Coleoid cephalopods through time (Warnke K., Keupp H., Boletzky S. v., eds)

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REFLECTIONS ON THE PHYLOGENETIC POSITION OF *SPIRULA* (CEPHALOPODA): PRELIMINARY EVIDENCE FROM THE 18S RIBOSOMAL RNA GENE

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ABSTRACT

In the scope of a study focusing on the phylogenetic position of Spirulida within the Coleoida DNA sequences of five cephalopod species (*Loligo vulgaris*, *Sepia officinalis*, *Sepietta* sp, *Illex coindetii*, *Eledone cirrhosa*) from the Mediterranean (Banyuls, France) as well as *Histioteuthis* sp., *Heteroteuthis* sp. and *Spirula spirula* from the West Atlantic Ocean (Fuerteventura, Canary Islands) were obtained and analyzed using different methods (NJ, MP and ML). Each method had a remarkable influence on tree topology. Only the MP tree supports the hypothesis of Engeser and Bandel (1988), suggesting that *Spirula* appears as the most ancient species of recent Decabrachia. This phylogeny is well supported by high bootstrap values (100%) and a high decay index (33).

INTRODUCTION

The Ram´s Horn Squid *Spirula* is one of the most unusual recent cephalopods especially considering its unique chambered shell. Based on the morphological observations on brain and statocysts by Young (1977), Engeser and Bandel (1988) regard *Spirula* as the most ancient form of the recent Decabrachia. A striking similarity also exists with the Ammonoidea, likely sister-group of the Coleoidea, in that the initial shell chambers look virtually identical (Bandel & Boletzky 1979). If the similar structure of the initial chamber in Ammonites and Spirulids is considered a synapomorphic feature within the Angusteradulata (Lehmann 1967), which is absent in all Coleoida other than *Spirula*, one may indeed expect to find the Spirulida in a relatively basal position.

However, in most systematic studies based on morphological characters, *Spirula* still raises various problems. Indeed, the phylogenetic position of this taxon changes depending on the set of characters used for the phylogenetic analysis (see Naef 1921-1923, Donovan 1977, Nesis 1987, Boletzky 1999). Molecular data, on the other hand, have also led to contradictory ideas on the position of the Spirulida and especially on the supposed sister group of *Spirula*. Bonnaud *et al.* (1994, 1996) as well as Carlini and Graves (1999) investigated relationships among coleoids using fragments of the 16S rRNA, COI and COIII genes. All of the sequences examined indicated significant levels of saturation, which is considered a signal for homoplasy. Consequently, Carlini *et al.* (2000) chose the highly conserved actin gene family to re-examine higher level relationships within the Coleoidea. Their results indicated that the containing group Sepioidea is probably not monophyletic, but no further clarification as to the position of *Spirula* was obtained.

In this paper we present new molecular data that tend to improve the data base for a recognition of the phylogenetic position of *Spirula* within the Coleoidea.

Table 1 PCR and sequencing primers

Name (F/R*)	Sequence (5' → 3')	References
F1f	gTCTCAAAgATTAAgCCATgCATg	Ohst unpublished, (modified)
F1r	gAATTACCgCggYTgCTggC	Wilmotte *et al.* 1993
F2f1	CTTATggCgCCRACgACCg	This work
F2f2	gAAACggCTACCACATCC	This work
F2r1	CCgTCAATTCCTTTAAgTT	Wilmotte *et al.* 1993
F2r2	CggTATCTgATCgTCTTCg	This work
F2r3	ggTATCTgATCgTCTTCg	This work
F2r4	AgTCTCgTTCgTTATCgg	This work
F3f	CTTAATTTgACTCAACACggg	This work
F3r	CATCTAAgggCATCACAgAC	This work
F4f	gTCTgTgATgCCCTTAgAT	Wilmotte *et al.* 1993, (modified)
F4r	CCTTgTTACgACTTTTACTg	This work

We used the relatively slowly evolving nuclear 18S rRNA gene, given its apparent suitability for the resolution of higher level taxa (Winnepenninckx *et al.* 1996, Winnepenninckx *et al.* 1998, Spears & Abele 2000, Mattern & Schlegel 2001, Giribet 2002).

