

MOLECULAR APPROACH OF DECABRACHIA PHYLOGENY: IS *IDIOSEPIUS* DEFINITELY NOT A SEPIOLID

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ABSTRACT: The taxonomic and phylogenetic position of *Idiosepius* among Decabrachia is reconsidered, based on cephalopod partial gene sequencing and on accessory nidamental gland symbiotic bacteria DNA analyses. Phylogenetic trees are established from partial ribosomal mitochondrial genes (16S rDNA and 12S rDNA) and cytochrome oxidase genes (CO I and CO III); they are analysed in relation with some diagnostic morphological characters and compared with the symbiotic bacteria 16S rDNA phylogenetic trees. The results obtained by both approaches show the idiosepiids as more closely related to teuthoids than to sepiolids. These new data lead us to propose that the taxon Teuthoidea gathers oegopsids (including Idiosepiidae), Myopsida, Sepiolida and Sepiadariidae.

INTRODUCTION

The original description of the pygmy squid *Idiosepius* (Steenstrup, 1881) presented this genus as a closed eyed Decabrachia deprived of a true gladius and having the normal appearance of a *Loligo* or *Loliolus*, with short arms common to sepiids and a similar locking apparatus. Steenstrup (1881) placed *Idiosepius* together with *Sepioloidea*, *Spirula*, *Sepiadarium* in the *Sepia-Loligo* family, in the large group of the myopsid or littoral cephalopods. Appellöf (1898) erected *Idiosepius* to a family level, Idiosepiidae. Presently, *Idiosepius* is mostly referred to as belonging to the family Idiosepiidae, within the Sepioidea or Sepiida, together with Sepiolidae, Sepiidae, Spirulidae (Voss, 1977 ; Nesis, 1987). Fioroni (1981) and Clarke (1988) consider Idiosepiidae as closely related to sepiolids. However, the taxonomic status of the pygmy squid has drawn new recent attention since Hylleberg and Nateewathana (1991a, b) described, contrary to the original description by Steenstrup (1881), the presence of a dorsal thin but clear gladius. Accordingly these authors suggest a relationship closer to teuthoids than to sepiolids. DNA sequence analyses brought out further evidence of this new position among teuthoids and allowed a first reevaluation of the taxonomic and

phylogenetic position of *Idiosepius* (Bonnaud *et al.*, 1997).

Beside the presence of a gladius, Hylleberg and Nateewathana (1991a, b) also described the presence of accessory nidamental glands (ANG) in female *Idiosepius*. These glands are present in most sepiids, sepiolids and myopsids and they could have a role during egg case formation. They are known to harbour various strains of symbiotic bacteria. In relation with the coevolution of hosts and symbionts, the phylogeny of bacteria strains might give indirect information on host phylogeny. A comparative analysis of the bacteria strains present in idiosepiid, sepiolid, sepiid and myopsid glands could thus shed light on the position of *Idiosepius* within the cephalopod group.

In the present paper, we include new DNA sequences to establish phylogenies of Decabrachia, and we use a new and indirect approach, by means of ANG symbionts, to examine the taxonomic status of *Idiosepius*.

MATERIALS AND METHODS

Specimens of *Idiosepius pygmaeus* and of *I. notoides* were caught off Australia (Queensland) and preserved in ethanol. DNA was extracted from

mantle tissue of these specimens and of 10 other cephalopod species. Partial ribosomal mitochondrial genes (16S rDNA, 12S rDNA) and cytochrome oxidase genes (COI and COIII) were amplified, cloned and sequenced as described in Bonnaud *et al.* (1994; 1997; 1998; 2003) or taken from Gene Bank (Table 1). Alignments were performed by eye using Se-Al (Rambaut, 1996), based on secondary structure for 12S and 16S sequences and based on amino acid translation for COI and COIII sequences. 16S and 12S rDNA sequences were analysed separately, COI and COIII nucleotide sequences were analysed together (COI sequences following COIII sequences). Alignment being uncertain in the most variable parts of 16S (loops), these regions and all the gaps were removed from the analysis. In this preliminary study, phylogenetic trees were obtained with neighbor-joining method.

