homology under parsimony

Molecular data were analysed with the computer program POY (Wheeler *et al*., 1996–2003) using the direct optimization method (Wheeler, 1996), with parsimony as the optimality criterion. Nodal support was calculated in POY using Farris's parsimony jackknifing procedure (Farris *et al*., 1996) for 100 replicates (using the commands: jackboot; replicates 100). Tree searches were conducted in parallel at Harvard University on a 19 dual-processor cluster (Darwin.oeb.harvard.edu) using pvm (parallel virtual machine). Commands for lad balancing of spawned jobs were used to optimize parallelization procedures (-parallel–dpm–jobspernode 2). Trees were built via a random-addition sequence procedure (10 replicates) followed by a combination of branch-swapping steps [SPR (subtree pruning and regrafting) and TBR (tree bisection and reconnection)] and tree fusing (Goloboff, 1999) in order to further improve on tree length minimization. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (-slop5–checkslop10). Phylogenetic trees were obtained using parsimony with a gap/ts/tv cost of various weighting. Several analyses were implemented with character transformations weighted differently to determine how various phylogenetic hypotheses were affected (sensitivity analysis *sensu* Wheeler, 1995). Each gene was analysed separately, using character transformations (indels/ts/tv) of equal weighting (111), and unequal weighting (121, 141, 211, 221, 241, 411, 421, 441). The parameter set that optimized the least amount of character incongruence was the equal weighted transformation (111) for all genes. *Histone* H3 and *pax-6* were the two exceptions that also had similar character incongruence values for the 211 and 411 transformations. The final tree was drawn with Tree View (Win32) and consensus trees were analysed in PAUP version 4.02b (Swofford, 1998). To determine nodal support all jackknife calculations were performed in POY using the procedure described in Nishiguchi *et al*. (2004).

Implied alignment under parsimony

PAUP*4.0b10 (Swofford, 1998) was used to perform maximum parsimony analyses on the sequence data that were aligned by eye. All parsimony searches were performed with 1,000 random sequence-addition replicated and TBR (tree bisection-reconnection) branch swapping. All characters were unordered and equally weighted. One thousand bootstrap replicates were performed to measure the support for each clade on the phylogenetic trees.

Alignment by eye and implied alignment under maximum likelihood

PAUP*4.0b10 (Swofford, 1998) was used to perform 100 full heuristic searches. Starting trees were generated by the neighbour-joining method (NJ) (Saitou & Nei, 1987). A $GTR + I + \Gamma$ likelihood model incorporating rate heterogeneity was used. Branch swapping was performed using TBR (tree-bisection-reconnection). Parameters were then re-estimated, and final branch swapping was performed using NNI (nearest-neighbour-interchange). ML bootstrap values of clade support were generated using the parameters estimated in the analysis, but with starting trees generated by the neighbour-joining method.

Alignment by eye and implied alignment under Bayesian analyses

MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to calculate marginal posterior probabilities using the $GTR + I + \Gamma$ model of nucleotide substitution. Model parameter values were treated as unknown and were estimated in each analysis. Random starting trees were used for the analyses and were run between 1 and 500,000 generations, sampling the Markov chain every 100 generations.

Three strategies were used to ensure that analyses were not trapped in local optima: (1) analysis was performed twice, starting with a different random tree and log-likelihood values at stationarity were compared for convergence

(Huelsenbeck & Bolback, 2001); (2) the topologies and clade posterior probabilities from each of the two analyses were compared for congruence (Huelsenbeck & Imennov, 2002); and (3) Metropolis-coupled Markov chain Monte Carlo (MCMCMC) was used with one cold and three incrementally heated Markov chains run simultaneously (default Mr Bayes heating values) to allow a more extensive exploration of parameter space (Huelsenbeck & Ronquist, 2001).

Stationarity was deemed to be reached when the average standard deviation of split frequencies, shown in MrBayes 3.1.2 was less than 0.01 (Ronquist & Huelsenbeck, 2003).

Tracer v1.3 (Rambaut & Drummond, 2003) was used to determine the correct 'burnin-in' for the analysis (i.e. the number of initial generations that must be discarded before stationarity is reached).

RESULTS

Sequence alignment

Alignment of the ODH, *pax*-6, COI and *histone* sequences required no insertion/deletion events (indels). Indels were introduced into aligned sequences of 12S rDNA, 16S rDNA, 28S rDNA, 18S rDNA and *rhodopsin* both by eye and 'dynamically' during the analysis using POY. The alignments of these genes where indels were required differed notably depending upon the alignment method (Table 3) (alignments available on request). A greater number of gaps were inserted using POY than by eye for the 12S rDNA, 16S rDNA and 28S rDNA genes (Table 3), whereas a greater number of insertions was used aligning by eye than by using POY for *rhodopsin* and 18S rDNA (Table 3). For each of these five genes requiring indels, regions that were deemed to be unalignable with confidence by eye were removed prior to analysis. In contrast, no sequence was removed from the POY analysis due to the fact that sequences are aligned simultaneously during analysis.

