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# Redescription and phylogenetic analyses of *Durchoniella* spp. (Ciliophora, Astomatida) associated with the polychaete *Cirriformia tentaculata* (Montagu, 1808)

Sauvadet Anne-Laure [1, *], Lynn Denis H. [2], Roussel Erwan [3], Le Panse Sophie [4], Bigeard Estelle [1], Schrével Joseph [5], Guillou Laure [1]

[1] Sorbonne Universités, Université Pierre et Marie Curie—Paris 6, CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff cedex, France
[2] Department of Integrative Biology, University of Guelph, N1G 2W1 Guelph, ON Canada
[3] School of Earth and Ocean Sciences, Cardiff University, Main Building, Park Place, Cardiff, Wales, UK
[4] CNRS, FR 2424, Service Informatique et Génomique, Station Biologique de Roscoff, 29680 Roscoff, France
[5] Muséum National d'Histoire Naturelle, Département RDDM, CNRS UMR 7245, CP 52, 61 Rue Buffon, 75231 Paris Cedex 05, France

* Corresponding author : Anne-Laure Sauvadet, email address : annelaure.sauvadet@gmail.com

**Abstract :**

Microscopic and phylogenetic analyses were performed on endocommensal astome ciliates retrieved from the middle intestine of a marine cirratulid polychaete, *Cirriformia tentaculata*, collected in the bay of Roscoff (English Channel, Northwest French coast) and on the Southwest English coast. Three morphotypes of the astome genus *Durchoniella* were identified, two corresponding to described species (the type species *Durchoniella brasili* (Léger and Duboscq, 1904) de Puytorac, 1954 and *Durchoniella legeriduboscqui* de Puytorac, 1954) while a third morphotype remains undescribed. Their small subunit (SSU) rRNA gene sequences showed at least 97.2% identity and phylogenetic analyses grouped them at the base of the subclass Scuticociliatia (Oligohymenophorea), as a sister lineage to all astomes from terrestrial oligochaete annelids. Ultrastructural examination by transmission electron microscopy and fluorescence in situ hybridization analyses revealed the presence of endocytoplasmic cocci and rod-shaped bacteria surrounded by a very thin membrane. These endocytoplasmic bacteria may play a role in the association between endocommensal astome ciliates and cirratulid polychaetes inhabiting in anoxic coastal sediments.

**Keywords** : Astomatida, Ciliophora, Cirratulidae, Endocytoplasmic bacteria, Microscopy, Molecular phylogeny

## Introduction

Unicellular ciliates are genetically and morphologically diverse (~8000 described species, Lynn 2008), mainly characterized by dimorphic nuclei and the presence of cilia at least once during their life cycle. Ciliates are typically active free-living grazers that generally have an oral cavity used during phagotrophy, although they can also perform pinocytosis and cell surface absorption (Corliss 1979; Lynn 2008). Most ciliates that live in association with metazoans are commensals, but parasitic interactions are also clearly established for some species (e.g. Ewing and Kocan 1992). Moreover, ciliates hosting prokaryotes are widespread (for review, Fokin 2004; Görtz 2001) and these associations seem to be frequent in distinct environments, including hydrothermal vents (Kouris et al. 2007) and anoxic habitats (e.g. Ott et al. 2005). Prokaryotic symbionts may play nutritive and defensive roles, or may also be used to modify surrounding and intrasecal environmental conditions (for review, Gast et al. 2009).

Astome ciliates were first observed in 1788 (Müller 1788). However, their anatomy and habitat were first described during the 20$^{th}$ century (Cépède 1910; de Puytorac 1954). They are endocommensals living mainly in association with annelids, frogs, and toads (de Puytorac 1954). No free-living astome species have been described so far. Astomes are all mouthless, and they use a thigmotactic field, or specialized structures in the form of hooks, spines or suckers, to attach themselves to their host. As astomes do not seem to damage host tissues, it has been suggested that the intestine of their host could represent a stable ecological niche that concentrates specific nutrients (de Puytorac 1954). However, their ecology is still poorly understood. Based upon morphological criteria, astomes are commonly placed within the subclass Astomatia in the class Oligohymenophorea (de Puytorac 1994;

Jankowski 2007; Lynn 2008). Jankowski (2007) and de Puytorac (1994) further divide the subclass into three orders – Hoplitophryida, Anoplophryida, and Haptophryida – while Lynn (2008) maintains a single order Astomatida with its included families. The analysis of a small subunit (SSU) rRNA gene sequence of *Anoplophrya marylandensis* Conklin, 1930, an astome retrieved from the intestine of a lumbricid annelid, confirmed their affiliation to the Oligohymenophorea (Affa'a et al. 2004). Nine other species of astomes from oligochaete hosts were sequenced subsequently confirming the monophyly of this subclass (Fokam et al. 2011).

The genus *Durchoniella* de Puytorac, 1954 includes four species (i.e. the type species *D. brasili*, *D. legeriduboscqui*, *D. cirratuli* and *D. dodecaceriae*), all retrieved from marine cirratulid polychaetes (Léger and Duboscq 1904; de Puytorac 1954; de Puytorac and Schrével 1965). The major criteria used for the identification of these species are the size and the general shape of cells, the number and arrangement of contractile vacuoles and the number of kineties. *Durchoniella brasili* (Léger and Duboscq, 1904) de Puytorac, 1954 and *D. legeriduboscqui* de Puytorac, 1954 are exclusively observed in the middle intestine of one host, *Cirriformia tentaculata* Montagu, 1808 (also commonly named *Audouinia tentaculata),* a widespread cirratulid polychaete along European coasts. This marine worm lives in sediments characterized by reduced and low-oxygen conditions (George 1964). *Cirriformia tentaculata* lives in the upper sediment layers and traps food particles falling onto the sediment surface with its tentacular filaments (Fauvel 1927). These filaments are also used to acquire oxygen. To avoid predation during low tide, worms bury themselves deeply into the sediment retracting their tentacles, encountering therefore chronic anoxic conditions (Bestwick et al. 1989).