The accessory nidamental glands of *Idiosepius pygmaeus* were dissected out aseptically. Total DNA extraction was performed using DNeasy Tissue Kit (Qiagen), and full length bacteria 16S rDNA gene (ca 1500bp) was amplified, cloned and sequenced as described in Grigioni *et al.* (2000). Bacteria sequences obtained from ANG of other cephalopod species (Pichon, unpublished, Grigioni *et al.*, 2000, Barbieri *et al.*, 2001) were also included in the phylogenetic analyses. Sequences were aligned using the ClustalW software (Thompson *et al.*, 1994). Phylogenetic trees were calculated using neighbor-joining algorithms with Kimura "2 parameters" model (Kimura, 1980). Bootstrap analyses (100 replicates) were performed for distance analyses (Felsenstein, 1993).

RESULTS AND DISCUSSION

Decabrachia phylogeny

Molecular results

16S and 12S rDNA sequences have been obtained for two Australian species of *Idiosepius*: *I. pygmaeus* and *I. notoides*. The sequences between these two species differ by ca. 7 % in 16S sequences and ca.10% in 12S sequences. Considering the small number of idiosepiid species and the relative homogeneity of the genus, this

percentage appears high. The geographical distribution of *I. pygmaeus* is larger than that of *I. notoides*. In the two species, the sequence variations between distant populations is not known. Thus, to evaluate the variation within this genus, sequences of specimens including all of the seven species identified at present from a large geographical area need to be compared.

16S rDNA sequence analysis (Figure 1) links the two species of *Idiosepius* to teuthoids. No clear evidence of relationships with oegopsids is evidenced. 16S sequences are too variable to obtain reliable nodes at the suprafamilial level. This gene is informative at the family level, as confirmed by the support of the relationship between a sepiolid and a sepiadariid.

12S rDNA sequence analysis (Figure 2) shows a clear link of *Idiosepius* with loliginids and sepiolids. With this gene, sepiolids (*Sepietta neglecta*) are linked to loliginids. Unfortunately, no oegopsid could be included in the analysis.

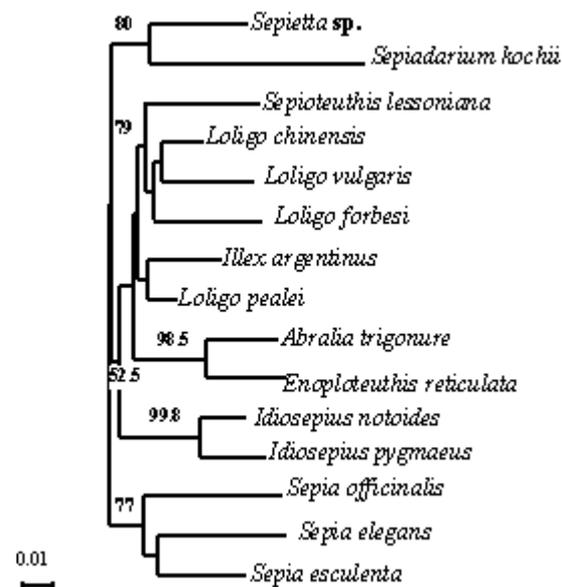


Figure 1. Phylogeny inferred by neighbor-joining analysis of 3' end of 16S rDNA sequences. Alignments of 554 nucleotides were performed by eye based on secondary structure. All the gaps and most variable parts were removed; 431 sites were taken into account in the analysis. Only bootstrap values higher than 50% are indicated.

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Table 1. List of the species included in this study. For each of them, the gene used and its accession number in EMBL databank is indicated, as well as the corresponding original reference.