Base composition heterogeneity

Chi-squared homogeneity tests of each of the genes shows that third positions of ODH, *rhodopsin* and COI have significant base frequency heterogeneity (Table 4). RY-coding the third positions of these genes was used to resolve base composition heterogeneity (Table 4). RY coding pools purines (adenine and guanine:R) and pyrimidines (cytosine and thymine:Y) into two-state categories (R,Y), and helps resolve bias resulting from differences in the relative frequency of either the two purines or pyrimidines (Phillips *et al.*, 2001).

A number of taxonomic groupings are robust to the different methods of coding, alignment and analysis. The following taxa are always monophyletic: Octopoda, Argonautoidea, Ommastrephidae and Oegopsida (Figs 1–9). Furthermore, in each topology the Bathyteuthoida is the sister taxon to the Oegopsida (Figs 1–9). Bayesian posterior probabilities provide the highest support for each of these clades (Figs 3, 6, 9).

Vampyromorpha and the Decapodiformes are sister taxa in the phylogenies resulting from parsimony, ML and Bayesian analyses of the sequences aligned using POY and also by eye (no RY) with variable levels of support (Figs 1–6). This relationship is also recovered from parsimony analysis of RY coded sequence (BS = 99) (Fig. 7). In contrast, ML and Bayesian analysis of RY coded sequence aligned by eye recovered a sister-taxon relationship between Vampyromorpha and Octopoda, i.e. the Octopodiformes (Figs 8, 9). However, these relationships are not highly supported by bootstraps or posterior probabilities (Figs 8, 9).

The placement of *Eledone* within the Octopoda differs depending upon alignment and analysis method. *Eledone* is the sister

Table 4. Chi-squared homogeneity test for base composition across all genes and codon positions.

Gene	Codon position	$\chi^2(P)$
12S rDNA	–	0.998
16S rDNA	–	0.997
18S rDNA	–	0.963
28S rDNA	–	1.000
COI	1st	1.000
COI	2nd	1.000
COI	3rd	**0.000**
COI (RY)	3rd	0.938
histone H3	1st	1.000
histone H3	2nd	1.000
histone H3	3rd	0.560
ODH	1st	1.000
ODH	2nd	1.000
ODH	3rd	**0.000**
ODH (RY)	3rd	0.999
pax-6	1st	1.000
pax-6	2nd	1.000
pax-6	3rd	0.945
rhodopsin	1st	0.994
rhodopsin	2nd	0.962
rhodopsin	3rd	**0.003**
rhodopsin (RY)	3rd	0.721

Tests were performed on variable sites only. $\chi^2(P) < 0.05$ are in bold.

Table 3. Comparison of alignment length of genes.

	Gene	Total base pairs in gene sequenced (no gaps) (bp)	Alignment method		
			POY (bp)	Eye (total alignment length) (bp)	Eye (unalignables removed, in analysis) (bp)
Mitochondrial	12S rDNA	417	573 (417)*	486 (417)*	283
	16S rDNA	528	627 (528)*	554 (528)*	427
Nuclear	18S rDNA	2,845	1,893 (1,842)*	3,202 (2,845)*	1,943
	28S rDNA	661	198 (191)*	166 (166)*	166
	rhodopsin	1,040	1,022 (991)*	1,032 (954)*	765

*Number in brackets indicates the starting sequence length without gaps. The portion of available sequence able to be aligned by eye was less for 28S and *rhodopsin* than by POY. A larger sequence fragment of 18S was attempted for alignment by eye, however a large proportion was unalignable and was removed prior to analysis.

Table 5. Phylogenetic relationships recovered by two alignment methods (by eye, dynamic homology/implied alignment using POY) and three analysis methods (P, parsimony; ML, maximum likelihood; Bayes, Bayesian).