The aim of the present study was to assess the phylogenetic position of *Durchoniella* species from the cirratulid polychaete *C. tentaculata*, morphologically identified based on the literature (Léger and Duboscq 1904; de Puytorac 1954; de Puytorac and Schrével 1965; Tchang 1931), and to re-investigate these species using light and transmission electron microscopy.

## Material and Methods

### Site description and sampling

Intestinal contents of *Cirriformia tentaculata*, collected between March 2009 and May 2010 from several different sites at Roscoff (Souris Island, SI; 03°59'W, 48°43'N; France), Plymouth and Portsmouth (04°9.80'W, 50°21.87'N and 01°1.90'W, 50°47.80'N; UK) were examined. Cirratulids were meticulously cleaned with 0.2 µm-filtered seawater by removing all grains of sand with forceps. Each worm was then placed individually in a 6-well plate filled with 10 mL filtered seawater and maintained up to eight weeks at room temperature with a daily seawater renewal without nutrient addition. Dissections were performed after worms were incubated for at least 30 min in 7% (w/v) $MgCl_2$ in filtered seawater, prepared at least 2 h in advance and stored at 4°C.

### Light, epifluorescence and electron microscopy

Living and fixed material extracted from worms were examined from freshly prepared slides and photographed with an Olympus BX51 epifluorescence microscope (Olympus Optical CO, Tokyo, Japan), coupled to a Spot RT-slider camera (Diagnostics Instruments, Sterling Heights, MI). From a 2 g/L stock solution of Neutral Red, cells were incubated 15 min in a 50 mg/L solution at room temperature, and rinsed twice with filtered-sterilized-filtered (F/S/F) seawater. DAPI colorations (0.225

µg/mL final concentration) were realized on glutaraldehyde (0.25% (v/v)) or paraformaldehyde (4% (w/v)) fixed cells in AF1 solution (Glycerol + PBS, CytiFluor, London, UK). For silver impregnation, cells were fixed in Champy's and then Da Fano's fluids (or glutaraldehyde for some cells) before staining as described elsewhere (Lynn et al. 1981). For scanning electron microscopy (SEM), cells were fixed for 4 h at room temperature in a fixative containing 4% (v/v) glutaraldehyde, 0.25 M sucrose, and 0.1 M sodium cacodylate pH 7.4. Samples were then rinsed in a series of buffer solutions containing graded concentrations of sucrose and post-fixed for 1 h at 4°C in 1.5% (w/v) $OsO_4$ in 0.2 M cacodylate pH 7.4 and 0.33 M NaCl. Dehydration was carried out in graded alcohol series and finally dried using a critical point dryer (CPD 030 Bal-Tec, Balzers, Lichtenstein). The dried cells were coated with gold in a sputter coater before observation with a JSM 5200 SEM (JEOL Ltd., Tokyo, Japan). The fixation process for transmission electron microscopy (TEM) was as described for SEM above. After dehydration, samples were then embedded in Epon™. Sections were made using a diamond knife on a Leica Ultracut UCT ultramicrotome (Leica Mikrosysteme GmbH, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and observed with a JEM 1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) or a Quanta 200 environmental SEM (FEI, Hillsboro, Oregon, USA).

**Bacterial FISH**

General probe Eub338 (Amann et al. 1990) was used to observe bacteria by in situ hybridization in both *Durchoniella* species. Non-sense probes (EUBN) were also used as negative controls and no signal was detected. All probes were synthesized with Cyanine-3 at the 5'-end (Thermo Fisher Scientific GmbH, Ulm, Germany). The probes, received in a lyophilized form, were dissolved in sterile deionised water

purified with the MilliQ system from Millipore (resistivity 18 MΩ) to a final concentration of 50 ng/µL and aliquots stored in the dark at −80°C.

Cells were individually collected with drawn glass pipette and placed on coated slides (Polysine® slides, Menzel GmbH, Brainschweig, Germany). Cells were fixed for 10 min in 4% (w/v) paraformaldehyde solution and dehydrated with 80% ethanol for 10 min. Cells were then incubated with proteinase K (5 µg/mL final concentration) and lysozyme (2 mg/mL final concentration) at 37°C for 1 h. Rinsing and pre-hybridization were performed for 10 min at 46°C in hybridization buffer (40% (v/v) formamide in 5X SSC, where 5X SSC = 83 mM NaCl, 83 mM sodium citrate, pH 7.0). Hybridizations with each fluorescent probe were performed on slides in a hybridization chamber (Evergreen Scientific, Los Angeles, USA) at 46°C for 10 min in 300 µL of hybridization buffer containing the probes at a final concentration of 5 ng/µL. After hybridization, cells were washed twice in a bath of 5X SSC at 48°C for 10 min. Slides were mounted in anti-fading reagent AF1 and stored at 4°C in the dark until they were observed.

**DNA extraction**

Before DNA extraction, worms were rinsed three times, all equipment (dissection tools, dissection boxes and jar) was incubated in DNA AWAY® (Molecular BioProducts™), ethanol, and sterilized for 30 min at 121°C. Single-cells of *Durchoniella* sp. were then isolated with sterilized drawn glass pipettes and rinsed individually three times in filtered/sterilized/filtered seawater. DNA of single-cells was extracted by a modified Guanidinium Isothiocyanate (GITC) protocol (Chomczynski and Sacchi 1987, 2006). Each fresh single-cell was ground in 50 µL of the GITC extraction buffer, and crushed with a tube-adapted piston pellet (Kimble Chase®) for at least one minute. Tubes were incubated for 20 min at 72°C, and quickly

centrifuged. One volume of cold isopropanol was added at -20°C overnight. After centrifugation for 15 min at 14,000 g at 4°C and one washing with 70% (v/v) ethanol, the DNA pellet was hydrated in sterile distilled water and stored at 4°C. The extraction product was used to amplify SSUrRNA genes.

