

# Taxonomy and new bacterial symbioses of gutless marine Tubificidae (Annelida, Oligochaeta) from the Island of Elba (Italy)

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## Abstract

In shallow sublittoral sediments of the north-west coast of the Island of Elba, Italy, a new gutless marine oligochaete, *Olavius ilvae* n. sp., was found together with a congeneric but not closely related species, *O. algarvensis* Giere et al., 1998. In diagnostic features of the genital organs, the new species differs from other *Olavius* species in having bipartite atria and very long, often folded spermathecae, but lacking penial chaetae. The Elba form of *O. algarvensis* has some structural differences from the original type described from the Algarve coast (Portugal). The two species from Elba share characteristics not previously reported for gutless oligochaetes: the lumen of the body cavity is unusually constricted and often filled with chloragocytes, and the symbiotic bacteria are often enclosed in vacuoles of the epidermal cells. Regarding the bacterial ultrastructure, the species share three similar morphotypes as symbionts; additionally, in *O. algarvensis* a rare fourth type was found. The divergence of the symbioses in *O. algarvensis*, and the coincidence in structural and bacteria-symbiotic features between the two taxonomically different, but syntopic host species at Elba are discussed.

**Key words:** Gutless oligochaetes, animal-bacteria symbioses, taxonomy, anatomy, distribution, ultrastructure, evolution, Mediterranean Sea

## Introduction

Gutless oligochaetes living in an obligate endosymbiosis with two or more types of bacteria were first described from shallow sediments in Bermuda (Giere 1981). Today, numerous species belonging to two tubificid genera, predominantly from the Caribbean and Australian regions (Erséus 1997, Giere et al. 1995), have been taxonomically, structurally and genetically analysed (Erséus 1984, 2002; Erséus et al. 2000; Dubilier et al. 1999). Previous to findings reported here, gutless, bacteria-symbiotic oligochaetes were known from Mediterranean waters on the basis of only two incomplete worms from Sicily (Italy) representing a single species, *Inanidrilus bononii* Erséus, 1984.

The present paper contains the description of two gutless oligochaete species based on mature specimens found at the shores of the Mediterranean Island of Elba, supplemented by ultrastructural details on their bacterial symbionts. One of the worm species is new to science.

## Material and methods

Numerous specimens of the two worm species studied were hand-collected (by a scuba diver) in shallow water (for the sampling site and dates see below) by qualitative sampling and subsequent inspection of various subsamples under the dissecting microscope (petri dishes with seawater). Decantation of the fragile worms was avoided to obtain complete, live specimens. Subdivision of mature individuals into (a) an anterior end plus genital region, (b) a median part, and (c) a posterior end, was necessary for subsequent work on the material by three different laboratories (taxonomy, electron microscopy, molecular biology), accounting for possible individual variation. For taxonomical descriptions, fully mature oligochaete specimens were stained in alcoholic paracarmine, dehydrated through an ethanol/xylene series, and whole-mounted in Canada balsam. Descriptions of the diagnostic features are based on microscopical examination (Zeiss Axioskop) of whole-mounted, complete specimens and/or the genital segments of selected animals. The type and other reference material is deposited in the Zoological Museum, University of Hamburg (ZMUH), and the Swedish Museum of Natural History (SMNH). For ul-

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trastructural work, immediate fixation was done in cold (4 °C) Trump's fixative buffered in sodium cacodylate (McDowell & Trump 1976). In the home laboratory, the material was washed in cacodylate, postfixed in 2% osmium tetroxide, dehydrated in an acetone series, and embedded in Spurr's resin. Ultrathin sections, mounted on copper grids and contrasted in aqueous uranyl acetate and lead citrate, were examined in a transmission electron microscope (Zeiss EM 902 A). For chemical element analysis (image electron energy loss spectrometry, I-EELS, and electron spectroscopic imaging, ESI), 30 nm thin sections, mounted on copper-grids, were examined in a TEM with a cooled vacuum chamber. The data acquired by the spectroscopic units (Zeiss) were analyzed with the software EsiVision Pro, AnalySIS 3.0. To obtain correct measurements of bacterial sizes, optimal sections were carefully selected.

Data on temperature and salinity were obtained from sediment cores directly in the field. Temperature was measured by an insertion electrode, and salinity recorded refractometrically in some drops of pore water. For sulphide measurement, pore water was syringe-sampled and sulphide was fixed (alkaline zincacetate) directly under water in the field. Total sulphide concentration of the pore water was subsequently measured photometrically using the methylene-blue method of Gilboa-Garber (1971) modified by Howarth et al. (1983). Grain size distribution of the sediment was calculated after fractionated sieving in the laboratory. Total organic carbon was assessed measuring ash-free dry weight after fixation of a fresh sediment sample in 70% ethanol and subsequent drying and combustion in the laboratory.