MATERIAL AND METHODS

Spirula spirula, *Histioteuthis* sp. and *Heteroteuthis* sp. were caught with an Isaacs-Kidd-Midwater-Trawl in deep water near Fuerteventura (Canary Islands, western Atlantic). *Loligo vulgaris*, *Illex coindetii*, *Sepia officinalis*, *Sepietta* sp. and *Eledone cirrhosa* were obtained from Banyuls-sur-Mer, Western Mediterranean (cf. Acknowledgements). All samples were preserved in 95–100% ethanol. Apart from *Spirula spirula* (two specimens) and *Sepia officinalis* (two specimens), single specimens were analysed for each representative species.

DNA was isolated following the Chelex method (Walsh *et al.* 1991) modified by Söller *et al.* (2000). Chelex supernatant was purified with the DNAeasy Kit (Quiagen, Hilden). Polymerase chain reaction (PCR) was performed in 50 µl reaction volumes containing 10 mM Tris-HCL, pH 8.8; 25 mM KCL; 2 mM $MgSO_4$; 0.2 mM each of dATP, dCTP, dGTP, dTTP; 0.2 µM of both forward and reverse primers; 2.5 U *Taq* polymerase (Pharmacia Biotech, Freiburg i. Br.), 1.25 U *Taq* Pwo (Peqlab, Erlangen), 1-10 µl DNA solution (purified Chelex supernatant), and 0.1 mg/ml BSA (MBI-Fermentas, St. Leon-Rot).

Primers (Table 1) were designed according to sequences of bacterial and eukaryotic SSU rRNA (http://oberon.rug.ac.be:8080/rRNA/primers/BS_Ist.html). In some cases the original primer sequence was modified. For amplification the gene was divided into four segments of lengths varying between 400 and 700 bp and were sequenced with various primers (Table 1). For long range PCR, primers F1f and F4r were used. PCR was performed on a thermocycler (Mini Cycler, MJ Research, Biozym Diagnostik GmbH, Oldendorf, Germany) and programmed for an initial denaturation during 2 min at 96°C, followed by 35 cycles each of 30 sec at 95°C (denaturation), 20 sec at 52°C (annealing) and 2 min at 72°C (extension). The final extension was 7 min at 72°C. The amplified products were purified with QiaQuick (Quiagen) spin colums and sequenced directly based on the Sanger method using the AmpliTaqFS-DyeDeoxy-Terminator chemistry and an ABI Prism 377 DNA sequencer according to the manufacturer´s recommendations (PE-Applied Biosystems, Weiterstadt). All sequences have been deposited in the EMBL database (accession numbers: *Eledone cirrhosa*, AJ606938; *Heteroteuthis* sp. AJ606940; *Histioteuthis* sp., AJ606941; *Illex coindetii*, AJ606936; *Loligo vulgaris*, AJ606939; *Sepia officinalis*, AJ606935; *Sepietta* sp., AJ606937 and *Spirula spirula*, AJ606934).

The gastropod *Nodilittorina punctata* (NP18SRRN1, Winnepenninckx *et al.* 1998) and the chiton *Liolophura japonica* (AJ18S, Winnepenninckx *et al.* 1993) from the EMBL data bank were used as outgroup representatives for all analyses. To test robustness of the topology additional outgroup representatives were used, the caudofoveatan mollusc *Scutopus ventrolineatus* (X91977, Winnepenninckx *et al.* 1996), and two crustaceans, *Leptodora kindtii*

Table 2 Pairwise p-distances (below diagonal) and ts/tv ratio (above diagonal) among the studied cephalopods

