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Phylogenetic characterisation of bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officinalis* (Cephalopoda: Decapoda)

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Abstract Female cuttlefish harbour a dense bacterial community in their accessory nidamental glands (ANG), as is also the case for the myopsid squids. Molecular approaches have been applied to explore this symbiotic association in the sepiid species *Sepia officinalis*. In situ localisation by *Bacteria*-specific probes in tissue sections of the ANG revealed the presence of a dense bacterial population in the lumina of the organ tubules. The phylogenetic identification of bacterial strains was realised by 16S rRNA gene sequencing analysis. None of the sequences obtained matched perfectly with known sequences in the database. However their similarity percentages allowed us to relate them to various bacterial groups including the taxa *Agrobacterium*, *Roseobacter*, *Sporichthya*, *Rhodobium–Xanthobacter* and *Clostridium*. Some bacterial species are common to both sepioids and myopsid teuthoids, others are different.

Introduction

Symbioses with prokaryotes are known to play an important role in the life of various organisms, and have been of major importance in evolutionary processes (Margulis 1993; Douglas 1994). Many cephalopods house symbiotic bacteria, the best known being the *Vibrio fisheri–Euprymna scolopes* light-organ association (Wei and Young 1989; Ruby and Lee 1998), and the accessory nidamental glands (ANG)–bacterial community association in loliginids (Bloodgood 1977; Barbieri et al. 1996). The ANG is a female organ related to the reproductive system, located adjacent to the nidamental glands of sepioids and myopsid squids. Colourless in immature animals, the ANGs become red-orange at maturity, due to the carotenoid pigments of their symbiotic bacteria (Bloodgood 1977; Van Den Branden et al. 1978). The nidamental glands are known to play a role in the production of the egg cases, but the role of the ANG in reproduction has still not clearly been established. It has recently been shown that in the squid *Loligo pealei* bacteria are transferred from the ANG to the egg cases where they might have a protective antimicrobial function (Barbieri et al. 1997). As early as 1918, observing a bacterial coating of the eggs of *Sepia officinalis*, Pierantoni (1918) deduced a possible vertical transmission of ANG bacteria from generation to generation. Upon hatching, the ANG–bacterial association is, however, not yet established in *Loligo* (aposymbiosis) (Kaufman et al. 1998), as is also the case for the *Vibrio fisheri–Euprymna scolopes* light-organ association (McFall-Ngai and Ruby 1991, 1998; Doino and McFall-Ngai 1995).

Symbiotic associations involving several bacterial groups are not well documented. They are rare among chemoautotrophic hosts (Dubilier et al. 1999). A phylogenetically diverse assemblage of bacteria is harboured in the glands of loliginids (Lum-Kong and Hastings 1992; Barbieri et al. 1996, 1997). Thus, the ANG represents a good model for the study of stable coexistence of multiple symbiotic species.

Recently developed molecular techniques allow the identification of bacterial communities, removing the bias of cultivation and resulting in a more comprehensive view of microbial associations. Specific oligonucleotide probes for in situ hybridisation (ISH) provide information on the localisation and the morphology of the bacterial strains, whereas the sequencing of small sub-unit ribosomal RNA (16S rRNA) genes allows determination of their phylogenetic position (Olsen et al. 1986).

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The objectives of the present study were to locate and to identify phylogenetically some of the bacterial populations present in the ANG of the cuttlefish *Sepia officinalis* using these molecular techniques. The results obtained are compared with those of loliginid symbionts, and some possible roles of the symbiosis are discussed.

Materials and methods

Organisms and samples

Adult *Sepia officinalis* were collected in the English Channel, off Luc-sur-mer, during 1997 and 1998. ANG samples were obtained from five sexually mature females. Prior to aseptic dissection, some individuals were kept alive in running-seawater tanks (three individuals) and some were frozen at −20 °C (two individuals).

Gram staining

A classical Gram test was carried out on histological sections of all individuals (Gerhard et al. 1994).