## Results

### 1. *Olavius ilvae* n. sp. (Figs 1, 2)

#### Type material

Holotype (ZMUH Ol. 14256): whole-mounted specimen (anterior part); ITALY, Island of Elba, north-west shore, semi-exposed bay close to Capo di Sant' Andrea, 42°48.4' N, 10°08.5' E, water depth about 4–8 m, sediment depth about 10–18 cm, collected in April 2000.

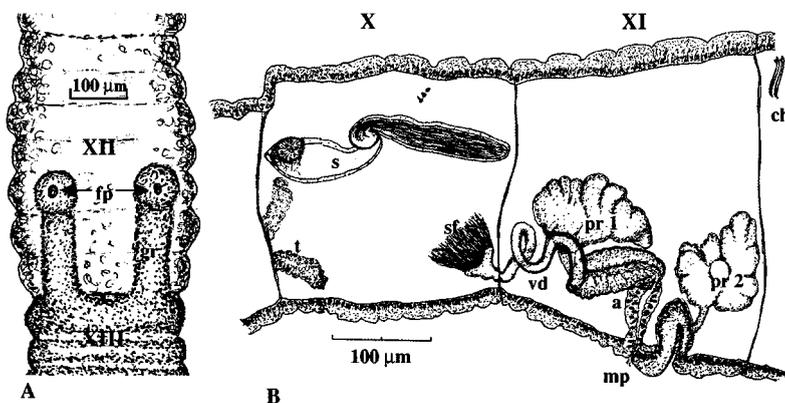
Paratypes (5); from type locality, collected in Aug. 1999: 1 complete worm and 1 anterior part (ZMUH Ol. 14257a, b) – 1 complete worm, 2 anterior parts (SMNH Type coll. Nos 5755–5757).

#### Etymology

The name is derived from (Latin) "Ilva", the Roman name of the Island of Elba.

#### Description

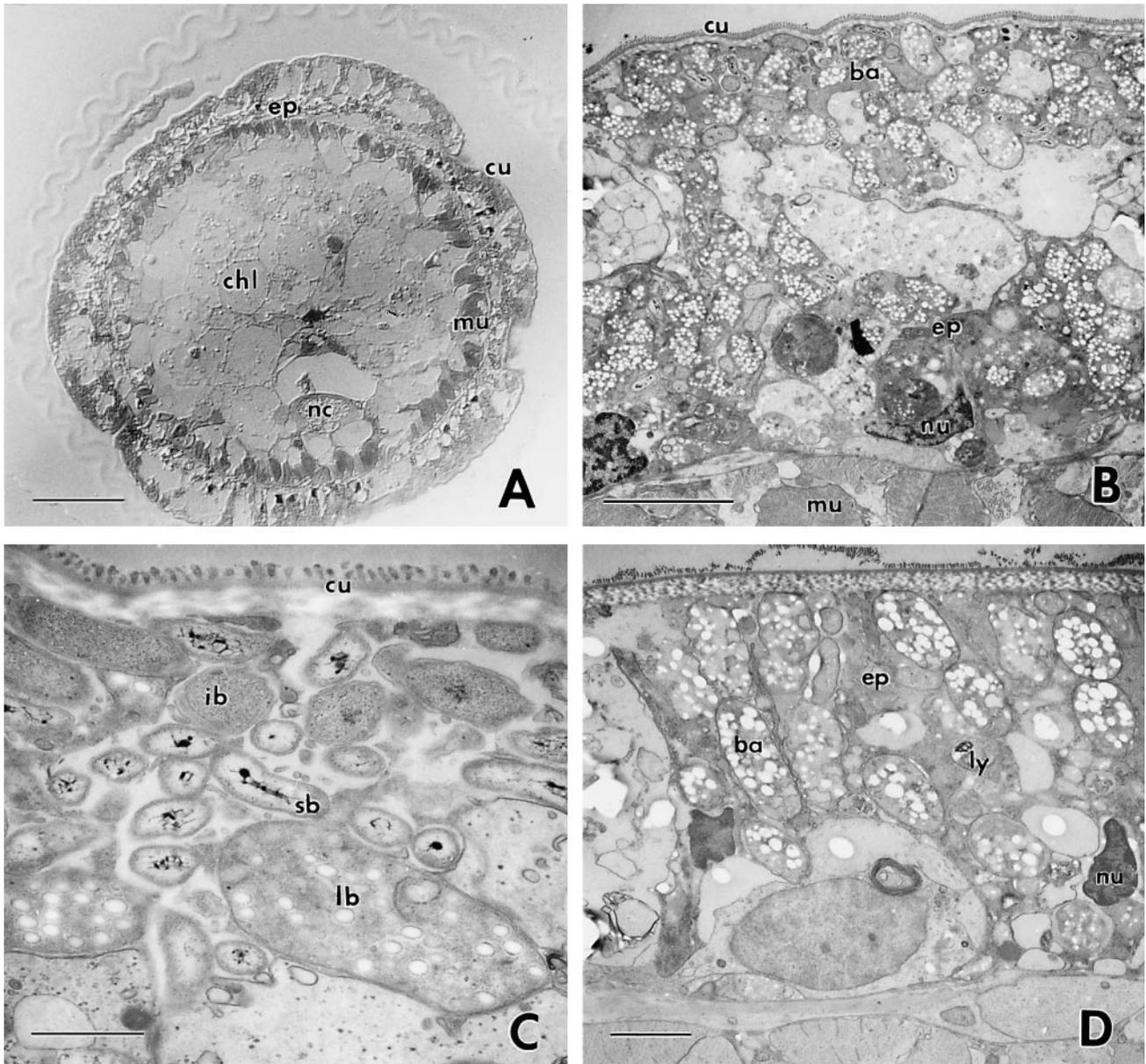
**External characters:** Length about 25 mm (live specimen), 22 mm (fixed, complete specimen), with about 80 segments, width about 0.3 mm in XI, 0.25 mm in post-genital segments. Colour shiny white in incident light. Prostomium rather elongate, sometimes pointed, depending on state of contraction; its wall fairly thick, its lumen filled with cells of refractory appearance (incident light). Clitellum inconspicuous, extending over 1/2 X-1/2 XII. Ventrally in XIII and XIV, subcuticular space densely filled with symbiotic bacteria and bulging forward, forming two granular ridges (see below) which extend anteriorly and enclose the paired female pores in XII (Fig. 1A). Elsewhere, body surface fairly smooth, secondary annuli not much pronounced, 7 per segment in most parts of body, in anteriormost segments only 2–3 per segment, near posterior end 3 per segment. Pygidium about as wide as last segment, elongate, terminally rounded and lacking caudal process. Somatic chaetae bifid, with upper tooth thinner than lower and with subdental ligament (Fig. 1B). Chaetae 25–40 µm long, 2.0–3.0 µm thick, 3–4 per bundle anteriorly, 2–3 per bundle in postclitellar segments. Penial chaetae absent. Male pores in latero-ventral position in posterior third of XI directly adjacent to involuted folds of body wall (simple copulatory sacs) forming extensile male papillae. Spermathecal pores paired, in line with dorsal chaetae, located closely behind intersegmental furrow IX/X.