Taxon	*Spirula*	*Sepia*	*Sepietta*	*Hetero-teuthis*	*Loligo*	*Illex*	*Histio-teuthis*	*Eledone*
Spirula spirula	-	0.99756	1.37158	1.61205	1.38045	1.32512	1.26186	1.04495
Sepia officinalis	0.04558	-	1.50419	1.61580	1.54121	1.75147	1.86690	1.07496
Sepietta sp.	0.03958	0.02153	-	1.63222	1.22097	1.49937	1.42294	1.08897
Heteroteuthis sp.	0.04980	0.02881	0.01289	-	1.60353	1.68642	1.65246	1.10701
Loligo vulgaris	0.04704	0.02348	0.01916	0.02398	-	2.00668	2.17071	1.10070
Illex coindetii	0.05335	0.03396	0.02465	0.03152	0.02992	-	1.70407	1.19385
Histioteuthis sp.	0.04379	0.02486	0.01800	0.02294	0.02360	0.01686	-	1.13323
Eledone cirrosa	0.14513	0.12012	0.11868	0.12332	0.12171	0.12652	0.11424	-

(AF144214, Spears & Abele 2000) and *Daphnia pulex* (AF014011, Crease & Colborne 1998). Sequences were aligned with Clustal V, using MegAlign (DNAstar Lasergene; GATC, Konstanz) and checked by eye. Analysis was limited to reliably aligned regions, which included 1719 nucleotide positions.

Putative phylogenetic relationships were analysed using the computer program PAUP*4.0b10 (Swofford 2002). The same program was used for further statistical analyses of the sequence data. In order to check the length distributions of tree skewness and the g_1 value (Hillis & Huelsenbeck 1992) a randomly sampled subset of 10^5 parsimony trees were calculated using the option "Evaluate Random Tree" in PAUP. Phylogenetic trees were calculated applying the following methods, Neighbour Joining (NJ) (Saitou and Nei 1987), Maximum Parsimony (MP) (Fitch 1971), and Maximum Likelihood (ML) (Felsenstein 1981). Parsimony analyses were performed with the "Exhaustive" search modus. Gaps were treated as a fifth character state. Bootstrapping (Felsenstein 1985) and branch support (Bremer 1988) were performed to evaluate the robustness of the tree. The Bremer support, or decay index, was calculated with PAUP combined with AutoDecay Version 3.0.3 (Eriksson & Wikström 1996). For the NJ analysis uncorrected distances (p-distances) were used following the instructions of Nei and Kumar (2000). The suggested simplest distance model was used for topologies of different distance analyses and yielded identical results (p, Jukes-Cantor, F81, Tajima-Nei, Kimura 2-parameter, F84, HKY85, Kimura 3-parameter and Tamura-Nei). With the help of log likelihood scores the program MODELTEST 3.03 (Posada & Crandall 1998) was used to choose the model of DNA evolution for ML analysis that best fits with the data.

RESULTS

The length of the analysed cephalopod sequences ranged from 1860 nucleotides in *Loligo vulgaris* to 2222 nucleotides in *Spirula spirula*. Sequences were rich in G/C content (G = 32%, C = 28%, T = 21%, A = 19%). Total alignment length of the nearly complete 18S rDNA sequences of eight cephalopods and two other molluscs (*Nodilittorina punctata* and *Liolophura japonica*) comprised 2583 sites. We found three hypervariable regions and therefore excluded large components of tentatively aligned regions. After removing 864 ambiguous positions the final alignment length was 1719 sites. The observed ts/tv ratio ranged from 0.9 to 2.2, with a mean of 1.44 (see Table 2).

Calculated p-distances were in the same range when *Spirula* was compared with other decabrachian taxa. P-distance between *Spirula* and *Sepia* was 4.6%, between *Spirula* and *Loligo* 4.7%, between *Spirula* and the Sepiolida approximately 4.5%. Comparison between *Spirula* and the Oegopsida (*Illex* and *Histioteuthis*) led to a distance value of approximately 4.9% (see Table 2).