**PCR and sequencing**

All PCR mixtures (30 µL final volume) contained 1X Taq DNA Polymerase buffer, 1 µL of dNTPs (20 mM each), 3 µL of $MgCl_2$ (25 mM), 0.1 µL of each primer (100 µM) and 0.25 µL of Taq DNA Polymerase (5 unit/µL, Promega®, Madison, Wisconsin). All amplifications were performed using a "GeneAmp PCR system" 9700® (Applied Biosystems™) or “MJ Mini Cycler” (Biorad™). Negative controls were also carried out with DNA extractions performed with no sample, with and without piston pellet. For all negative controls, no PCR products were detected.

Eukaryotic SSUrRNA genes were amplified with primers EukMK_63F/EukMK_1818R (5'-ACG CTT GTC TCA AAG ATT A-3'; 5'-ACG GAA ACC TTG TTA CGA-3'; M. Kawachi, unpublished) with the following conditions: one cycle of 5 min at 95°C, 30 cycles of 1 min at 95°C, 1.5 min at 57°C and 1.5 min at 72°C and one cycle of 10 min at 72°C. PCR cycles were as follows: one cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 49°C and 2 min at 72°C and one cycle of 6 min at 72°C. Three independent PCR products were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and cloned into *Escherichia coli* (One Shot F10’, Promega™) using the pCR2.1-TOPO TA vector system (Invitrogen®) following the manufacturer’s instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Positive PCR products were purified (ExoSAP-IT® For PCR Product Clean-Up, USB™) and sequenced in both directions with

specific primers using the Big Dye Terminator Cycle Sequencing Kit 3.0 (PE Biosystems™) and an ABI PRISM model 377 (version 3.3) automated sequencer.

**Phylogenetic analyses**

Sequences were edited in the BioEdit 7.0.5.3 program and aligned using CLUSTALW2 version 2.0.10 (Hall 1999; Larkin et al. 2007). To determine the first phylogenetic affiliation, each sequence was compared with sequences available in the NCBI database (National Center for Biotechnology Information) using BLAST (Altschul et al. 1990). For the Oligohymenophorea tree (Fig. 5), a general time-reversible (GTR+I+G) model with gamma distributed rates and a proportion of invariable sites was selected by hierarchical Likelihood Ratio Tests (hLRT), via jModeltest 0.1.1 (Posada 2008) and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by Maximum Likelihood (ML) and Bayesian inference (BI) (Posada and Crandall 1998). ML was conducted using PhyML 3.0 (Guindon et al. 2005) and the robustness of the inferred topology was tested by bootstrap resampling (100). BI was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) and started with a random tree, run for 356 000 generations in four chains (Standard deviation = 0.009) and a burn-in of 890 generations in order to ensure the use of only stable chains. Neighbour Joining (NJ) and Maximum of Parsimony (MP) trees were inferred using PAUP 4.0b10 via PaupUp graphical interface (Swofford 2000; Calendini and Martin 2005). The robustness of the inferred topologies was supported by bootstrap resampling (1000) with NJ and MP; values over 50% are shown on the tree. The ML trees gave the same topologies as the BI trees. Trees were visualised and labelled with TreeDyn (Chevenet et al. 2006).

### Voucher slides

Three Chatton-Lwoff silver-stained cell slides are deposited in the protist collection of the National Museum of Natural History, Paris, France. The reference numbers are: ZS123 for *Durchoniella legeriduboscqui* genotype Dld1-G12 (isolate 1, Glutharaldehyde tube 12); ZS124 for *Durchoniella legeriduboscqui* genotype Dld2-G13-1 (isolate 2, Glutharaldehyde tube 13-1); and ZS125 for *Durchoniella brasili* 5.

### Nucleotide sequence accession numbers

The SSU rRNA gene sequences are available from the GenBank database under the following accession numbers: FN998987 to FN999022.

## Results

### Identification of *Durchoniella* species retrieved from *Cirriformia tentaculata*

*Cirriformia tentaculata* specimens were retrieved from anoxic sediments (Fig. 1A), between 3 to 15 cm depth, from the bay of Roscoff, France (English Channel) or from the English coast near Plymouth and Portsmouth. The sediments were characterized by a black colour (Fig. 1B) and sometimes a strong hydrogen sulphide odour. Six 20-g samples from the sediments and the interstitial water surrounding the worms were analysed by light microscopy. Amoebae, copepods, eggs similar to those retrieved from *Cirriformia*, as well as unicellular cells were observed in the interstitial waters and in the sediment matrix. However, no free *Durchoniella*-like cells were identified.

*Durchoniella*-like cells from the host intestine fell into two morphotypes/genotypes that we were able to assign to previously described species, and a third morphotype/genotype for which we have been unable to obtain sufficient cells for morphometric analysis. Cells were observed in the middle intestine of *C. tentaculata*, not fixed to the epithelial tissue of the host and were always located around the

alimentary bolus (Fig. 1C, D). After dissection, a large number of vigorously swimming ciliated cells of distinct sizes was usually observed. Swimming behaviour of these ciliates was clearly different, the largest cells swimming faster than the smallest ones. However, some cells remained stuck in a viscous gel-like liquid.

***Durchoniella brasili* (Léger and Duboscq, 1904) de Puytorac, 1954 (Figs. 2A-G, 3A-J, 4D-E; Table 1)**. It was the smallest species described so far and was often truncated or rounded at the posterior end (Fig. 2A, B, E; Fig. 4D). Based on 34 living cells, the somatic length ranged in vivo between 75-170 µm, the somatic width between 29-103 µm. The number of contractile vacuoles on 20 cells ranged between 2-6 (Table 1).