Species name	Gene	Accession number	References
<i>Idiosepius pygmaeus</i>	16S		present study
	12S		present study
	COI	AF000046	Carlini and Graves (1999)
	COIII	X97959	Bonnaud <i>et al.</i> (1997)
<i>Idiosepius notoides</i>	16S	AJ001647	Hudelot <i>et al.</i> (unpublished)
	12S		present study
<i>Abralia trigonure</i>	16S	X79585	Bonnaud <i>et al.</i> (1994)
	COI	AF000025	Carlini and Graves (1999)
	COIII	X97948	Bonnaud <i>et al.</i> (1997)
<i>Enoploteuthis reticulata</i>	16S	X79573	Bonnaud <i>et al.</i> (1994)
	COI	AF000039	Carlini and Graves (1999)
	COIII	X97956	Bonnaud <i>et al.</i> (1997)
<i>Illex argentinus</i>	16S	X79595	Bonnaud <i>et al.</i> (1994)
<i>Loligo vulgaris</i>	16S	X79586	Bonnaud <i>et al.</i> (1994)
	12S		Bonnaud <i>et al.</i> in prep
<i>Loligo reynaudii</i>	COI	AF075406	Anderson (2000)
	COIII	X97960	Bonnaud <i>et al.</i> (1997)
<i>Loligo forbesi</i>	16S	X79584	Bonnaud <i>et al.</i> (1994)
	12S		present study
	COI	AF075402	Anderson (2000)
	COIII	X97962	Bonnaud <i>et al.</i> (1997)
<i>Loligo bleekeri</i>	ALL	AB009838	Tomita <i>et al.</i> (1998)
<i>Loligo pealei</i>	16S	AF11079	Anderson (2000)
<i>Loligo chinensis</i>	16S	AF369955	Zheng <i>et al.</i> (unpublished)
<i>Loligo opalescens</i>	12S		present study
<i>Sepioteuthis lessoniana</i>	16S	X79572	Bonnaud <i>et al.</i> (1994)
	12S		present study
<i>Sepia officinalis</i>	16S	X79570	Bonnaud <i>et al.</i> (1994)
	12S		Bonnaud <i>et al.</i> (in prep)
	COI	AF000062	Carlini and Graves (1999)
	COIII	X97954	Bonnaud <i>et al.</i> (1997)
<i>Sepia elegans</i>	16S	X79591	Bonnaud <i>et al.</i> (1994)
<i>Sepia esculenta</i>	16S	AF369114	Zheng <i>et al.</i> (unpublished)
<i>Sepia orbignyana</i>	12S		Bonnaud (unpublished)
<i>Sepia pharaonis</i>	12S		Bonnaud (unpublished)
<i>Sepia smithi</i>	12S		Bonnaud (unpublished)
<i>Euprymna scolopes</i>	COI	AF075417	Anderson (2000)
	COIII	AY149448	Kimbell <i>et al.</i> (unpublished)
<i>Heteroteuthis hawaiiensis</i>	COI	AF000044	Carlini and Graves (1999)
	COIII	X97952	Bonnaud <i>et al.</i> (1997)
<i>Sepiadarium kochii</i>	16S		present study
<i>Sepietta neglecta</i>	12S		present study

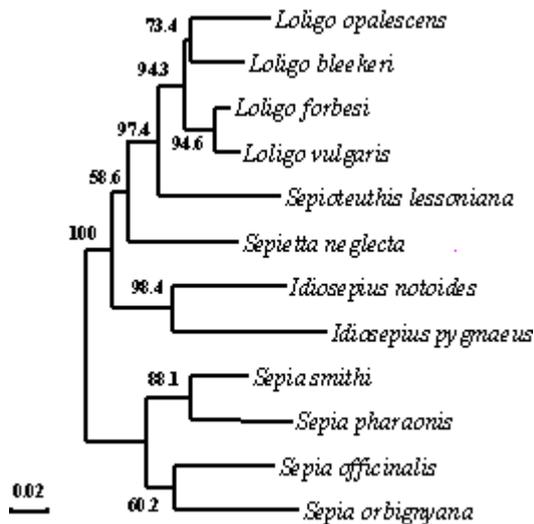


Figure 2. Phylogeny inferred by neighbor-joining analysis of 12S rDNA sequences. Alignments of 446 nucleotides were performed by eye based on secondary structure. All the gaps and most variable parts were removed; 327 sites were taken into account in the analysis. Only bootstrap values higher than 50% are indicated.

The COI-COIII genes analysis (Figure 3) confirms results previously obtained with COIII alone: *Idiosepius* is related to oegopsids.

Thus, in all cases idiosepiids are linked with loliginids and oegopsids: they are teuthoids. The base of teuthoids is poorly resolved with some genes, therefore sepiolids do not appear as a robust sister group of one or another of teuthoid groups. This could indicate a rapid radiation from a « teuthoid » ancestor.

The above molecular results are analysed in relation with some morphological characters mentioned as diagnostic criteria in species identification (Table 2).

Morphological criteria

Oegopsids show many specific (derived?) characters and based on these characters, a close relationship with loliginids is not evident.