As a result of the NJ analysis *Spirula* forms a clade

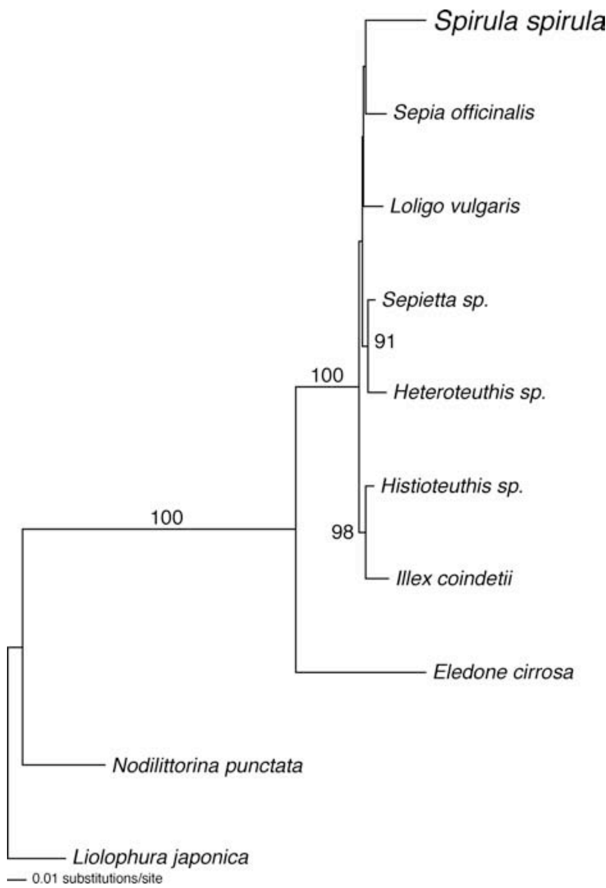

Fig. 1 NJ tree calculated with uncorrected p-distances of coleoid 18S rDNA sequences. Rooting was completed using the sequences of *Nodilittorina punctata* and *Liolophura japonica*. Bootstrap values (> 50%) obtained from 1000 bootstrap replications are shown above nodes

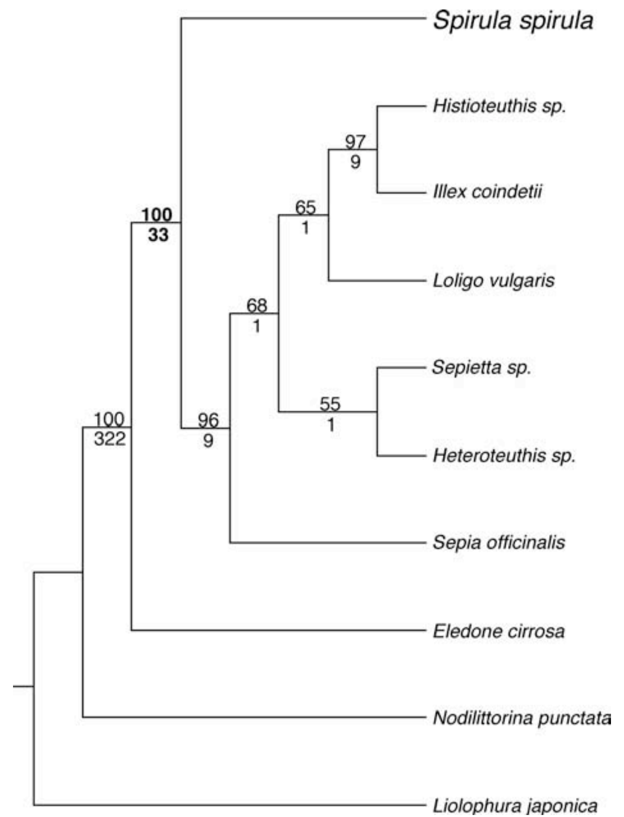

Fig. 2 MP tree constructed on the basis of unweighted coleoid 18S rDNA sequences by exhaustive search. Gaps treated as fifth character state (parsimonious-informative character = 527; variable parsimonious-uninformative character = 231; number of trees retained = 1; tree length = 1160; consistency index = 0.890; retention index = 0.818). Rooting was completed using the sequences of *Nodilittorina punctata* and *Liolophura japonica.* Bootstrap values (> 50%) obtained from 1000 bootstrap replications are shown above nodes, decay indices are below nodes

with *Loligo, Sepia* and the Sepiolida (S*epietta* and *Heteroteuthis*) that is distinct from the oegopsid clade (Fig. 1). The phylogenetic relationships with respect to *Spirula* were not resolved, as indicated by low bootstrap values. However, separation between the Decabrachia and the Octobrachia as well as the monophyly of the Sepiolida and the Oegopsida were well supported.