Alignment method	POY			By eye					
				No RY			RY		
Analysis method	POY	ML	Bayes	POY	ML	Bayes	POY	ML	Bayes
Vampyromorpha(Decapodiformes)	√	√	√	√	√	√	√	X	X
Vampyromorpha(Octopoda)	X	X	X	X	X	X	X	√	√
Octopoda	√	√	√	√	√	√	√	√	√
Argonautoidea	√	√	√	√	√	√	√	√	√
((*Japetella*, *Graneledone*)*Eledone)*	√	√	√	√	X	X	√	X	X
(*Eledone*((*Japetella,Graneledone*)(*Haliphron,Argonauta*))	X	X	X	X	√	√	X	√	√
Decapodiformes	√	√	√	√	√	√	√	√	√
(Oegopsida)(remaining Decapodiformes)	X	X	X	X	√	√	X	√	√
Polyphyletic Sepioidea	√	√	√	√	√	√	√	√	√
Ommastrephidae	√	√	√	√	√	√	√	√	√
Oegopsida	√	√	√	√	√	√	√	√	√
Bathyteuthoida(Oegopisda)	√	√	√	√	√	√	√	√	√
Spirulida(Bathyteuthoida(Oegopisda))	√	√	√	√	X	X	X	X	X
Idiosepiidae(Sepioidea(Myopsida(Spirulida(Bathyteuthoida(Oegopisda)))))	√	X	X	X	X	X	X	X	X
Sepioidea(Myopsida(Spirulida(Bathyteuthoida(Oegopisda))))	√	√	√	X	X	X	X	X	X
Myopsida(Spirulida(Bathyteuthoida(Oegopisda)))	√	√	√	X	X	X	X	X	X
(*Heteroteuthis, Idiosepius*)	X	√	√	X	X	X	X	X	X
(*Sepioteuthis*, *Idiosepius*)	X	X	X	√	X	X	X	X	X
(*Sepia, Idiosepius*)	X	X	X	X	√	√	√	√	√
((*Sepia, Idiosepius*)*Sepioeuthis*)	X	X	X	X	√	√	√	X	√
(((*Sepia, Idiosepius*)*Sepioeuthis*)*Spirula*)	X	X	X	X	√	√	√	X	√
((((*Sepia, Idiosepius*)*Sepioeuthis*)*Spirula*)*Heteroteuthis*)	X	X	X	X	√	√	√	X	√
(Oegopsida,Bathyteuthoidea)(Sepioidea, Myopsida*)	X	X	X	X	√	√	X	√	√

*Myopsida falls within Sepioidea in this topology.
The data aligned by eye have been analysed for both nucleotide data and RY coded data. √, the relationship is supported, X, the relationship is not supported.

taxon to a clade containing *Japetella* and *Graneledone* in each of the phylogenies resulting from the POY alignment, and also parsimony analysis of the sequence data aligned by eye, both RY coded and not RY coded (Figs 1–4,7). High support for this relationship is evident on each of these phylogenies. However, *Eledone* is highly supported as being basal within the Octopoda in analysis of sequence data aligned by eye (both RY coded and not RY coded) and analysed using ML (no RY, BS = 100; RY, BS = 100) and Bayesian analyses (no RY, PP = 1.00; RY, PP = 1.00) (Figs 5, 6, 8, 9).

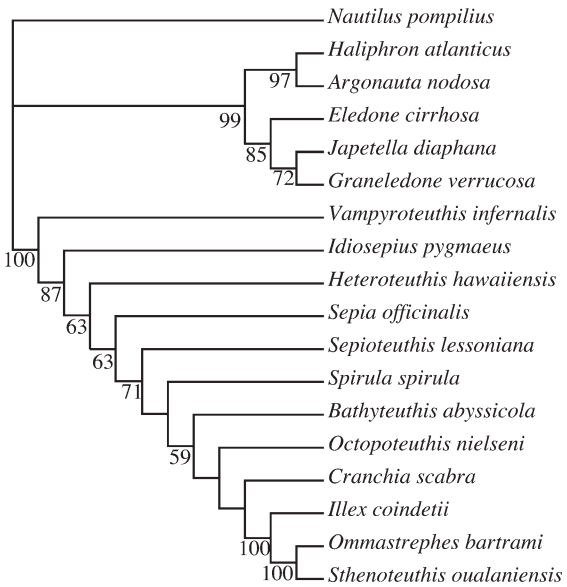

Figure 1. Parsimony topology of coleoid cephalopod relationships obtained using direct optimization using POY. Jackknife support values are indicated beneath each node.

Higher-level decapodiform relationships differ markedly between the various methods of alignment, coding, and analysis (Figs 1–9). Phylogenies generated from ML and Bayesian analyses of sequences aligned by eye (both RY coded and not RY coded) demonstrate Decapodiformes to be divided into two monophyletic groups, one containing the Oegopsida, and the second containing the remaining decapodiforms (i.e. Myopsida, Spirulidae, Sepiidae, Sepiolidae and Idiosepiidae) (Figs 5, 6,8,9). This division is highly supported by bootstrap support (no RY, BS = 98; RY, BS = 98) and posterior probabilities (no RY, PP = 0.99; RY, PP = 0.97) (Figs 5, 6,8,9). Within these topologies *Sepia* and *Idiosepius* are sister taxa, thereby rendering 'Sepioidea' (including Sepiidae, Sepiadariidae and Sepiolidae) polyphyletic (Figs 5, 6, 8, 9).

In contrast, a clade containing *Heteroteuthis* and *Idiosepius* is basal within decapodiforms in ML and Bayesian analysis (PP = 0.90) of sequence data aligned using POY (Figs 2, 3). *Heteroteuthis* alone is basal in phylogenies resulting from parsimony analysis of sequences aligned by eye, both RY coded (BS = 100) and not RY coded (BS = 100) (Figs 4, 7).