The elongated uneven macronucleus has extensions in glutaraldehyde-fixed material that appeared to be part of an external layer that surrounds an elongate and smooth inner region (Figs. 2A, 3A). The macronucleus was between 65-144 µm in length and 10-33 µm in width on 12 living cells (Table 1).

The total number of somatic kineties on fixed cells ranged between 65-81 and 61-78 respectively on glutaraldehyde (n=12) and Champy's-DaFano's (n=16) fixed material (Table 1). It was however difficult to accurately count the number of lateral kineties on silver-stained cells.

In the few cells where the anterior suture region was visible, it had a pattern of four points with the right ventrolateral one being the most prominent (Figs. 2B, 4D-E). The anterior skeletal structure was a large pyramid-shaped structure with four basal extensions and a non-chitinous papilla at the anterior end of the cell (Fig. 2B, F; Fig. 4D-E).

***Durchoniella legeriduboscqui* de Puytorac, 1954 (Figs. 2A-G, 3A-J, 4A-C; Table 1)**. Based on 21 living cells, the somatic length ranged in vivo between 180-350 µm,

the somatic width between 75-90 µm. The number of contractile vacuoles on 15 cells ranged between 6-15 (Table 1).

The elongated uneven macronucleus has extensions in glutaraldehyde-fixed material that appeared to be part of an external layer that surrounds an elongate and smooth inner region (Figs. 2C, 3B). The macronucleus was between 277-340 µm in length and 49-97 µm in width on 6 living cells (Table 1).

The total number of somatic kineties on fixed cells ranged between 97-121 and 103-128 respectively on glutaraldehyde (n=46) and Champy's-DaFano's (n=8) fixed material (Table 1). As noted above, we cannot assume that our estimates are accurate as there is some uncertainty in counting the numbers of “lateral” kineties. We report a minimal number of somatic kineties as 97–128 when we could count those on the entire cell; if we double the number of inferior kineties, which is roughly the relation between inferior or ventral and superior or dorsal kinety numbers, our range is 96–134 (Table 1).

In cells where the anterior suture region was visible, it had a pattern of three points with the right ventrolateral one being the most prominent (Fig. 2D, 4A-C). The anterior skeletal structure was a large tripod-shaped structure with three basal extensions and a non-chitinous papilla at the anterior end of the cell (Fig. 2D, G; Fig. 4B).

In both species*, v*egetative divisions took place by binary fission (*D. brasili*, Fig. 3C, D; *D. legeriduboscqui*, Fig. 3E, F). However, only micronuclei already divided and macronuclei under elongation were observed (Fig. 3D, F). Conjugation was only rarely observed in *D. brasili*, once in more than 20,000 *Durchoniella* cells analysed over more than a year (Fig. 3G-J). Two pairing cells were observed, still motile, of equal size and attached at the same level by their apical extremities, forming an

acute angle (Fig. 3G-I). At this stage, four micronuclei per cell were identified by DAPI staining (Fig. 3J).

**Phylogenetic analyses of *Durchoniella* species based on Small Subunit (SSU) rRNA gene sequences**

From 60 *C. tentaculata* worms dissected, the total DNA was extracted from 115 ciliate individuals and the SSUrRNA gene was directly sequenced after PCR amplification. We assigned 74 of them to the morphotype corresponding to the smallest *Durchoniella* morphotype. This was supported by SSUrRNA sequences that differed by less than 0.4% (based on 1626 bp) among the most divergent isolates (representative sequence FN998990, Fig. 5). The remaining 41 cells, assigned to *D. legeriduboscqui* based on somatic length and shape, were collected from Plymouth, Portsmouth, and Roscoff (Table 1). The SSUrRNA sequences of six isolates morphometrically characterized from these localities were identical to the SSUrRNA sequences of 27 other cells (based on 1626 bp and represented by sequence FN998995, Fig. 5). Since it was the most common morphotype and genotype of the larger *Durchoniella*-like cells, we assigned these 33 cells to *D. legeriduboscqui*, the only other large *Durchoniella* species from *C. tentaculata* previously described to have one row of contractile vacuoles. The remaining eight cells were assigned to *Durchoniella* sp. The SSUrRNA sequences of these cells (represented by sequence FN998993, Fig. 5) differed by 2.8% from sequences of *D. legeriduboscqui* (represented by sequence FN998995, Fig. 5). They morphologically resembled *D. legeriduboscqui* based on cell size, cell shape, and numbers of contractile vacuoles, which were also distributed in a single row.

Using ML and BI methods, *D. brasili*, *D. legeriduboscqui*, and *Durchoniella* sp. were shown to form a monophyletic clade in the subclass Astomatia, and this clade is

sister to the clade formed by the ten other astome sequences retrieved from the intestinal contents of oligochaete annelids (Fokam et al. 2011) (Fig. 5). The relationship between all of these astome species was supported by a posterior probability of 1.0, ML bootstrap of 94%, NJ bootstrap of 100%, and MP bootstrap of 93%. They formed a cluster associated with the subclass Scuticociliatia within the class Oligohymenophorea, a position supported by a posterior probability of 0.98, ML bootstrap of 92%, NJ bootstrap of 93%, and MP bootstrap of 65% (Fig. 5).

***Microscopic evidence of endocytoplasmic bacteria***

Transmission electron microscopy (TEM) revealed numerous prokaryotic-like bodies that were not observed using scanning electron microscopy (SEM), light microscopy or differential interference contrast (DIC) microscopy. Fluorescence in situ hybridization (FISH) analysis using a general bacterial probe (EUB338) confirmed the presence of these bacteria in the cytoplasm of all *D. brasili* and *D. legeriduboscqui* (Fig 6A, B). No positive signal was observed using the general archaeal probe ARCH915. These numerous endocytoplasmic bacteria were freely distributed throughout the cytoplasm of the *Durchoniella* cells (Fig. 6C). Although bacteria were not incorporated in food vacuoles or in association with nuclei or other eukaryote organelles, all the bacteria observed by TEM were surrounded by an electron-translucent halo delimited by a very thin membrane (Fig. 6D, white and black arrow; Fig. 6E, black arrowheads). All bacteria observed had two membranes (Fig. 6D, black arrows), contained granular cytoplasm and nucleoid-like structures (Fig. 6D-G). Rod-shaped and coccoid bacteria, 2-3 µm long and 0.5-0.8 µm of diameter, were observed by TEM (Fig. 6D-G) and by FISH (Fig 6A, B). Division of these endocytoplasmic bacteria was suggested by TEM observations (Fig. 6G). Bacteria were not motile inside *Durchoniella* cells and no flagellum was observed.