**Fig. 1.** *Olavius ilvae* n. sp. **A.** Schematic drawing of ventral segments XII and XIII, with thick bacterial layer and two genital ridges (gr) reaching to female pores (fp). **B.** Camera lucida drawing of diagnostically relevant genital segments X and XI and of postgenital chaetae; lateral view. a – atrium, ch – chaeta, mp – male pore, pr 1 – anterior prostate, pr 2 – posterior prostate, s – spermatheca, sf – sperm funnel, t – testis, vd – vas deferens.

*Light-microscopical internal characters* (Figs 1B, 2A): Epidermis with numerous massive strands in direct contact with inner cuticular layer, nuclei mostly in basal position. Numerous glandular cells interspersed in epidermal cytoplasm. Dermal layer (including bacteria and cuticle) varying in width, locally thin. Alimentary canal and nephridia absent; ventral nerve cord, ensheathed in a

muscular ring, and two to three blood vessels traverse body cavity longitudinally. Longitudinal muscles (internal of circular musculature) with myosarc portions well developed and extending into body cavity. Remaining coelomic body cavity more or less filled with a rich and fluffy chloragocytic layer leaving only little free lumen (Fig. 2A).



**Fig. 2.** *Olavius ilvae* n. sp. **A.** Typical cross section through postgenital segment. chl – chloragocytic cells, cu – cuticle, ep – epidermis, mu – muscular layer (longitudinal), nc – nerve cord (light micrograph, scale bar: 50  $\mu$ m). **B.** Cross section through body wall with subcuticular layer containing bacteria (ba), epidermal (ep) and muscular (mu) cells. nu – nucleus of epidermal cell (electron micrograph, scale bar: 5.0  $\mu$ m). **C.** Three different subcuticular bacterial morphotypes. ib – intermediate bacterium, lb – large, oval, sulfur containing bacterium, sb – small bacterium (electron micrograph, scale bar: 1.0  $\mu$ m). **D.** Cross section through body wall, showing position of bacteria (ba) in vacuoles of epidermal cytoplasm (ep). ly – lysis stage of bacterium in deeper position, nu – nucleus of epidermal cell (electron micrograph, scale bar: 2.0  $\mu$ m).

Male genitalia (Fig. 1B) paired, sperm funnel about 40 µm wide, 20 µm deep; vas deferens non-muscular, coiled, about 230 µm long, about 12–15 µm wide, entering apical end of atrium. Atrium: ental part 100 µm long, 40 µm wide, oriented horizontally, spindle-shaped and thick-walled, finely granulated, muscular structures not discernible; ectal part 80 µm long, about 35 µm wide, vertical, tapering, with conspicuous nuclei. Anterior prostate gland elongate to oval, lobed, attached to apical part of atrium; posterior prostate attached by long stalk to distal end of vertical part of atrium. Spermathecae tubular, up to about 300 µm long, extending through all of X, with marked bend in middle part separating anterior duct (125–150 µm long, 45 µm wide) from posterior slender ampulla (about 130–150 µm long, 20–30 µm wide); ampulla filled with sperm (tails longitudinally oriented, not coiled). In mature specimens, sperm sac extending backwards into XV, three to four eggs at a time in XIV to XVII.