The position of Spirulidae within the Decapodiformes is highly dependent upon the method of alignment and analysis. Spirulidae are the sister taxon to a clade containing the Oegopsida and Bathyteuthoidea in all three analyses where sequences were aligned using POY, although support was only obtained

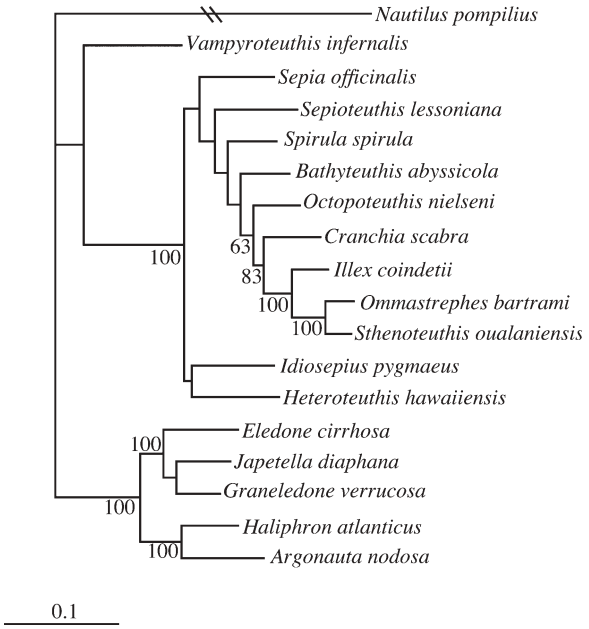

Figure 2. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were obtained from implied alignments using POY. Bootstrap support values are indicated beneath each node.

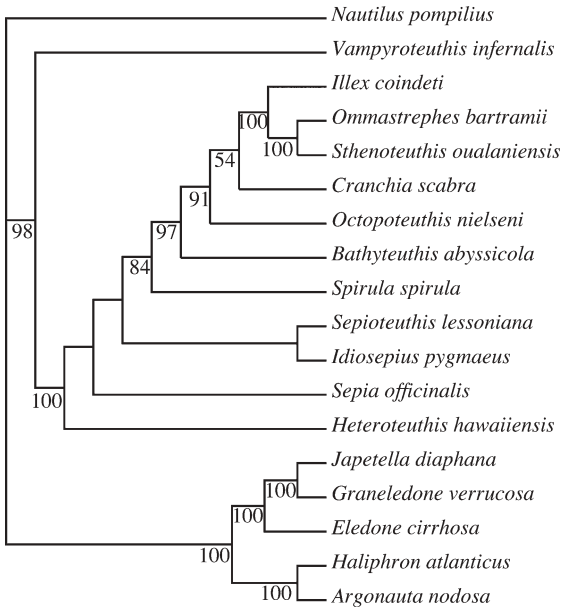

Figure 4. Parsimony topology of coleoid cephalopod relationships. Sequences were aligned by eye. Boostrap support values are indicated beneath each node.

for this relationship from the Bayesian analysis (PP = 1.00) (Figs 1–3). Interestingly, this same arrangement results from parsimony analysis of sequence data aligned by eye, not RY coded (BS = 84) (Fig. 4). In contrast, Spirulidae are the sister taxon to a clade containing Idiosepiidae, Sepiidae and Myopsida in the topologies resulting from ML and Bayesian analysis (PP = 0.99) of data aligned by eye (not RY coded) (Figs 5, 6) and in parsimony and Bayesian analysis (PP = 0.92) of RY coded data aligned by eye (Figs 8, 9).

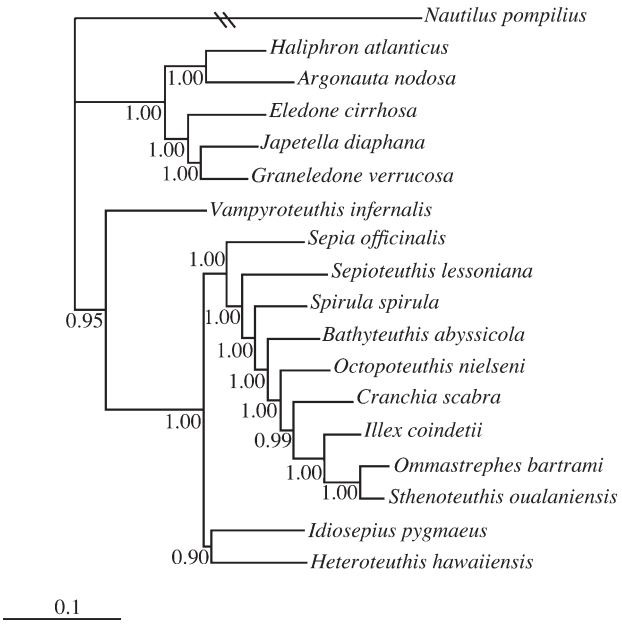

Figure 3. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were obtained from implied alignments using POY. Bayesian posterior probabilities are indicated beneath each node.