In situ hybridisation

For in situ hybridisation, ANG were processed as described by Amann et al. (1990) with some modifications. Briefly, ANG were fixed at 4 °C over 4 to 24 h (depending on size) in a solution of 3% paraformaldehyde in phosphate-buffered saline (PBS: 0.13 *M* NaCl, 7 m*M* Na_2HPO_4, 3 m*M* NaH_2PO_4, pH 7.2). After washing at room temperature in PBS (once for 30 min and three times for 1 h), they were post-fixed in 50% ethanol in PBS for at least 1 h at room temperature, and then stored at −20 °C. Before embedding in paraffin, the samples were dehydrated in 100% ethanol for 1 h, and then overnight. The histological sections (7 μm) were thoroughly disembedded before rehydration and hybridisation.

The protocol for whole-cell hybridisation was adapted from Hahn et al. (1992) and Zarda et al. (1997). Specific hybridisation of eubacterial cells was performed with the fluorescent (Cy3 or Fluorescein) labelled probe EUB 338 (5′-GCT GCC TCC CGT AGG AGT-3′). This probe is complementary to a conserved region on the 16S rRNA molecule of bacteria belonging to the domain *Bacteria*. The hybridisation was carried out in 9 μl hybridisation buffer (0.9 *M* NaCl, 20 m*M* Tris/HCl, 30% *N*-*N*-dimethylformamide, 0.01% SDS) and 1 μl of probe (25 ng μl^{-1}) during 90 min at 48 °C. Washing lasted 20 min at 46 °C (1.02 *M* NaCl, 20 m*M* Tris/HCl pH 7.2, 10 m*M* EDTA pH 8, 0.01% SDS). In situ hybridisation was also performed with α Proteobacteria (5′-CGT TCG YTC TGA GCC AG-3′), β Proteobacteria (5′-GCC TTC CCA CTT CGT TT-3′), γ Proteobacteria (5′-GCC TTC CCA CAT CGT TT-3′), and Gram-positive (5′-TAT AGT TAC CAC CGC CGT-3′)-specific labelled probes. The same protocol was used with an *N*-*N*-dimethylformamide concentration according to Wagner et al. (1995).

Ten microlitres of a 0.0001% solution of DAPI (DNA intercalating dye 4′,6-diamidino-2′-phenylindole solution, Sigma) was applied; the sample was incubated for 7 min, then rinsed with distilled water and air dried.

Samples were mounted with Citifluor immersion oil solution (Chemical Laboratory, The University Canterbury, England) and immediately observed with a Leitz epifluorescence microscope, equipped with a high-pressure mercury bulb using filter sets I2 (Leica) for fluorescein (360/40, 400DCLP, 460/50), F41–007 for Cy3 (535/50, 565LP, 610/75) and A513824 for DAPI (340–380/425 nm).

Amplification and cloning of 16S rRNA genes

ANG samples for PCR analysis were stored in TE buffer pH 8 (10 m*M* Tris/HCl pH 7.2, 1 m*M* EDTA) at −20 °C until used. Total DNA was prepared according to the method described by Kocher et al. (1989). Briefly, after Lysozyme and Proteinase K lysis, phenol purification and ethanol precipitation, purified DNA was resuspended in 50 μl of TE and stored at −20 °C.

PCR was conducted with dNTP, *Taq* polymerase and buffer (Eurogentec S.A.) in a ThermoJet thermocycler (EquiBio) with a denaturing step of 94 °C for 5 min, 32 cycles of 94 °C (30 s), 55 °C (30 s) and 72 °C (1 min) and a final elongation step of 72 °C for 7 min. The amplification primers were specific for bacteria, either 27F–1385R pairs (respectively *Escherichia coli* Position 9: 5′-GAG TTT GAT CCT GGC TCA G-3′ and Position 1385: 5′-CGG TGT GTR CAA GGC CC-3′) or UNIL–UNIR pairs (respectively *E. coli* Position 3: 5′-ATT CTA GAG TTT GAT CAT GGC TCA-3′ and Position 1418: 5′-ATG GTA CCG TGT GAC GGG CGG TGT A-3′), and produced almost the entire 16S rRNA gene fragment. Each PCR product was checked by electrophoresis in 1.5% agarose gel to have the expected length of ∼1500 bp. Purified PCR products (QIAquick PCR Purification Kit, Quiagen Inc.) were inserted into plasmid vector pCR 2.1-TOPO and transformed into competent cells of *E. coli* using the commercial kit TOPO TA Cloning (Invitrogen) following the instructions of the manufacturers.

Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) analysis was carried out on the bacterial 16S rDNA obtained by PCR, using two tetrameric restriction enzymes, *Hae*III and *Mbo*I, following the instructions of the manufacturer (Promega Corp.). PCR products were digested in specific buffer (Promega Corp.) for 2 h at 37 °C. Digested DNA fragments were electrophoresed through a 2% agarose gel.

16S rRNA gene sequencing and analysis

Minipreps of the clones were amplified by PCR using either 27F–1385R or UNIL–UNIR primers. PCR products were purified with QIAquick PCR Purification Kit and were prepared for sequencing using the Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer) on a Perkin Elmer GeneAmp 2400 thermal cycler, according to the respective manufacturer's instructions. Excess terminators were removed by spin-column purification (Centri-Sep Columns, Princeton Separation Inc.) before preparing and loading the samples onto the Abi PRISM Ready Reaction Kit on an ABI Prism 310 automated sequencer (Perkin Elmer) as specified in the manufacturer's protocol.

Both strands of the 16S rRNA gene in 16 clones were fully sequenced. The sequences of the first 500 bp (5′ extremity of the gene) were also obtained for 33 clones (5 clones for Individual 3, 7 clones for Individual 5, 11 clones for Individual 19, 5 clones for Individual 27 and 5 clones for Individual 28).

Sequences were aligned using the MegAlign software (DNASTAR Inc., Windows 95) with a subset of bacterial 16S rRNA sequences obtained by comparison with the EMBL GenBank database using the FASTA algorithm (Pearson and Lipman 1988). Phylogenetic trees were calculated using parsimony (PAUP 3.0, Swofford 1991) and neighbour-joining algorithms (Jukes and Cantor distance); bootstrapping (1000 replicates) was performed for distance analyses to test each topology for robustness (MUST 1.0; Philippe 1992).

Results

The ISH with the fluorescently labelled probe EUB338 enabled us to observe precisely the morphological

characteristics of the symbiotic bacteria in the tissues of cephalopods, despite some autofluorescence caused by the high concentration of mucus characteristic of these molluscs. By this technique, rod-shaped and coccoid-shaped bacteria were identified, both occurring together in high densities in the ANG tubules (Fig. 1). Gram staining and Gram-positive ISH allowed recognition of these two morphological forms as Gram positive (the majority of the bacterial population) or Gram variable. ISH also allowed determination of the part of the bacterial population representing the α Proteobacteria group. No β or γ Proteobacteria were present in the ANG of *Sepia officinalis*.

Whether the 16S rRNA gene could be used to discriminate the different genotypes corresponding to the morphotypes was tested by enzymatic digestion. The RFLP analysis of the amplicon obtained after PCR using universal primers for the bacterial 16S rRNA gene gave ca. ten fragments, ranging from 50 to 700 bp in length. The total length always exceeded 1500 bp, indicating that more than one genotype was detectable. Amplicons were therefore cloned in *Escherichia coli* in order to separate each bacterial population. The full-length insert of ~1500 bp was detected in 33 clones. It was completely sequenced in 16 clones and partially sequenced in the others. For each sequence, a query was made to the EMBL GenBank database using the FASTA algorithm (Pearson and Lipman 1988), in order to obtain the close relatives within the database. None of the sequences of the clones matched perfectly with known organism sequences in the database (Table 1). Two complete sequences, corresponding to DNA chimeras, were eliminated.