*Electron-microscopical characters* (Fig. 2B–D): As in other species of gutless oligochaetes, body wall with layer of symbiotic endobacteria underneath cuticle, starting in V, gradually becoming denser in VII–VIII, and continuing backwards until pygidium which lacks bacteria. With changing width of epidermal cell extensions bacterial number per section much varying, in regions with large glandular cells (see above) only few bacteria present. Endobacteria also encountered in youngest specimens.

Different from many other gutless oligochaetes, this species harbours three clearly distinguishable morphotypes of symbiotic bacteria (Fig. 2C). Most frequent is a large, oval type (length 2.3 µm ± 0.5 µm SD; width 1.3 µm ± 0.18 µm SD; n = 10) with double membrane typical of gram-negative bacteria. These bacteria always contain numerous electron light vesicles without membranes, and some membrane-bound globules in which sulfur was monitored (electron spectroscopic element analysis). A second type of bacteria, rod-shaped or slightly bent, usually located in a more ectal, peripheral position; mostly small (length 1.3 µm ± 0.2 µm SD; width 0.4 µm ± 0.04 µm SD; n = 10). Sometimes also longer rods present, about 1.9 µm long; cell membrane double, cytoplasm with chromatic fibres arranged in reticular, often centrally condensed pattern, but vesicular structures rarely found in cytoplasm. Third bacterial morphotype intermediate in size (length 1.6 µm ± 0.2 µm SD; width 0.6 µm ± 0.1 µm SD; n = 10) and shape, mostly found in external half of the bacteria-containing layer; its cytoplasm with a fine-reticulate pattern, usually lacking electron-dark chromatic aggregations and vesicular structures; its cell wall rather solid (40 nm thick).

Bacteria mostly enclosed in epidermal cytoplasm which forms narrow vacuoles around them (Fig. 2D). Only directly underneath cuticle bacteria sometimes in

extracellular position or only partly enclosed by cytoplasm.

Bacterial fission stages (cross-division) regularly found in large, oval bacteria, occasionally also in small and intermediate types. Finger-print-like structures of lytic bacteria mostly in ental parts of epidermal cells.

### Remarks

This new species is unique within *Olavius* in that its atrial openings are not inside copulatory sacs, but instead are located immediately anterior to a pair of epidermal invaginations (Fig. 1B) which possibly but not conclusively are homologous to the copulatory sacs of all congeners. *Olavius ilvae* n. sp. also has unusually slender (and long) spermathecae which are morphologically distinct in that they are clearly bipartite (each with a large wide duct followed by a more slender ampulla) and often characteristically twisted at the middle (Fig. 1B). In addition, the bipartite condition of the atria, with oval ampullae followed by ducts that are distinctly set off, is unusual within *Olavius*, and otherwise restricted to some species within a distinct clade once recognized as a subgenus, *Olavius (Coralliodriloides)* Erséus, 1984 (see Erséus 1984, 1993; Erséus & Davis 1989); all species of this clade, however, lack prostate glands and do not appear to be the closest relatives of *O. ilvae* n. sp.

*Olavius ilvae* n. sp. lacks penial chaetae, but this condition seems to have evolved in several lineages within the genus: the same applies to *O. comorensis* (Erséus, 1981); *O. crassitunicatus* Finogenova, 1986; *O. rallus* Erséus, 1991; *O. tannerensis* Erséus, 1991; *O. vacuus* Erséus, 1990; *O. verpa* Erséus, 1986; all '*Olavius (Coralliodriloides)*'; and one new species described separately (Erséus, 2002).

## 2. *Olavius algarvensis* Giere, Erséus & Stuhlmacher, 1998

*Olavius algarvensis* Giere et al., 1998: 210–212, figs 1–5.

### New material examined

8 specimens (ZMUH O1. 14258 a–h), 12 specimens (SMNH Main coll. Nos 46253–46264), all from type locality of *Olavius ilvae* n. sp., collected in Aug. 1999 or March/April 2000, respectively.

At Elba, occurring syntopically with *O. ilvae* n. sp., numerous gutless oligochaetes were found resembling *O. algarvensis* from the Atlantic shore of southern Portugal. However, the worms as well as their symbionts showed some features deviating from the Portuguese material, and these differences are pointed out here.