## Discussion

### *Durchoniella* species

The somatic length, somatic width, and the number of contractile vacuoles of our isolates identified as *D. brasili* and *D. legeriduboscqui* fell within or overlapped the ranges provided by de Puytorac (1954) but never reached the maximum length observed by Léger and Duboscq (1904) for *D. brasili* (Table 1). The macronucleus width again overlapped the range provided by de Puytorac (1954). Our observations on the anterior skeletal structures on both species were consistent with the description of this author.

In the case of *D. brasili*, the number of somatic kineties was also similar to that provided by de Puytorac (1954), but corresponded to half the number reported by Léger and Duboscq (1904) or Tchang (1931) (Table 1). Like *A. brasili*, which was named after colleague L. Brasil, *D. legeriduboscqui* de Puytorac, 1954 was initially assigned to the genus *Anoplophrya* by Léger and Duboscq (1904). Tchang (1931) recollected what he called *A. brasili* in France, in the same region as in 1904, and, using Chatton-Lwoff silver impregnation, redescribed it with the same genus assignment as Léger and Duboscq (1904). De Puytorac (1954) based his analysis of *D. legeriduboscqui* on this latter redescription along with his own observations. In our case, the number of somatic kineties never reached the number of 138–140 provided by de Puytorac (1954), Léger and Duboscq (1904), and Tchang (1931) (Table 1). However, these authors provided no evidence of variability in this feature nor reported how many cells were measured.

### Systematic position of astomes

*Durchoniella brasili* and *D. legeriduboscqui* were placed by de Puytorac (1994) within the class Oligohymenophorea, subclass Astomatia, order Hoplitophryida, family

Radiophryidae, and subfamily Durchoniellinae de Puytorac, 1972, a classification supported by Jankowski (2007) down to the family level. This systematic position of astomes is still controversial, as de Puytorac considered the group polyphyletic (de Puytorac 1954, 1994; de Puytorac et al. 1979), whereas Lynn (2008) classified all astomes within the order Astomatida. The common presence of a thigmotactic field and an apical structure in astomes suggests a common ancestry with the Hysterocinetidae (de Puytorac 1994; Lynn 2008; Ngassam et al. 1994). However, our phylogenetic analyses with two thigmotrich sequences (i.e. Hemispeiridae and Ancistridae; Gao et al. 2010, 2012; Fig. 5) demonstrated that thigmotrichs and astomes are not sister taxa as both thigmotrich sequences clustered robustly within the Scuticociliatia.

The first phylogenetic study of a SSUrRNA gene sequence retrieved from an astome suggested that this group belonged to the class Oligohymenophorea, and placed astomes sister to the Scuticociliatia (Affa'a et al. 2004). The analysis of nine other SSUrRNA gene sequences from astomes associated with terrestrial oligochaetes confirmed this placement and additionally suggested the monophyly of the subclass Astomatia. The tree topology in the present analysis supported, with high statistical confidence levels, the monophyly of *D. brasili*, *D. legeriduboscqui*, and *Durchoniella* sp. with all other members of Astomatia within the class Oligohymenophorea. *Durchoniella* SSUrRNA gene sequences grouped, however, in a monophyletic clade with robust statistical support from the other available sequences of astomes that are assigned to the families Radiophryidae (*Eudrilophrya*, *Metarocoelophrya*, *Metaradiophrya*), Anoplophryidae (*Almophrya*, *Anoplophrya*, *Njinella*) and Clausilocolidae (*Paraclausilocola*). This result suggests that Durchoniellinae is not a subfamily of the Radiophryidae, but could represent a novel order within de

Puytorac's classification, or a family within Lynn's classification. As SSUrRNA gene sequences of hysterocinetid ciliates are not available, it is still not possible to decide conclusively on the phylogenetic origin of astomes. Presuming astome genera and species have been identified correctly, the SSUrRNA gene sequences from *D. brasili* and *D. legeriduboscqui* grouped into distinct clusters (data not shown), confirming the initial morphological diagnosis of two distinct species. Given the genetic similarity of these two *Durchoniella* morphospecies, we believe the eight diverging sequences belong to the genus *Durchoniella* probably as a new species, but more evidence should be provided for its characterization.

**A third "Russian nesting doll": the bacteria**

Endosymbiotic bacteria in astomes was first reported in specimens retrieved from the oligochaete *Lumbriculus variegatus* (Hovasse 1945), and subsequent research showed that several astomes also harbour bacteria (Hovasse 1946). This endosymbiotic association does not seem to be required for all astomes as several species are free from bacteria, such as *Spirobuetschiella chattoni* and *Cepedella hepatica* (Hovasse 1946). Bacteria were not previously observed in *Durchoniella* from *C. tentaculata* sampled at Roscoff using an acid fuchsin-azure II stain (Hovasse 1946), whereas we observed endocytoplasmic bacteria in *D. brasili* and also in *D. legeriduboscqui* by combining FISH staining and transmission electron microscopy.

Microscopic observation using DAPI showed that the endocytoplasmic bacteria were equally distributed in the *Durchoniella* cells during conjugation (data not shown). The same observation was also made during binary fission using SYBR green staining and FISH analysis (data not shown), suggesting that bacterial symbionts are acquired by vertical transmission. However, further molecular genetic analyses of the biogeographic distribution would be required to verify this particular point. While the

ecology of this association remains to be explored, these endocytoplasmic bacteria may play a role in the association between endocommensal astome ciliates and cirratulid polychaetes inhabiting in anoxic coastal sediments.