Decabrachia taxa possess specific features and the characters considered in identification keys are often subject to convergences or parallel

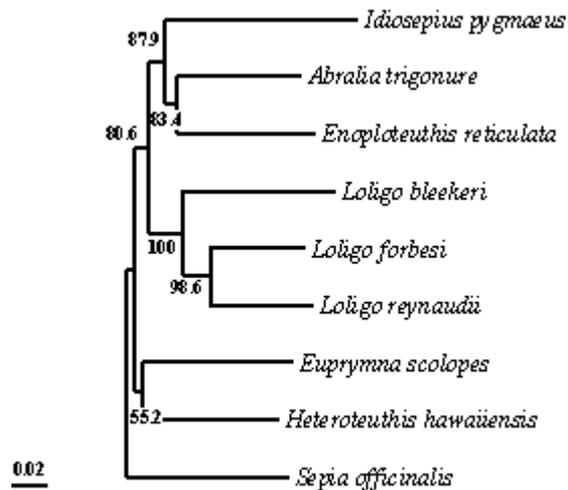


Figure 3. Phylogeny inferred by neighbor-joining analysis of COI and COIII sequences. The two sequences of the same species were added to each other. Alignments were performed based on amino-acid translation. 1055 nucleotides were taken into account. Only bootstrap values higher than 50% are indicated.

evolutions. Discussion about the polarization of the characters is necessary to map morphological characters on phylogenies.

The gladius (shell not calcified) is the strongest character that allows grouping of all Decabrachia except sepiids, with regression to loss in some groups as sepiolids. This is congruent with the most parsimonious hypothesis of shell evolution: from a cuttlebone, loss of calcification, leading in “squid” lineage to a gladius, followed by regression and loss of the gladius in some groups. However, paleontological data do not support this hypothesis, the first cuttlebone being identified only in the late tertiary, after the first gladius (Donovan and Toll, 1988).

Comparison of morphological and molecular data

Based on our molecular results a consensus tree is built (Figure 4) and related to morphological criteria: it shows idiosepiids linked to oegopsids but they do not share any derived character except late appearance of tentacles during development.

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Table 2. Some of the morphological characters used in specimen identification A : Gladius, presence (0), regressed or absent (1); B : Eye, opened (0), closed (1); C : Ventral septum, absence (0), presence (1); D : Nuchal cartilage, absence (0), presence (1); E : Accessory nidamental glands, absence (0), presence (1); F : Functional oviduct, left (1), both (0); G : Digestive gland, bilobed (0), unilobed (1); H : Posterior salivary glands, two (0), one (1); I : Caecal sac, absence (0), presence (1); J : Tentacles at birth, absence (0), presence (1). ? not known by the authors.

	A	B	C	D	E	F	G	H	I	J
Sepiidae /	0	0	0	1	1	0	0	1	1	
Oegopsida	0	1	0	0/1	0	0/1	0	1	1	0
Idiosepiidae	0	0	1	1	1	1	0	1?	0	0
Sepiolidae	1	0	1	0/1	1	1	0	0	0	1
Sepiadariidae	1	0	1	1	1	1	?	?	?	1
Myopsida	0	0	0	1	1	1	1	1	1	1

This characteristic shown in *Idiosepius* by Boletzky (1996) is observed also in some oegopsids as ommastrephids (Nesis, 1987). This heterochronic development could be a synapomorphy of oegopsids and idiosepiids, and confirm the close relationships of these taxa. Developmental characters might be informative in a comparative and evolutionary perspective: they need to be explored and taken into account in phylogenetic analyses.

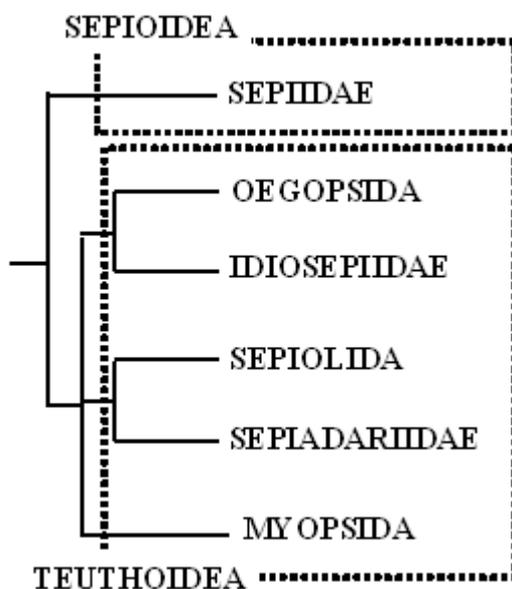


Figure 4. Consensus tree built from molecular phylogenies. This hypothesis on the relationships between Decabrachia lead to reevaluate the taxonomy in the light of morphological characters.