### Description

*External characters*: Length between 12 and 20 mm (fixed, complete specimens), with about 120–140 seg-

ments, width about 0.27 mm in XI, 0.25–0.27 mm in post-genital segments. Prostomium elongate (about 100  $\mu$ m long), with rounded tip and rather thin wall. Body surface rugged, body width varying. Clitellar glands fairly conspicuous, extending from  $\frac{1}{2}$  X to XII. Massive bacterial pad ventrally in XIV, and two ridges extending forward to female pores in XII (as in *O. ilvae* n. sp., see above). Penial chaetae (9)10–11(12) per bundle, about 70  $\mu$ m long, 2.5–3  $\mu$ m thick, in oblique position behind male papillae; within each bundle, posterior chaetae shorter than anterior ones (Fig. 3). Spermathecal pores paired, in line with dorsal chaetae, located mid-way between intersegmental furrow IX/X and dorsal bundle of chaetae.

**Light-microscopical (internal) characters** (Figs 3, 4A): Strands of epidermal cells often in direct contact with cuticle varying in length, thus width of epidermal layer irregular. Numerous glandular cells interspersed between ordinary epidermal cells (Fig. 4A). Sperm funnel 42  $\mu$ m wide, 15  $\mu$ m deep. Vas deferens 125  $\mu$ m long, about 10  $\mu$ m wide, non-muscular, entering atrium more or less apically. Atrium slender, almost perpendicular to worm axis, 80–100  $\mu$ m long, 20  $\mu$ m wide, with narrow lumen, its irregular, thick walls with conspicuous nuclei. Anterior prostate large, lobed, about 110  $\mu$ m long, 70–90  $\mu$ m wide; posterior prostate in upright position, 95  $\mu$ m long, about 55  $\mu$ m wide. Spermathecae 130  $\mu$ m long, about 40  $\mu$ m wide, duct short, its narrow lumen set off from ampullar part; the latter elongate, filled with straight masses of sperm. Two to four eggs in XV–XVI, seminal vesicle extending back to about XV. Body cavity filled with fluffy chloragocytic cells often extending to myosarc of longitudinal muscle cells; thus, width of coelomic lumen irregular and narrow.

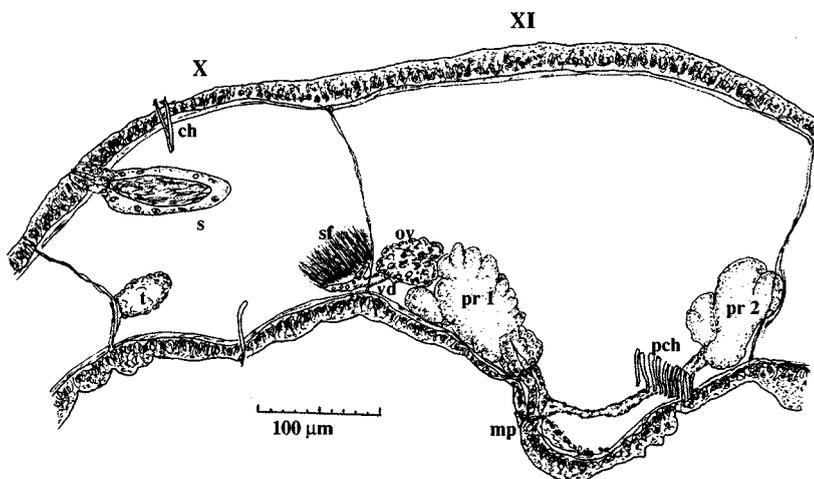
**Electron-microscopical characters** (Fig. 4B–F): Generally more than three bacterial morphotypes present. Large oval bacteria, with cell wall structure and vesicular cytoplasmic appearance as in *O. ilvae* sp. n. (see

above), but somewhat larger in size (length 2.6  $\mu$ m  $\pm$  0.3  $\mu$ m SD; width 1.4  $\mu$ m  $\pm$  0.1  $\mu$ m SD; n = 10). Small, rod-shaped or crescent-shaped bacteria with cytoplasm regularly containing condensed chromatic material (length 1.1  $\mu$ m  $\pm$  0.1 SD; width 0.4  $\mu$ m  $\pm$  0.05  $\mu$ m SD; n = 10). Third bacterial type (Fig. 4C: ib) mostly somewhat larger and stouter (length 1.5  $\mu$ m  $\pm$  0.2  $\mu$ m SD; width 0.6  $\mu$ m  $\pm$  0.1  $\mu$ m SD; n = 10) than rod-shaped type mentioned above, but more varying in size, and with bilayered cell wall typical for gram-negatives; cytoplasm mostly with homogeneous fine-reticulate structure, rarely with condensed electron-dark structures. A fourth, almost filiform type (length 1.8  $\mu$ m  $\pm$  0.6  $\mu$ m SD; width 0.4  $\mu$ m  $\pm$  0.1  $\mu$ m SD; n = 10) rarely encountered (Figs 4D, E); its thick (27 nm) cell wall locally dented or contorted, its cytoplasm reticulate. This morphotype characterized by wide variation in length (SD about 30% of mean). Not all these various bacterial types could be found in each section, in a few host specimens only two bacterial morphotypes could be found, namely the large oval one and the medium-sized, stout (third) type.