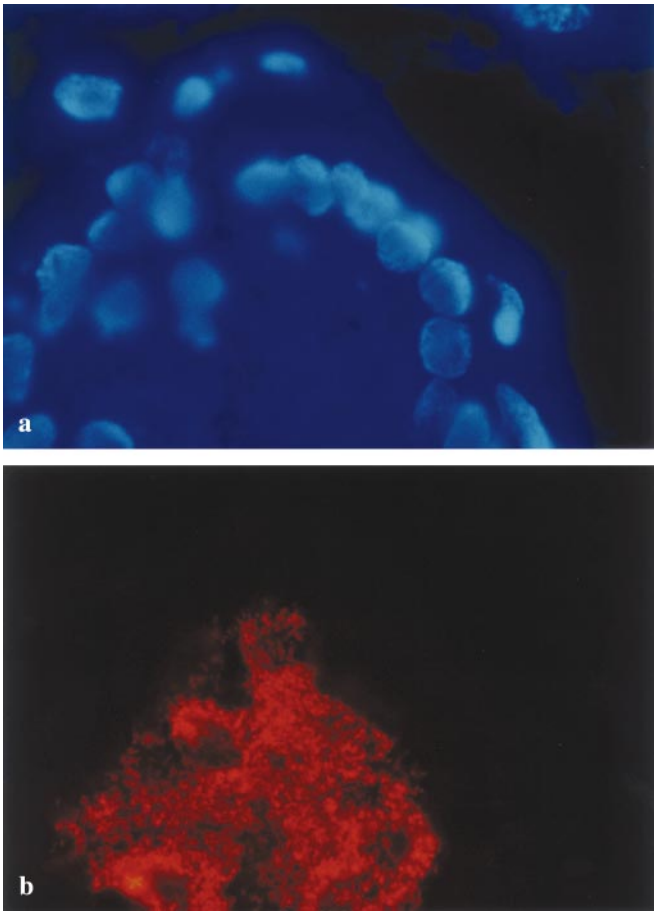

Fig. 1 *Sepia officinalis*. Identical field epifluorescence micrographs of an accessory nidamental gland section showing: **a** DAPI-stained cells and **b** bacteria hybridised with a Cy3-labelled EUB probe

Phylogenetic trees were obtained by parsimony and by neighbour-joining methods (Swofford 1991; Philippe 1992). The position of our clones in the phylogenetic trees did not change whether parsimony or neighbour-joining methods were used, or whether 500 bp or ~1500 bp sequences were analysed, only the distances were sometimes slightly different. Fig. 2 illustrates the partial 16S rRNA sequence analysis of all clones and reveals that, within each group, clones share nearly identical group sequences. Most of the groupings are solidly supported by bootstrap values (17 values higher than 99% and only 13 below 60%, out of a total of 48 values). Fig. 3 shows a better discrimination of our *Sepia officinalis* bacterial clones (~1500 bp) and gives more information on their phylogenetic position among their closest relatives in the domain *Bacteria*, especially for the *Rhodobium–Xanthobacter* group clones. In fact, by analysing only 500 bp, these clones are difficult to relate to a known bacterial group. With the 1500 bp analysis, instead, the group is homogenous despite the low bootstrap value (64%) and the low percentage of similarity with known taxa (Table 1).

The majority of our clones (15) showed a high percentage of similarity with the *Agrobacterium* group, representing nitrifying and denitrifying bacteria of the α Proteobacteria phylogenetic group (Fig. 3; Table 1). Two subgroups are pointed out, one being more closely related to *A. atlanticum* and the other to *A. meteori*.

A high percentage of similarity was also found between six clones and the *Roseobacter* group, which includes phototrophic bacteria sometimes reducing nitrates, belonging to the α group of Proteobacteria (Fig. 3; Table 1). Bootstrap values demonstrate that our clones (6) and *Loligo opalescens* symbionts are monophyletic.

The other clones were less clearly related to *Sporichthya* (Gram positive, eubacteria), *Clostridium* (Gram positive, eubacteria) and *Rhodobium–Xanthobacter* (α Proteobacteria, eubacteria) groups, showing percentages of similarity ranging from 78 to 92% (Table 1; Fig. 3). The low percentages of similarity demonstrate that sequences pertaining to these three bacterial groups are unique to *Sepia officinalis* and differ from those of symbionts from other species and of free-living bacteria. The strains grouping with the *Sporichthya*, *Clostridium* and *Rhodobium–Xanthobacter* groups could only be detected from fresh material.