The case of sepiadariids remains enigmatic as it could be the sister group of sepiolids: sepiolids and sepiadariids are also linked together, as idiosepiids and oegopsids. Based on COI sequences, Carlini and Graves (1999) suggested an affinity between *Sepiadarium* and *Idiosepius* although the node was not supported. Surprisingly, COI is less informative than 16S for low-level Decabrachia phylogeny because of a fast turn over (probably due to functional constraints). 16S rDNA sequences, more discriminant, allow the detection of temporal events such as the emergence of a specific lineage sepiadariid/sepiolid from a “squid ancestor”: the grouping of *Sepiadarium* and *Sepietta* with our molecular data is reinforced by morphological characters such as the common regression of the gladius. The loss of the capacity to produce a gladius is however only partial in sepiolids which possess a shell sac during early stages of development.

As far as *Spirula* is concerned, previous results (Bonnaud *et al.*, 1994; 1997) obtained with the phylogenetic analyses of mitochondrial 16S and CoIII genes, always associate it with oegopsids, although this position is never solidly supported. The taxonomic position of *Spirula* thus needs further studies.

Symbiotic bacteria phylogeny

Bacteria 16S rDNA sequences have been obtained from the accessory nidamental glands of the Australian *Idiosepius pygmaeus*. As in all the

other cephalopod species investigated so far, various bacteria strains appear to be present in idiosepiids, including Gram labile (mainly the *Rhodobium* α proteo-bacteria) and Gram positive bacteria (Grigioni *et al.*, 2000).

The Gram positive bacteria group

The phylogenetic trees of Gram positive bacteria are rooted with *Rhodobium* strains (Figure 5). Two groups of cephalopod strains are identified. One group (group A) gathers two clusters of Gram positive bacteria, each is monophyletic and robust (bootstrap value: 100). No closely related strains from the environment or from other hosts referenced in the Gene Bank are linked with these cephalopod symbionts. All the strains are strongly

specific of cephalopod taxa (high bootstrap values for the two clusters of the group). They correspond to cephalopod taxonomy at high hierarchical levels, *i.e.* the bacteria strains relationships correspond exactly to Decabrachia relationships. One cluster strongly associates bacteria strains isolated from various sepiids ANG, the other cluster strongly relates *Idiosepius* strains to loliginid and sepiolid strains.

The other group (group B) gathers some *Sepia* species and reference strains (*Geodermatophilus*, *Sporichthya*) from the environment.

The Rhodobium group

The *Rhodobium* bacteria phylogeny evidences five groups (Figure 6). Three clusters (1) (2) (3)