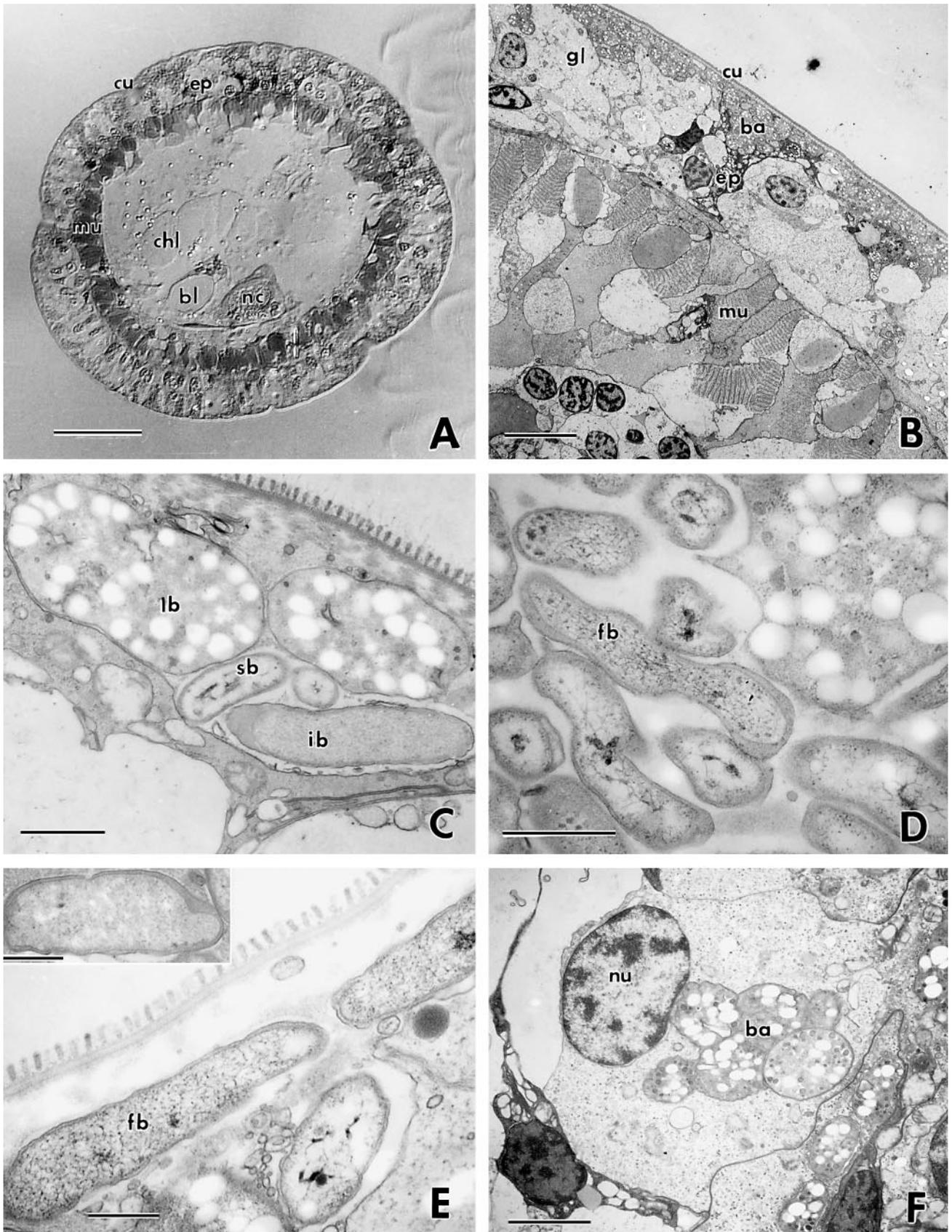
Bacteria mostly in vacuoles, formed by epidermal cytoplasm and its cell extensions (Fig. 4B, F), extracellular only in most peripheral position underneath cuticle. Lytic bacterial stages fairly frequent in ental parts of epidermal cells, whereas bacteria of regular appearance and with division stages in more ectal positions.

#### Remarks

In the original description of *O. algarvensis* found in Portugal (Giere et al. 1998), some details of the genital organs were only tentatively described, largely due to the submature status of the material. The present specimens are slightly different in their diagnostic features from the previous description. The fact that many of the worms were fully mature may at least partly explain their larger body width, higher number of penial



**Fig. 3.** *Olavius algarvensis*, Elba; lateral view. Camera lucida drawing of diagnostically relevant genital segments X and XI. a – atrium, ch – chaetae, mp – male pore, ov – ovary, pch – penial chaetae, pr – prostate, s – spermatheca, sf – sperm funnel, t – testis, vd – vas deferens.



chaetae, as well as their larger (and sperm-filled) spermathecae and atria. However, one consistent difference between the two populations remains: the reduction of the coelomic lumen by richly developed muscular and chloragocytic cells in the Elba form. This anomaly is greater than normally conceived as intra-specific variation in Tubificidae, but interestingly, the same feature is noted also for the distantly related, but syntopic, *O. ilvae* n. sp. (see above). The partial occlusion of the coelomic cavity may be an anatomical response to the specific combination of symbionts that appear to be associated with both *Olavius* species at Elba (see Discussion below).