All MP trees calculated from the data set with changing outgroup representatives show the same topology (data not shown). The corresponding g_1 values (for example *Nodilittorina punctata* and *Liolophura japonica* as outgroup, g_1 = -3.37) were clearly below the 99% critical value (Hillis & Huelsenbeck 1992). The MP tree calculated with *Nodilittorina punctata* and *Liolophura japonica* as outgroup representatives is shown in figure 2. *Spirula* appears at the base of the Decabrachia, followed by *Sepia* (see Fig. 2). The separation between *Spirula* and the other Decabrachia is well supported by a bootstrap value of 100% and a high decay index (33). The Oegopsida and the Sepiolida each indicate monophyletic clades. While the monophyly of the Oegopsida is well supported by a high bootstrap value (97%) and a decay index of 9, the support for the Sepiolida is only low (55%). As a further result of the MP analysis the Octobrachia (represented by *Eledone cirrhosa*) are clearly separated from the Decabracia by a bootstrap value of 100% and an extremely high decay index (322).

Based on the log likelihood scores the program MODELTEST 3.03 recommended the general time reversible substitution model (GTR+G) with (gamma) corrections for among-site rate variation for the ML analysis. Based on this model the ML analysis provided a tree in which the Decabrachia were paraphyletic due to the position of *Eledone* (data not

shown). As seen in Fig. 3 the internal structure of the coleoids could not be resolved with Felsenstein´s bootstrap test.

DISCUSSION

The question of the phylogenetic position of Spirulida was deliberately approached with a limited number of species representing major groups. In fact the Spirulida are themselves represented by a single extant species. That the species used are really reliable representatives of their respective containing groups can be taken from the literature (Naef 1921-1923, Nesis 1987, Young & Vecchione 1996).

DNA sequences of the 18S rDNA from cephalopods seem to be unusually long. Although the gene has not been completely sequenced, the lengths vary between 1860 and 2222 nucleotides. Normally, 18S rDNA possesses between 1700 and 1900 nucleotides (Crease & Taylor 1998). Unusually long 18S rDNA's with over 2000 nucleotides caused by hypervariable regions (Mattern & Schlegel 2001) were described for various metazoa (Crease & Colborne 1998, Held & Wägele 1998, Giribet *et al.* 2001, Giribet 2002). In our alignment we found three hypervariable regions accounting for the length differences.

In this study, the methods used for tree construction (ML, NJ, MP) had a strong influence on tree topology. The phylogenetic relationships as shown in the ML (Fig. 3) tree appear quite unlikely because all previous studied morphological data are in disagreement with the ostensible position of *Eledone* among the Decabrachia (e.g. Neaf 1923, Nesis 1987, Boletzky 1999). Moreover, monophyly of the Decabrachia was supported by various molecular studies using both NJ and MP analyses (Bonnaud *et al.* 1994, 1996, Carlini & Graves 1999, Warnke *et al.* 2001). Carlini and co-workers (2000) who analysed the actin genes I and II with the ML method also found evidence for the monophyly of the Decabrachia although different topologies were observed between the genes. Apperently the ML method can support very different topologies. Mattern and Schlegel (2001) suggested that discrepancies in the phylogenetic resolution of 18S rDNA sequences from different models are caused by different characters of the conserved and variable