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## Captions

**Fig. 1A-D.** Localisation of species of the astome ciliate *Durchoniella* from the cirratulid polychaete *Cirriformia tentaculata*. **(A)** *Cirriformia tentaculata*, with “a”, the anterior part and “p”, the posterior part. Scale bar = 1 cm. **(B)** Example of a sampling site at Roscoff. Scale bar = 25 cm. **(C, D)** Semi-thin transverse sections of *C. tentaculata* with dorsal (d) and ventral (v) sides indicated. The wall of the digestive tract is represented by the black arrows; the gut of the worm in C is empty (white arrow). Scale bars = 100 µm. Longitudinal section of a specimen of *Durchoniella brasili* (top) and *D. legeriduboscqui* (bottom) were enlarged in parts of semi-thin sections. Scale bars = 20 µm.

**Fig. 2A-G.** *Durchoniella brasili* **(A, B, E, F)** and *Durchoniella legeriduboscqui* **(C, D, E, G)**. DIC of living specimens **(A, C)**, after Chatton-Lwoff silver preparation **(B, D)**, and in SEM **(E-G)** with a zoom on fixative apparatus of the anterior end **(F-G)**. Mi: Micronucleus; Ma: macronucleus; CV: Contractile vacuoles, revealed with neutral red staining in A. Bars: 20 µm (A), 70 µm (B), 50 µm (C–E), 10 µm (F), 5 µm (G).

**Fig. 3A-J.** *Durchoniella brasili* **(A, C, D, G, H, I, J)** ,and *Durchoniella legeriduboscqui* **(B, E, F)**. Drawings of macronuclei based on silver impregnation **(A, B)**. DIC Nomarski observation (with DAPI coloration) during binary fission, arrows indicate fission furrow **(C-F)**. Conjugation process, with zoom on conjugation junction and nuclear content of the two conjugants **(G-J)**. Bars: 30 µm (A), 50 µm (B), 20µm (C-G, J), 10 µm (H-I).

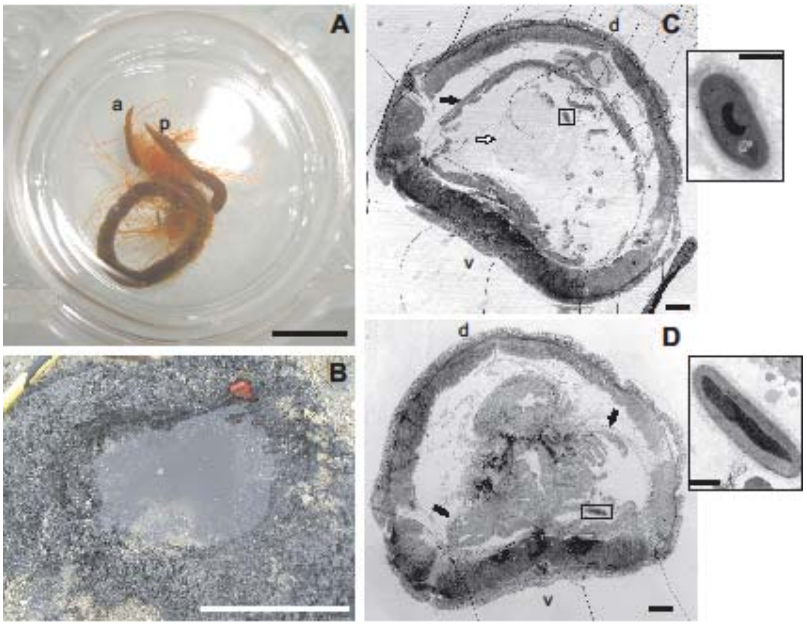
**Fig. 4A-E.** Micrographs of Chatton-Lwoff silver-stained *Durchoniella legeriduboscqui* **(A-C)** and *Durchoniella brasili* **(D-E)** isolated from the intestine of the cirratulid polychaete *Cirriformia tentaculata* at Roscoff (France). Ventral views **(A, D)**. Anterior

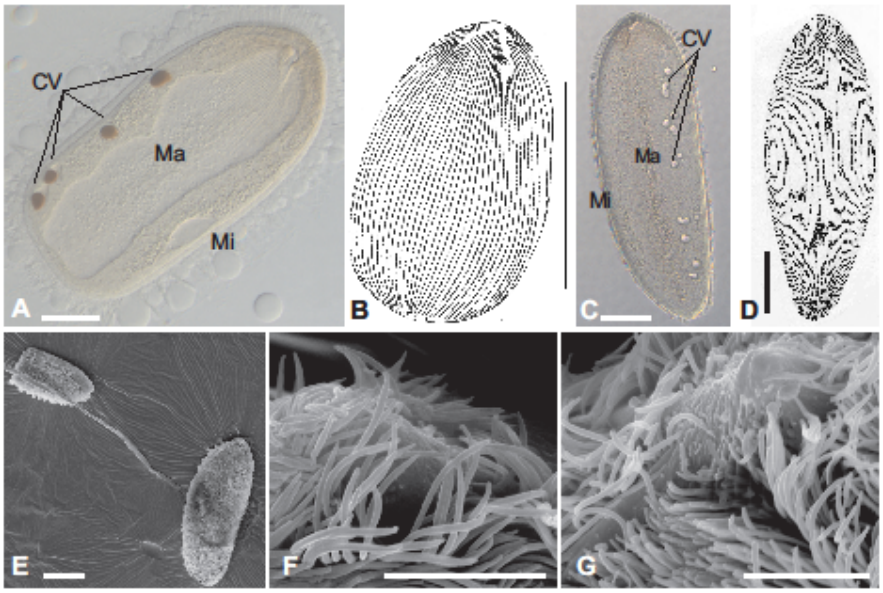
secant systems **(B, E)**. Posterior secant system **(C)**. Bars: 50 µm (A), 10 µm (B, C, E), 65 µm (D).