### Distribution and habitat

*Olavius ilvae* n. sp. and *O. algarvensis* were found syntopically in both spring and summer, in shallow subtidal siliceous sand of a small, sheltered bight adjacent to an area with seagrass stands. In all samples mature specimens of *O. ilvae* n. sp. were much rarer than those of *O. algarvensis* (approximately one *O. ilvae* among 30 individuals of *O. algarvensis*), with juvenile and submature worms prevailing in both populations. The siliceous sediment was relatively rich in debris (0.8 wt% organic C) and consisted of coarse sand (MD: 760  $\mu\text{m}$ ) that was only moderately sorted (sorting coefficient 0.82) due to presence of some gravel. A visible chemocline was not developed, free sulfide could not be recorded. The sediment depth preferred by the oligochaetes was 10–18 cm.

Summer temperatures in the water of the bight were about 26 °C (in winter they can decrease to 13 °C; C. Lott, pers. comm.), summer salinity was about 37 ‰. The meiofauna accompanying the gutless oligochaetes consisted predominantly of nematodes with a considerable number of thiobiotic Stilbonematinae and Linhomoeidae. Bacteria-symbiotic ciliates of the genus *Kentrophorus* were also fairly common in the sediment layer at 10–15 cm depth. It seems that the occurrence of the gutless oligochaetes around Elba is rather restricted, since numerous sediment samples from other locations never contained these worms.

### Discussion

In all gutless oligochaetes previously studied, a multiple obligate endosymbiosis with extracellular bacteria in the subcuticular space between cytoplasmic strands of epidermal cells has been established (Giere & Langheld 1987, Giere et al. 1995). In *O. ilvae* n. sp., however, the bacterial position differs considerably in that the extensions of the epidermal cells almost completely enclose the bacteria in tight vacuoles. Thus, except for the most peripheral ones, the bacteria become endobacteria rather than ectobacteria.

The large oval bacteria with ‘spotted’ vesicular cytoplasm, always present in gutless oligochaetes, dominate the bacterial layer in *O. ilvae* n. sp. as well. Based on the appearance, size range and the analytical evidence of sulfur in the bacterial cytoplasm, this morphotype is again assumed to belong to the sulfur-oxidizing  $\gamma$ -Proteobacteria.

In contrast to these ubiquitous ‘primary symbionts’ of gutless oligochaetes, the additional, ‘secondary’ symbionts are more variable in taxonomic affiliation and function (Dubilier et al. 1999, 2001). In the symbiosis of *O. ilvae* n. sp., significant differences in size and the divergence in cytoplasmic patterns suggest that there are at least two additional distinct morphotypes involved.

Based on the ultrastructural evidence, *O. algarvensis* from Elba is remarkably different from the conspecific worms occurring at the Algarve coast (cf. Giere et al. 1998). Not only are the bacteria in the Elba specimens mostly surrounded by epidermal cytoplasm, i.e. they are intracellular rather than extracellular, the suite of bacteria is also clearly different. Even the most consistent, large oval morphotype differs significantly in size (mean length 2.6  $\mu\text{m}$   $\pm$  0.3  $\mu\text{m}$  SD, as opposed to 1.6  $\mu\text{m}$   $\pm$  0.4  $\mu\text{m}$  SD in the Algarve material). While the worms from the Algarve coast had only one additional bacterial partner (small, rod-shaped bacteria), in most of the Elba animals we discerned what appear to be three additional morphotypes. On average, the rods are of similar size, but they differ in their frequently bent shape. More importantly, it is the existence of a third, intermediate type

**Fig. 4.** *Olavius algarvensis*, Elba. **A.** Typical cross section through postgenital segment. bl – blood vessel, chl – chloragogue cells, cu – cuticle, ep – epidermis, mu – muscular layer (longitudinal), nc – nerve cord (light micrograph, scale bar: 50  $\mu\text{m}$ ). **B.** Cross section through body wall with bacteria (ba) under cuticle (cu). Epidermal (ep) and muscular (mu) cells, gl – glandular cytoplasm (electron micrograph, scale bar: 10  $\mu\text{m}$ ). **C.** Three different subcuticular bacterial morphotypes. ib – intermediate bacterium, lb – large, oval, sulfur containing bacterium, sb – small, rod- or crescent-shaped bacterium (electron micrograph, scale bar: 1.0  $\mu\text{m}$ ). **D.** Section through two filiform bacteria (fb) representing the fourth morphotype (electron micrograph, scale bar: 0.5  $\mu\text{m}$ ). **E.** Sections through two filiform bacteria directly underneath cuticle (electron micrograph, scale bar: 0.5  $\mu\text{m}$ ). Inset: another filiform morphotype showing well contorted cell wall (electron micrograph, scale bar: 0.5  $\mu\text{m}$ ). **F.** Section through epidermal cell with cluster of endocellular bacteria enclosed in vacuoles. nu – nucleus of epidermal cell (electron micrograph, scale bar: 2.0  $\mu\text{m}$ ).