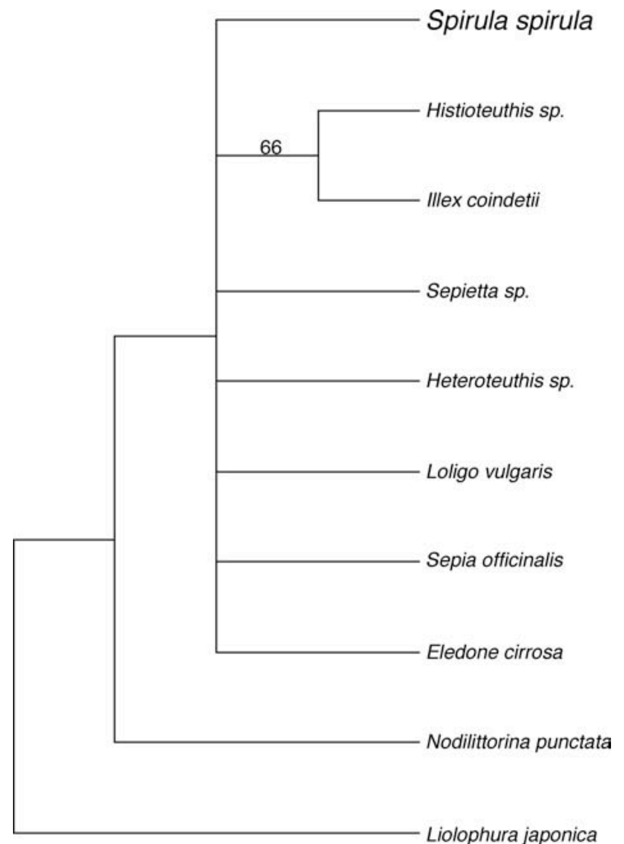

Fig. 3 ML-bootstrap tree of coleoid 18S rDNA sequences estimated with the general time reversible substitution model (GTR+G) with corrections for among-site rate variation. Rooting was completed using sequences of *Nodilittorina punctata* and *Liolophura japonica.* Bootstrap values (> 50%) obtained from 100 bootstrap replications are shown above nodes

regions (AT content, length, mutation rate).

Because the variable regions (considered to be loops) have high evolution rates, the alignments of such regions may not be optimal. Therefore, these regions were excluded from the analysis. The exclusive use of conservative sites, however, may result in a lack of phylogenetic information and thus, an erroneous phylogenetic reconstruction (Wägele & Stanjek 1995, Winnepennickxs & Backeljau 1996) which may be the reason for the insufficient resolution of our ML tree.

Likewise, bootstrap values did not support the *Spirula* node in the NJ tree. The NJ method using unweighted characters is a basically phenetic approach. Thus, the essential disadvantage of the ML and the NJ analyses is that they cannot distinguish between plesiomorphic and apomorphic characters in the sense of Hennig (1966), see Mattern and Schlegel (2001).

Since the character-based phylogenetic tree of the

MP analysis demonstrates that *Spirula* is basal within the Decabrachia, and is supported by a high bootstrap value and a high decay index (even with changing outgroup representatives), our molecular data favour the hypothesis of Engeser and Bandel (1988). In other words, this suggests that *Spirula* represents the sister group to all other recent Decabrachia. Interestingly, Doguzhaeva *et al.* (1999) used ultrastructural analysis of fossil coleoids to conclude that the Spirulida already existed in the Late Carboniferous. However, this structural evidence should not be overestimated for assessment of *absolute* age. Thomas Becker rightly remarked during a discussion (Berlin, September 2002) that extant *Spirula* clearly represents the Decabrachia, whereas Palaeozoic forms such as *Shimanskya* lack clear evidence of diagnostic features and are better assigned to stem-group taxa from which both the Decabrachia and Octobrachia may have been derived.

In any case, the results of this work remain preliminary because the sequences are not of the complete length of the 18S rRNA gene and the analysis is not able to eliminate all doubt concerning the topologies which have been found. Therefore, it is important to complete the sequences and to further analyse this gene region. Moreover, to test the hypothesis of Engeser and Bandel (1988), additional molecular and morphological data would be useful.

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