Table 1 List and general characteristics of bacterial strains genetically close to the symbionts of *Sepia officinalis*. *Roseobacter* sp. J2W, *Roseobacter* sp. J8W and α Proteobacterium str. 303 were described from loliginid accessory nidamental glands

Bacterial species	Bacterial groups	Similarity (%)	Systematic position	Implication in the nitrogen cycle
Roseobacter denitrificans, *R. litoralis*, *R. algicola*, *R. gallaeciensis*, *R.* sp. J2W, *R.* sp. J8W, α Proteobacterium str. 303	*Roseobacter*	95–98	α Proteobacteria	Reduction of nitrates
Agrobacterium meteori, *A. atlanticum*	*Agrobacterium*	94–99	α Proteobacteria	Nitrification/ Denitrification, Reduction of nitrates
Geodermatophilus obscurus obscurus, *G.* sp. G1S, *G.* sp. G18, *Sporichthya polymorpha*	*Sporichthya*	89–92	Gram positive	Nitrogen fixation
Rhodobium orientis, Xanthobacter tagetidis, X. agilis	*Rhodobium–Xanthobacter*	88–89	α Proteobacteria	Reduction of nitrates
Clostridium sp., *C. cellulovorans*, uncultured eubacteria, unidentified bacteria	*Clostridium*	78–80	Gram positive	Nitrogen fixation

Discussion and conclusions

The existence of a bacterial community in the ANG of the cuttlefish *Sepia officinalis* was confirmed by ISH, Gram staining and 16S rRNA sequencing. A mixed bacterial population was found in the lumina of the ANG tubules, as described for *Loligo pealei* by Bloodgood (1977) by electron microscopy (TEM, SEM). In *S. officinalis*, rod- and coccoid-shaped bacteria, Gram positive or variable, occurred together in the ANG tubules.

The gene encoding the bacterial small sub-unit rRNA isolated from the ANG of five adults of *Sepia officinalis* helped resolve the taxonomic position of the symbiont populations. None of our sequences matched perfectly with any known organism in the database, but close resemblance in sequences allowed us to group our clones with known taxa of bacteria.

The bacterial symbionts from ANG of the Sepioidea *Sepia officinalis* can be compared to those from the myopsid Teuthoidea *Loligo pealei* (Barbieri et al. 1996, 1997) and *L. opalescens* (Kaufman et al. 1998, unpublished). One bacterial group is common to *Sepia* and *Loligo*, the others are more specific: *Agrobacterium*, *Sporichthya*, *Rhodobium–Xanthobacter* and *Clostridium* groups are found in *Sepia* only; *Roseobacter* is common to both cephalopod taxa; and *Alteromonas*, *Vibrio*, *Rhodovulum* (Barbieri et al. 1996) and *Shewanella* (Barbieri et al. 1997) are present only in *Loligo*. Barbieri et al. (1997) attribute an antimicrobial function to *Alteromonas* and *Shewanella*, identified in both ANG and internal egg jelly membranes of loliginids. These bacteria were not detected in *Sepia* ANG, but antimicrobial activity by other strains cannot be excluded.

Gram-positive bacteria are evidenced only in fresh *Sepia* samples. No comparison can be made with *Loligo*, since no data on Gram staining has been reported in recent articles. By light microscopy, Gram-positive strains represented the majority of the bacterial populations in the ANG of *S. officinalis*, but their percentage in our clonal library is low. This could be explained by the difficulty of DNA extraction from Gram-positive bacteria (Gerhard et al. 1994), evidenced in the present study by their apparent absence in the clones obtained from frozen samples. *Loligo* specimens were fresh (Barbieri et al. 1997), suggesting a species- rather than a sampling-related difference in bacterial groups between the two cephalopod taxa.

Clones clustering with the *Roseobacter* group were observed in both *Sepia* and *Loligo*. Our *Roseobacter* clones are closer to the strains *Roseobacter* sp. J2W and *Roseobacter* sp. J8W found in the egg capsule sheath of *L. opalescens* (Kaufmann et al. unpublished), than to the strain "α Proteobacterium str. 303" found by Barbieri et al. (1997) in the ANG of *L. pealei*.