and a rare fourth type with contorted cell wall that qualitatively separate the symbiotic pattern of the Elba population from that of the original *O. algarvensis*. While the structure of the intermediate morphotype resembles that of the third morphotype reported for *O. ilvae* n. sp. (Fig. 2C: ib), the contorted membrane of the fourth type corresponds well to bacteria found in *O. loisae* Erséus, 1984 and identified as aberrant Spirochaeta (Dubilier et al. 1999: fig. 1). This difference at the bacterial level is unique, as it occurs in hosts that apparently belong to the same species.

Dubilier et al. (2001), analysing *O. algarvensis* material from the same location at Elba, found on the basis of molecular studies a novel form of syntrophism which linked the sulfide oxidation of the large  $\gamma$ -bacteria to the sulfate reduction of  $\delta$ -bacteria. The latter corresponded to the intermediate morphotype described here (see fig. 1 in Dubilier et al. 2001). At the time of that study, the existence of a third (small rods or crescents) and fourth morphotype (with contorted cell wall) had not yet been ascertained. The affiliation and function of these additional morphotypes remain, as yet, unknown.

Triple bacterial endosymbioses have previously been documented by structural and molecular analyses for several Pacific species of gutless oligochaetes (Dubilier et al. 1999, Giere & Krieger 2001), but to our knowledge they are unknown in symbioses from other marine hosts. The Elba population of *O. algarvensis* would be the first gutless oligochaete with four ultrastructurally discernible bacterial symbionts.

Comparing, in terms of evolutionary trends, the variations of the symbiotic pattern in the two populations of *O. algarvensis* on the one hand and the sympatric gutless worms from Elba (*O. algarvensis* and *O. ilvae* n. sp.) on the other, two aspects emerge.

The first deals with the origin of the secondary symbionts. Colonization of European shallow sediments by gutless Oligochaeta is assumed to have originated from one of their radiative centres, the subtropical western Atlantic shores (Erséus 1990, Giere et al. 1995). Thus, settlement in the shore sediments of the eastern Atlantic Ocean (Portugal) was probably previous to that in the geologically young ( $5.5 \times 10^6$  y) Mediterranean Sea. If this scenario is correct, the eastern Atlantic form of *O. algarvensis*, in the process of colonizing the Tyrrhenian Sea, must have altered its suite of endosymbiotic bacteria. Even if the primary symbiont, the sulfur oxidising large oval type, remained the same (despite the difference in size), uptake of a different, small morphotype and addition to the assemblage of two more bacterial types would be major evolutionary events with only a relatively short time available for functional integration. A high variability in the nature and probably also function of the secondary symbionts has been found also in other gutless oligochaete species (Dubilier et al. 1999,

Giere & Krieger 2001). This would support the earlier assumption that the secondary symbionts may have been acquired via environmental transmittance, whereas the primary symbionts seem to be 'inherited' vertically via the eggs. Environmental transmittance of bacteria has been proven in several bacteria symbioses of marine animals. It is characteristic for one of the 'classical' vent animals, the giant tube worm *Riftia pachyptila* (see Cary et al. 1993), as well as for the sulfur-oxidizing gill bacteria of the shallow-water bivalve *Codakia orbicularis* (see Gros et al. 1996). The symbiosis of deep-sea cephalopods with luminescent *Vibrio fischeri* bacteria is also based on environmental transmittance (Ruby & Asato 1993, Nyholm et al. 2000). In *O. algarvensis*, the advantages and pathways of the suggested combination of transmittance pathways (vertical plus environmental) remain as yet speculative, but the fact that conspecific host populations have altered their symbiotic pattern within an evolutionarily short time underlines a high variability and a considerable adaptive potential of this symbiosis.

The second aspect to consider concerns some striking shared features of the taxonomically so clearly different species of gutless oligochaetes, *O. ilvae* n. sp. and *O. algarvensis*, living syntopically at the same Elba site. Three peculiar traits separate them from the numerous other gutless oligochaete species studied. Firstly, their body cavities do not have wide lumina but within each worm are to a large extent constricted by a rich musculature and filled by chloragocytic cells. Secondly, their bacterial associations share three well-definable and rather similar morphotypes; and yet, structural variations in some of the bacterial types do not preclude the existence of even additional (shared?) symbionts. Thirdly, in both species most of the bacteria are enclosed in the cytoplasm of the epidermis, i.e. most of them are endocellular in vacuoles. At present, we have no explanation for this structural congruence in these clearly different species. It remains to be shown to what extent the bacterial symbionts of the two syntopic host species are also congruent in molecular biological characters. Moreover, molecular studies of the hosts could help clarify the relationship between the disjunct populations of *O. algarvensis*.

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