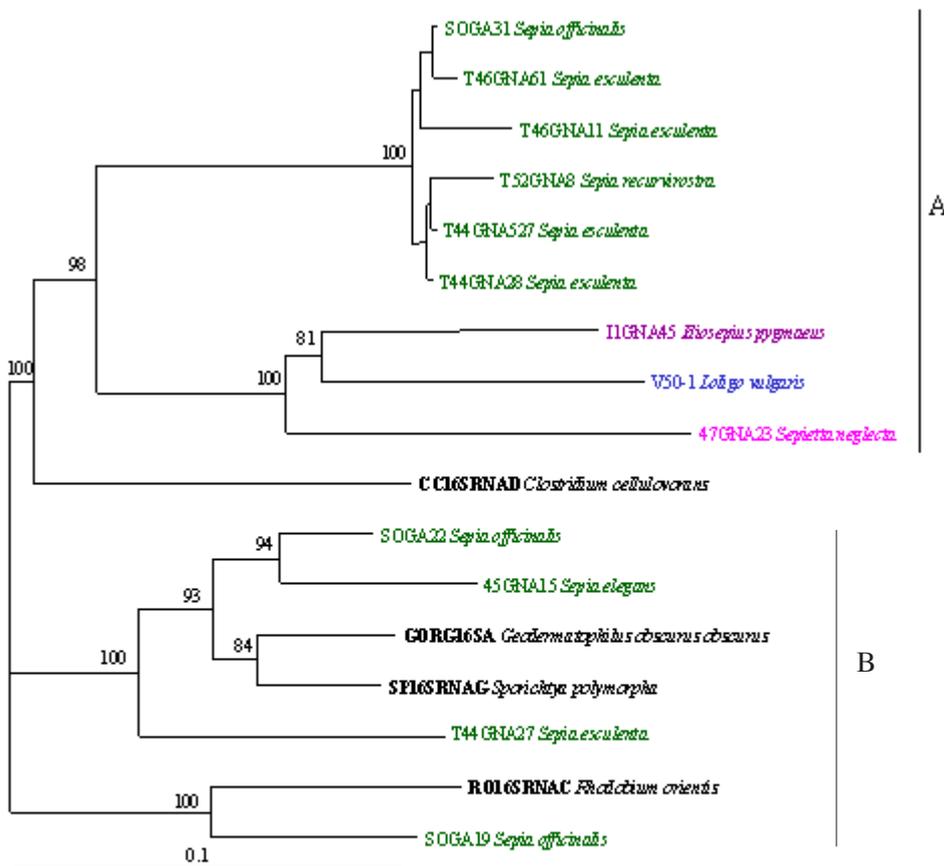


Figure 5. Phylogeny inferred by neighbor-joining analysis of ca 1500 nucleotides 16S rDNA sequences of Gram positive bacteria strains. All sites are taken into account. Only bootstrap values higher than 50% are indicated.

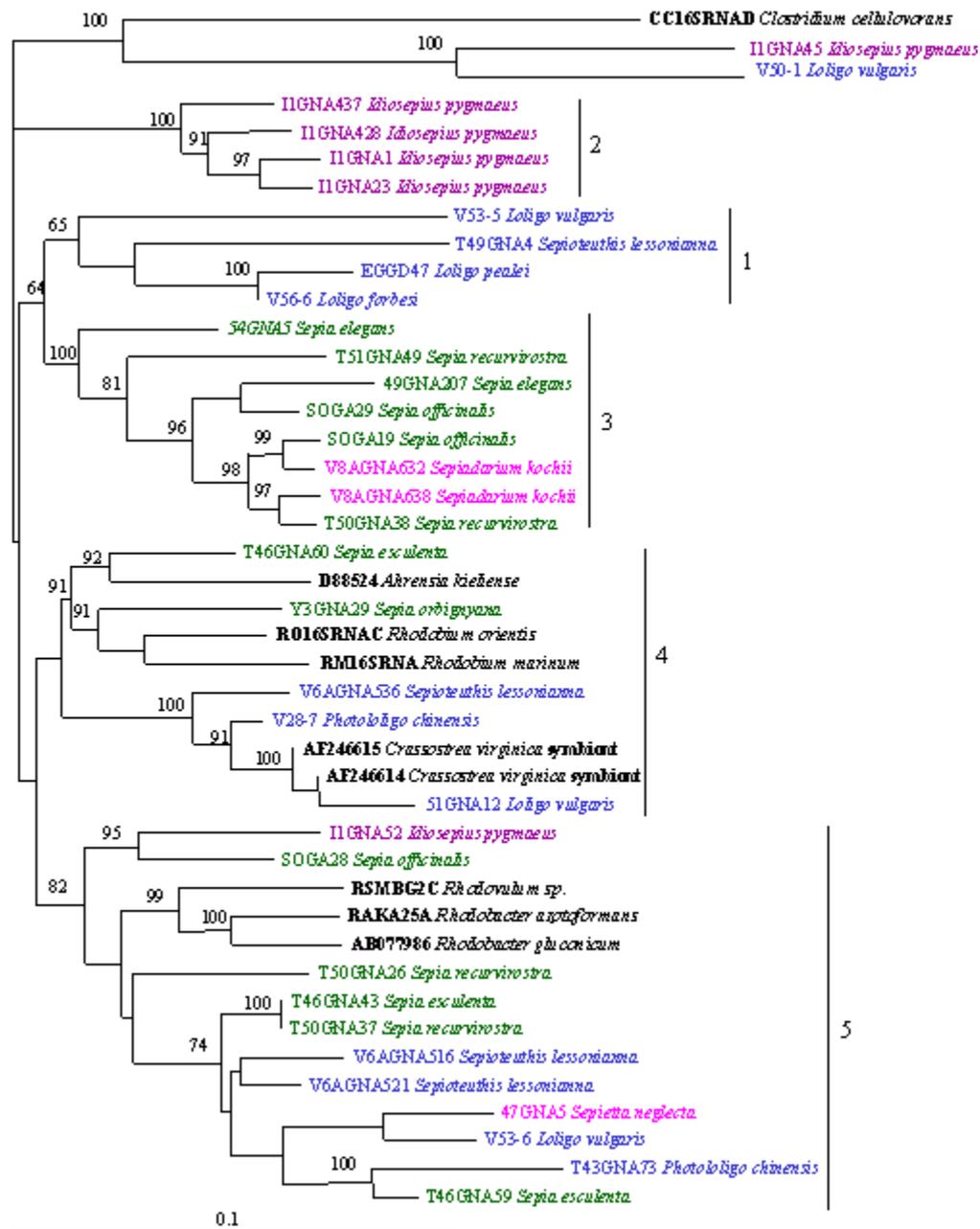
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Figure 6. Phylogeny inferred by neighbor-joining analysis of 1500 nucleotides 16S rDNA sequences of *Rhodobium* bacteria strains. All sites are taken into account. Only bootstrap values higher than 50% are indicated.