DISCUSSION

The present study is the largest molecular analysis of cephalopod phylogeny to date, with regard to sequence length, and provides a thorough comparison of the effect of commonly used alignment and analysis methodologies on the resulting higher-level phylogenetic relationships.

The different alignment, analysis and coding methods used within this study produced a range of considerably different topologies. Only the clades Octopoda, Argonautoidea, Decapodiformes, Oegopsida, Ommastrephidae and a sister-taxon

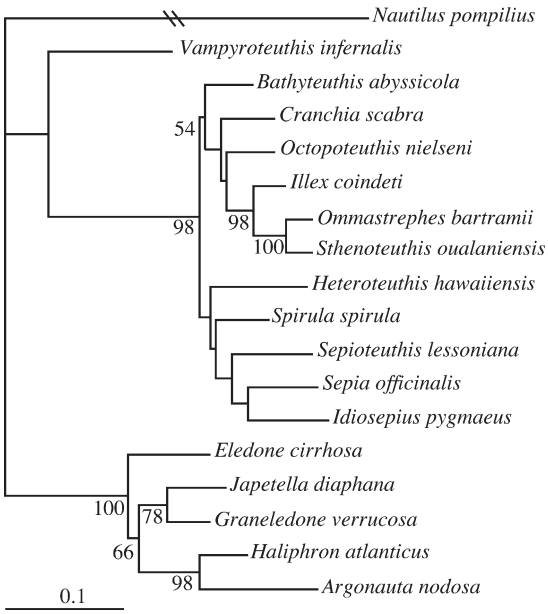

Figure 5. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye. Bootstrap support values are indicated beneath each node.

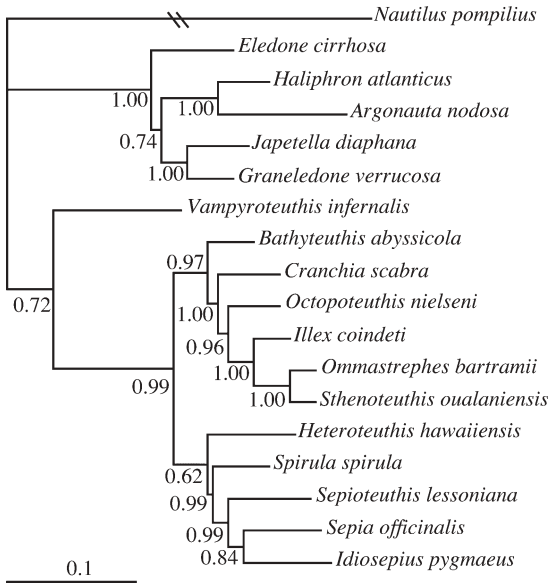

Figure 6. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye. Bayesian posterior probabilities are indicated beneath each node.

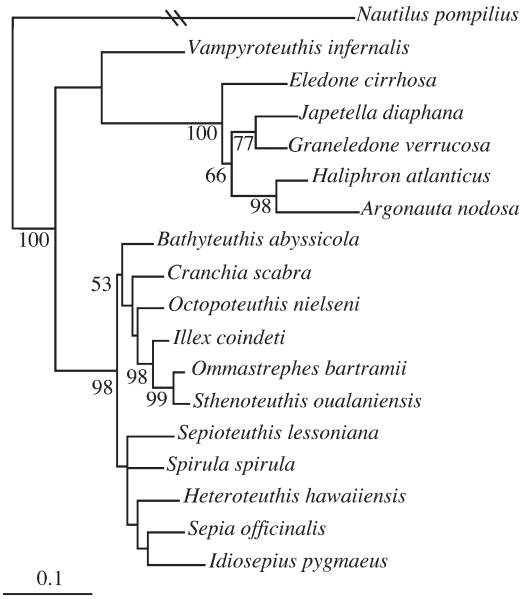

Figure 8. ML topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Boostrap support values are indicated beneath each node.

relationship between Bathyteuthidae and Oegopsida are robust to the alignment and analysis methods used.

Alignment methods

It is not surprising that different alignments can affect the resulting phylogeny, as the process of alignment aims to recover the evolutionary history of the sequences and therefore provides the very data upon which the algorithm performs (Giribet, Desalle & Wheller, 2002). For protein coding genes, the method of sequence alignment is usually insignificant, since in theory they should all produce the same alignment, i.e. an alignment without indels. However, as we demonstrate here, alignment methods for rDNAs, and both coding (i.e. *rhodopsin*) and non-coding genes can differ in their resulting sequence alignment and phylogenies.