Symbiotic bacteria are known to interact with the host: bacteria respond to sexual state since they change from white in immature females to bright red in sexually mature ones. Bloodgood (1977) suggests that putative sexual hormones affect the functioning of the ANG and that material secreted by ANG epithelial cells may alter the metabolism of the bacteria. Bacteria belonging to *Roseobacter* and also to *Rhodobium–Xanthobacter* are phototrophic and could be responsible for the red-orange colour of the ANG in mature animals, due to carotenoid accumulation. These bacteria are culturable; their metabolic activities and interactions with the host are presently being investigated under various experimental conditions.

Besides *Roseobacter* and *Rhodobium–Xanthobacter*, other strains are also present in the ANG. What is the role of such a multiple bacterial infection? The ANG are connected to the reproductive system, and their activity

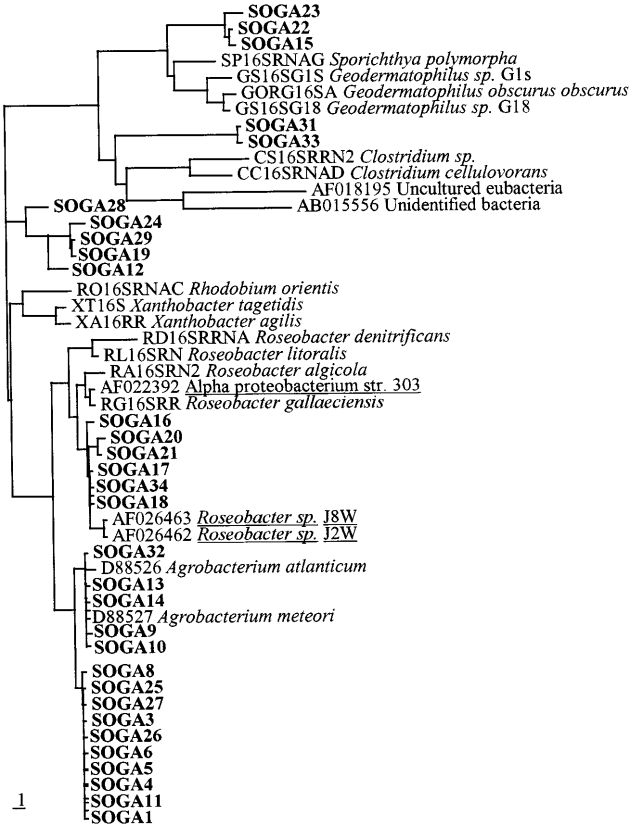

Fig. 2 *Sepia officinalis*. Distance tree obtained after neighbour-joining analysis of the 5′ end 16S rDNA sequences (500 bp). All sites are taken into account. EMBL accession numbers come before designations of bacterial taxa. Our clones (*bold*) have the following accession numbers: **SOGA1**: AJ244780, **SOGA3**: AJ244781, **SOGA4**: AJ244782, **SOGA5**: AJ244783, **SOGA6**: AJ244784, **SOGA8**: AJ244786, **SOGA9**: AJ244787, **SOGA10**: AJ244788, **SOGA11**: AJ244789, **SOGA12**: AJ244785, **SOGA13**: AJ244790, **SOGA14**: AJ244791, **SOGA15**: AJ244792, **SOGA16**: AJ244793, **SOGA17**: AJ244794, **SOGA18**: AJ244795, **SOGA19**: AJ244796, **SOGA20**: AJ244797, **SOGA21**: AJ244798, **SOGA22**: AJ244799, **SOGA23**: AJ244800, **SOGA24**: AJ244801, **SOGA25**: AJ244802, **SOGA26**: AJ244803, **SOGA27**: AJ244804, **SOGA28**: AJ244805, **SOGA29**: AJ244806, **SOGA31**: AJ244807, **SOGA32**: AJ244808, **SOGA33**: AJ244809, **SOGA34**: AJ244810. Loliginid ANG bacterial clones are underlined