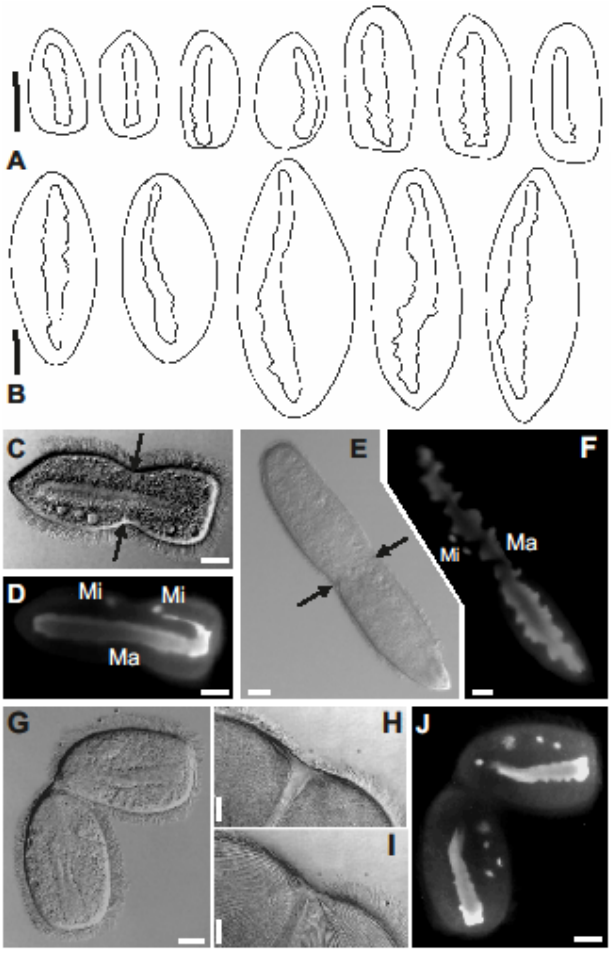
**Fig. 5.** Bayesian analysis of the small subunit rRNA gene sequences of representatives of the class Oligohymenophorea. *Durchoniella* sequences are in bold. Main haplotypes retrieved from *Durchoniella* species are represented by one sequence sharing ≥99% similarity with all other sequences. Each astome isolate was named with the initial of the city (R for Roscoff), the initial and the number of the site (SI for Souris Island), the worm number (Iy), and the initial and the number of the *Durchoniella* species (Db: *D. brasili*, Dl: *D. legeriduboscqui* and D: *Durchoniella* sp.). Maximum Likelihood bootstrap, posterior probability of Bayesian method, and Maximum Parsimony and Neighbour-Joining bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, MP, NJ). White dots correspond to values between ≥80%. Outgroup sequences, not shown, were three ciliophoran sequences (Phyllopharyngea, AY378112; Colpodea, M97908; and Karyorelictea, GQ16715).

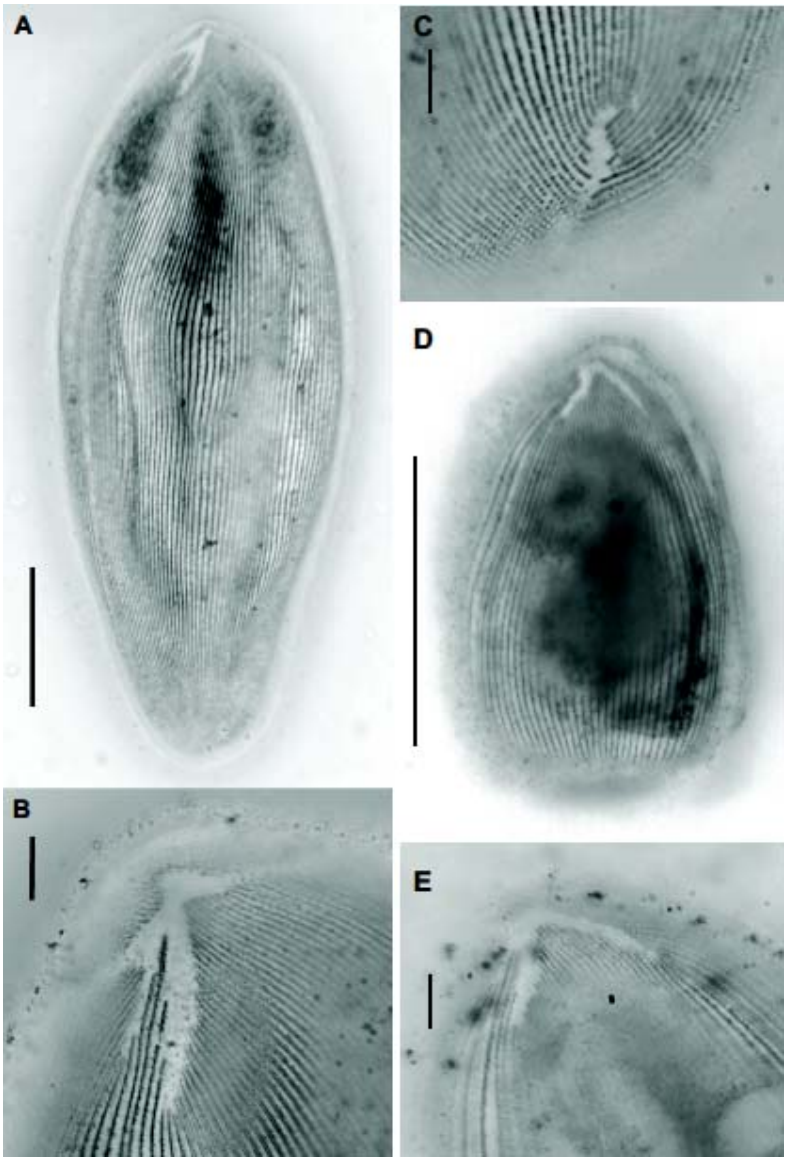
**Fig. 6A-G.** Epifluoresence **(A-B)** and transmission electron microscopy micrographs **(C-G)** of endocytoplasmic bacteria of *Durchoniella* species. Results of FISH with EUB338 general bacterial probe on *D. brasili* **(A)** and *D. legeriduboscqui* **(B)** showing bacteria most abundant in the ectoplasm of the host astomes. **(C)** Inset of an entire cell (scale bar = 5 µm) shows where the zoom was made. Note the numerous bacteria. **(D)** Single bacterium (scale bar = 200 nm) with an enlarged view of the boxed region, CM: inner cytoplasmic membrane, CW: outer envelope (black arrows), and translucent halo membrane (white and black arrow). **(E)** Detail of coccoid bacteria. Note the electron-translucent halo around bacteria (arrowheads). **(F)** Rod-