There is debate in the literature regarding the best method of sequence alignment. Proponents of aligning sequences by eye (using secondary structural information) claim that

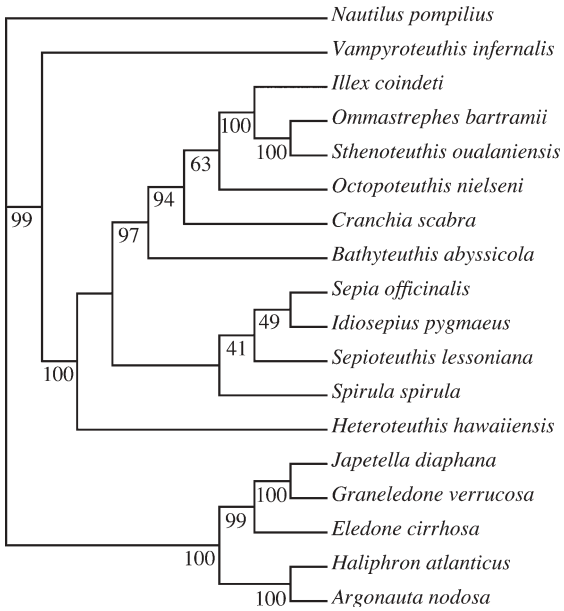

Figure 7. Parsimony topology of coleoid cephalopod relationships. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Bootstrap support values are indicated beneath each node.

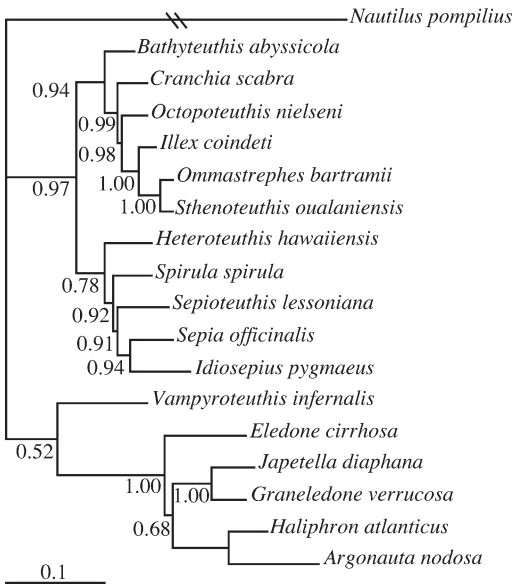

Figure 9. Bayesian topology of coleoid cephalopod relationships obtained using GTR + I + Γ. Sequences were aligned by eye, and third positions of *rhodopsin*, COI and ODH were RY coded. Bayesian posterior probabilities are indicated beneath each node.

they are 'both philosophically and operationally superior' (Kjer *et al*., 2007), whereas proponents of computational methods claim that alignments performed by eye are subjective and therefore not repeatable (Giribet & Wheeler, 1999).

Proponents of the method of direct optimization using POY claim that it avoids the problem of alignment by generalizing phylogenetic character analysis to include insertion/deletion events (indels), with the sequence data proceeding directly to phylogenetic reconstruction, obviating the necessity to create gap characters. Indels do not appear as states, but as transformations linking ancestral to descendent nucleotide sequences (Giribet & Wheeler, 1999; Giribet *et al*., 2002). POY assumes that shorter trees are better trees and that aligning nucleotides together based on state is parsimonious and algorithmically less costly. Kjer *et al*. (2007) argues that this is not justified in structurally conserved molecules such as rDNAs, where conserved structures in the molecules are more important than the states of the nucleotides.

There has been intense disagreement over the relative merits of manual alignment and direct optimization (Kjer, 1995; Wheeler, 1995; Shull *et al*., 2001; Belshaw & Quicke, 2002; Gillespie, Yoder & Wharton, 2005) but few rigorous comparisons of these methods. Recently, Kjer *et al*. (2007) compared the phylogenies obtained by three phylogeneticists who independently aligned and analysed the same 16S rDNA datset by eye (using rDNA secondary structure and analysed by parsimony) and using direct optimization within POY. Interestingly, although all three alignments by eye differed at some positions, each alignment produced nearly identical topologies. In contrast, when using POY, none of the three phylogeneticists converged on the same parameters or the same tree. Kjer *et al*. (2007) suggest that the reason for this is that gap cost to change ratios (used within POY) are arbitrary, and this allows different researchers to obtain different results.