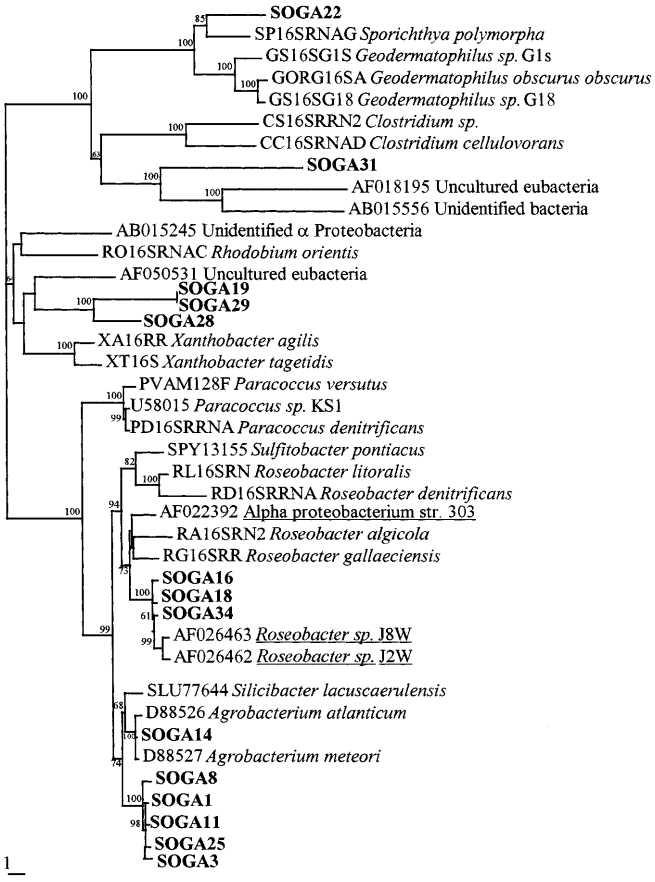

Fig. 3 *Sepia officinalis*. Distance tree obtained after neighbour-joining analysis of 1500 bp 16S rDNA sequences. All sites are taken into account. Only bootstrap values higher than 60% are indicated. Bacterial taxa and *S. officinalis* clones identified as in Fig. 2

is related to the presence of bacterial symbionts. They might be responsible for symbiosis transmission. It has been shown in *Loligo* that ANG bacteria are present in the egg jelly membranes and have a role of protection, but the hatchlings appear to be aposymbiotic (Barbieri et al. 1997; Kaufman et al. 1998). In *Sepia*, eggs also have a bacterial coating, apparently originating from the ANG (Pierantoni 1918), although this needs to be confirmed. But vertical transmission in this case seems possible, since bacteria are present in *Sepia* embryos (Grigioni et al. in preparation), which is in agreement with the possibility of maternal transmission of bacterial symbionts, via the ANG according to Pierantoni (1918).

However, ANGs are not yet differentiated at birth (Kaufman et al. 1998; Von Boletzky personal communication), which means that vertical transmission involves another symbiotic association. In adult *Sepia*, bacterial symbionts are known from other organs, in particular from the renal sacs (Grigioni et al. 1999). These might be involved in nitrogen waste recycling, as recently suggested for the marine gutless oligochete *Olavius loisae* (Dubilier et al. 1999). The buoyancy of *Sepia* is due to the presence of gas (mainly N_2) in the cuttlebone chambers (Denton 1974). This species is known for a low ammonia excretion rate, leading to the hypothesis of a symbiotic, bacteria-driven transformation of ammonia nitrogen into gaseous nitrogen in shelled cephalopods (Boucher-Rodoni and Mangold 1994). Bacteria capable of such a transformation are present in the ANG. One of the roles of the latter could then be to transmit these symbionts (necessary already in the embryo) to the following generation in the shelled *S. officinalis*. Identification of the symbionts present in the embryos and the renal sacs is currently underway to confirm this hypothesis.

The present results identify by molecular methods the ANG bacteria of *Sepia officinalis* and allow a new hypothesis concerning the role of these glands, which needs to be confirmed by further studies.

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