are specific to cephalopods at the family level (Loliginidae (1); Idiosepiidae (2); Sepiidae and Sepiadariidae (3)). These clusters are not significantly linked to each other, which might be due to a fast evolutionary rate in α -proteobacteria.

Two other clusters (4) (5) gather cephalopod symbionts with other mollusc symbionts and free living bacteria, suggesting that ANG bacteria colonization occurs from the environment.

From bacteria analyses, it appears that some symbiotic bacteria groups are coherent with cephalopod taxonomy: the two phylogenies are congruent. Both Gram positive and *Rhodobium* bacteria phylogenies allow identification of strains specific to cephalopod taxa. The specificity of bacteria symbionts suggests vertical transmission and co-evolution for some strains. Cephalopod strains also present in other organisms or found in the environment rather suggest horizontal transmission. These bacteria strains possibly acquired from the environment could indicate a coadaptation of a bacteria for a self function (adaptation). This recognition of specific partners, observed in the light organs of sepiolids (McFall-Ngai and Ruby, 1991; 1998; Nishigushi *et al.*, 1998), is a mechanism which needs further insight. Moreover, again like for the luminous organs, the ANG are not yet developed at birth (Boletzky, pers.com.), which raises the question of symbiont transmission, both vertical and horizontal, in an organ that is not yet formed.

CONCLUSIONS

The present results obtained from cephalopod partial genes sequencing and from symbiotic bacteria DNA analyses give new information on the taxonomic status of *Idiosepius*: it is definitely removed from sepiolids and included in teuthoids. The phylogenetically closest squid remains however to be defined by enlarging the teuthoid sample.

However, the genus *Idiosepius* includes other species that need to be explored at the molecular level in an attempt to have a better understanding of the evolution of this genus.

This study reconsiders also the evolution of Decabrachia: myopsids, oegopsids, idiosepiids,

sepiadariids and sepiolids are closely related and have probably evolved from a teuthoid ancestor possessing a gladius. This lineage has probably exploded in a short period of time by fast adaptative radiation leading to the present biodiversity of Decabrachia families possessing a gladius.

The results obtained from symbiotic bacteria DNA sequences show that both Gram positive and *Rhodobium* bacteria phylogenies identify strains specific to cephalopod taxa. The Gram positive strains confirm the taxonomic grouping of *Idiosepius* within the teuthoids, as supported by mitochondrial genes analyses. Gram positive strains might help investigating co-evolution of host and symbionts, and the functional meaning of the ANG. The specificity of bacteria symbionts suggests vertical transmission and co-evolution for these strains. Cephalopod strains also present in other organisms or found in the environment suggest a horizontal transmission.

As a consequence of the results obtained by both approaches, we propose that the taxon Teuthoidea includes Oegopsida (including the family Idiosepiidae), Myopsida (loliginids), Sepiolida and its sister group Sepiadariida. Therefore, Sepioidea is restricted to the Sepiidae family which is currently being revisited (Bonnaud, unpublished data).

In this study, we have shown that molecular characters, *i.e.* gene sequence analysis, are now usable at different levels: first by considering partial genome of the species of interest and second through symbiotic organisms DNA sequencing. In addition, developmental characters and functional studies would also be helpful to establish a reliable classification of Decabrachia.

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