Sequence alignments resulting from POY have been reported to be 'gappy' in some studies (Pons & Vogler, 2006) with the program inserting a greater number of indels than other methods when utilizing indel costs of 1. Similarly, in the present study POY inserted a greater number of gaps in the 12S rDNA, 16S rDNA and 28S rDNA alignments, even though several parameters were explored using POY (costs of 1, 2 and 4 for indels, transitions and transversions, respectively). In addition, no sequence data were removed from the POY alignments used in the analysis. In contrast, a notable proportion of the sequence alignments of 12S rDNA, 16S rDNA, 18S rDNA and *rhodopsin* was removed after alignment by eye as it was deemed to be unalignable and would contribute noisy signal to the analysis. Therefore the starting information present in both datasets differed. The sequence data that were deemed 'unalignable' when aligning by eye are by their nature 'variable' and would therefore have an important contribution in the POY alignments in determining the resulting phylogenetic relationships. Differences in phylogenetic relationships observed in this study between the two alignment methods is largely due to the deleted sequences. Over 60% of the sequence information in both the datasets was constant (i.e. not variable) further demonstrating the significance of these variable sites. Despite these obvious differences in output, both of these methods of sequence alignment are widely accepted and appear in the cephalopod (Table 1) and wider literature today. It is likely that debate will continue regarding the best method of sequence alignment and while this continues to be the case, it may be beneficial to employ more than one method of alignment in phylogenetic studies.

Analysis methods

There is considerable debate in the literature regarding methods of phylogenetic analysis (e.g. Giribet, 2003). Parsimony methods have the benefit of being relatively easy to understand and require few assumptions about the evolutionary process (Page & Holmes, 1998). However they have been shown to produce the wrong topology under the most realistic models of evolution (e.g. long branch attraction; Huelsenbeck & Hillis, 1993).

ML methods allow the incorporation of sophisticated models of sequence evolution and allow statistical tests of different evolutionary hypotheses (i.e. likelihood ratio testing Felsenstein, 1981) yet require very large computational resources. Furthermore ML methods have been shown to be susceptible to long branch repulsion and long branch attraction under some circumstances (Pol & Siddall, 2001).

Bayesian methodologies (differing from likelihood methods only in the use of a prior distribution of the quantity being inferred, which is typically the tree) have the advantage over ML methods of being computationally efficient. They allow very complex models of sequence evolution to be implemented and also can efficiently analyse large datasets. Bayesian methods have been criticized however, for producing unrealistically high posterior probability support (Suzuki, Glazko & Nei, 2002; Simmons, Pickett & Miya, 2004).

In the present study, the majority of topologies resulting from the three analysis methods on the implied aligned data (from POY) are very similar. The exception to this is the position of *Idiosepius*. In contrast, the method of analysis had a greater effect on the data aligned by eye. In many cases ML and Bayesian methods of analysis produced the same or very similar topology for both RY coded and non-RY coded data, while the parsimony analysis produced a different topology. This is the case for the relationships of octopod taxa, and the relationship between the Oegopsida and the rest of the decapodiforms. It is unsurprising that ML and Bayesian analysis methods produce more similar topologies than parsimony analysis, because both are based on the same probabilistic model of evolution. In contrast, parsimony analysis is based on the idea that the preferred phylogenetic tree is the one that requires the fewest evolutionary changes.

Discussion of phylogenetic relationships

Order Vampyromorpha: *Vampyroteuthis infernalis* is the only species within the order Vampyromorpha. It possesses a number of unusual characteristics including two pairs of fins in juveniles (one pair in adults) and a second pair of arms modified into retractile filaments. Traditionally Vampyromorpha and Octopoda have been suggested to be sister taxa due to embryological, developmental (Naef, 1928; Young & Vecchione, 1996; Boletzky, 2003) and morphological similarities, such as sperm morphology (Healy, 1989) and the presence of radial sucker symmetry (Lindgren *et al*., 2004). However, the vampyromorph gladius is known to be morphologically similar to that of decapodiforms (Toll, 1982, 1998). Previous molecular studies have found support for both a sister taxon relationship between Vampyromorpha and Octopoda (Bonnaud *et al*., 1997; Carlini & Graves, 1999; Lindgren *et al*., 2004; Strugnell *et al*., 2004,2005) and Vampyromorpha and the Decapodiformes (Bonnaud *et al*., 1997; Lindgren *et al*., 2004). This present study found support for both of these relationships. The majority of alignment and analysis combinations support a sister-taxon relationship between Vampyromorpha and Decapodiformes. Only ML and Bayesian analysis of the 'by eye' alignment of RY coded data support a sister-taxon relationship between Vampyromorpha and Octopoda. RY coding rectified the base composition heterogeneity identified in the third positions of

COI, *rhodopsin* and ODH and thus is possible that this contributed to the Vampyromorpha and Octopoda sister-taxon relationship. RY coding also would have aided in reducing the effect of saturation (Phillips & Penny, 2003). However, parsimony analysis of the same dataset recovered a vampyromorph and decapodiform sister-taxon relationship. These results suggest that this relationship is unstable. The lineage Vampyromorpha is supposed to be at least 162 Myr from fossil evidence (Fischer & Riou, 2002) and has been estimated from fossil and molecular data to be potentially 252 Myr (Strugnell *et al*., 2006). The ancient diversification of this lineage provides support for the supposition that the molecular data used within this study are likely to be saturated at this level (Strugnell *et al*., 2005). Furthermore, the numerous extinction events throughout the Coleoidea during this time may contribute to the obscuring of affinities of Vampyromorpha (Lindgren *et al*., 2004).

Order Octopoda: *Eledone* was traditionally placed within the subfamily Eledoninae because it possesses an ink sac, a single row of suckers and large eggs (Robson, 1929). The taxonomic value of these characters has been debated; the presence of an ink sac has been suggested to be a function of depth (Robson, 1931; Voss, 1988; Allcock & Piertney, 2002) and sucker arrangement has been suggested to be a plastic character (Naef, 1921–1923; Voight, 1993a; Allcock & Piertney, 2002). Allcock & Piertney, (2002) suggested that sub-familial level assignment within the Octopodidae is 'a totally artificial classification with no evolutionary basis.' *Eledone* has been included in relatively few molecular studies (Bonnaud *et al*., 1997; Lindgren *et al*., 2004; Warnke *et al*., 2004). The present study recovered two differing placements for *Eledone*. All parsimony analyses, and also ML and Bayesian analyses of the POY alignment, show a sister-taxon relationship between *Eledone* and a clade containing *Japetella* and *Graneledone*, thus grouping together all species with a single row of suckers. In contrast ML and Bayesian analyses of data aligned by eye show *Eledone* to be basal within the Octopoda. This relationship was also recovered by Strugnell (2004), using a subset of the genes used within the present study, but with additional octopod species. *Eledone* possesses a number of morphological features supporting a basal position within the Octopoda, including the absence of a ligula (Naef, 1921–1923). It must be noted that there are relatively few octopod taxa included within the present study. The inclusion of additional taxa such as *Benthoctopus*, *Bathypolypus* and members of the suborder Cirrata would likely improve stability and resolution of octopod relationships.

Suborder Oegopsida and the family Bathyteuthidae: The suborder Oegospida contains squids that possess a gladius and lack a cornea. Molecular studies by Bonnaud *et al*. (1994, 1997), Carlini & Graves (1999), Carlini *et al*. (2000) and Lindgren *et al*. (2004) have suggested that the suborder may be polyphyletic, the later three studies reporting *Spirula* to fall within the Oegopsida. In contrast, Strugnell *et al*. (2005) supported a monophyletic Oegopsida. The present study also strongly supports a monophyletic Oegospida, since all alignment and analysis combinations supported this grouping. It is possible that the datasets in the previous studies that suggested a polyphyletic Oegopsida have been too small, and thus contained insufficient information to recover this relationship. All alignment and analysis combinations also support a sister-taxon relationship between the Oegopsida and the family Bathyteuthidae. This supports previous molecular studies by Carlini *et al*. (2000) and Strugnell *et al*. (2005) and also agrees with Naef's (1921–1923) suggestion that the Bathyteuthidae possess 'primitive characters for all Oegopsida'.

Suborder Myopsida and Sepioidea: Traditionally Spirulidae, Sepiidae, Idiosepiidae and Sepiadariiae/Sepiolidae have been grouped together in the suborder Sepioidea (Naef, 1921–1923), while the suborder Myopsida was grouped with the suborder Oegopsida in the order Teuthoidea on the basis of similar gladii and tentacular clubs (Naef, 1916, 1921–1923). However, the Myopsida has also been suggested to be derived from the 'Sepioidea' line based on a number of characteristics including possession of a cornea, suckers with circularis muscle, beak without angle point and a vena cava ventral to the intestine (d'Orbigny, 1845; Berthold & Engeser, 1987; Engeser, 1997; Haas, 1997, see Young *et al*., 1998, for a more detailed discussion). Molecular studies have suggested a close relationship between the Myopsida and some or all members of the Sepioidea (Carlini *et al*., 2000; Lindgren *et al*., 2004; Strugnell *et al*., 2005), although the precise relationship has varied depending upon the genes and analyses used. The present study also suggests a closer relationship between the Myopsida and the Sepioidea than the Myopsida and the Oegopsida, although the exact configuration of this is dependent upon the alignment method and analysis employed. In the phylogenies resulting from data aligned using POY, Myopsida was consistently the sister taxon to a clade containing Spirulida, Bathyteuthoidea and Oegopsida, with the remaining Sepioidea taxa falling outside this clade. However, in the phylogenies resulting from ML and Bayesian analyses of data aligned by eye (RY coded and not RY coded) the Myopsida fell within Sepioidea, together forming a sister taxon to a clade containing the Oegopsida and Bathyteuthidae.

These results clearly show that differing alignment and analysis strategies commonly used in coleoid cephalopod phylogenetics can produce notably different phylogenetic relationships. Researchers are far from agreeing on a single 'best' strategy of phylogenetic analysis, because the advantages and disadvantages of competing strategies are not yet clear. Until such a time, we advocate the use of a variety of different alignment and analysis strategies in phylogenetic analysis.

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