shaped bacterium. **(G)** Possibly dividing cell of endocytoplasmic bacterium. Bars: 10 µm (A), 20 µm (B), 500 nm (C), 100 µm (D), 200 nm (E, F, G)







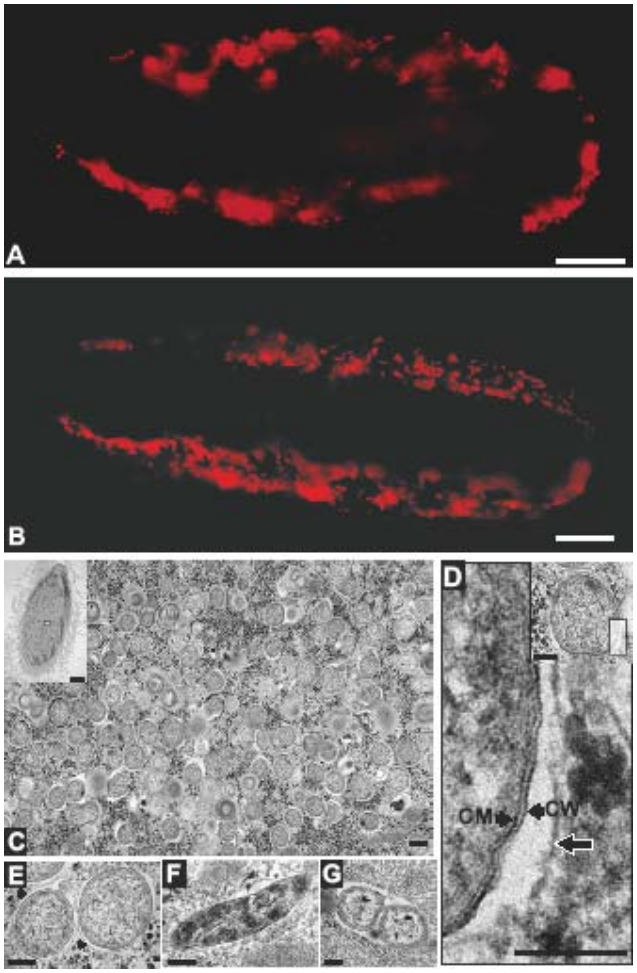


**Table 1.** Morphometric characterization of *Durchoniella* species from the intestine of the cirratulid polychaete *Cirriformia tentaculata* in the present study compared to those presented by Léger and Duboscq (1904) and de Puytorac (1954). Measurements of lengths and widths are given in micrometres with mean ± standard deviation followed by range and sample size, where known.

| ***Durchoniella*** **species** | **Treatment** | **Somatic** | | **Macronuclear** | | **Kineties** | | **No. contractile** | **Source** |
|---|---|---|---|---|---|---|---|---|---|
| | | **Length** | **Width** | **Length** | **Width** | **No. Inferior** | **No. Total** | **vacuoles** | **(location)** |
| ***D. brasili*** | Living | 80-450 | ND | ND | ND | 70 | ND | 4-5 | Léger & Duboscq (1904) (Calvados, France) |
| | Living | 90–195 | 60–70 | ND | 10–20 | ND | 78 (n=7) | 3–5 | de Puytorac (1954) (Luc-sur-Mer, France) |
| | Living | 114±26.2 (75–170; 34) | 51±18.4 (29–103; 34) | 96±22.1 (65–144; 12) | 18±9.4 (10–33; 12) | ND | ND | 2–6 (n=20) | This study (Roscoff, France) |
| | Glutaraldehyde fixed C&L[a] | 110±10.3 (86–124; 12) | 43±9.3 (38–51; 12) | 82±1.8 (59–95; 12) | 12±1.8 (11–16; 12) | 36±2.7 (33–41; 12) | 70±4.1 (65–81; 12) | 2–6 (n=5)[b] | This study (Roscoff, France) |
| | Champy's-DaFano's fixed C&L[a] | 101±13.9 (76–136; 33) | 58±9.9 (41–80; 33) | 78±12.8 (61–110; 19) | 15.5±2.0 (13–23; 19) | 39±3.3 (32–45; 24) | 73±4.0 (61–78; 16) | ND | This study (Roscoff, France) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***D. legeri*** | Living | 180–350 | 75–90 | ND | ND | ND | 138–140 | 6–14 | de Puytorac (1954) |
| ***duboscqui*** | | | | | | | | | (Luc-sur-Mer, France) |
| | Living | 314±52.5 | 119±36.4 | 314±20.8 | 75±19.8 | ND | ND | 6–15 | This study |
| | | (230–400; 21) | (68–183; 21) | (277–340; 6) | (49–97; 6) | | | (n=15) | (Roscoff, France) |
| | Glutaraldehyde | 256±53.2 | 74±7.4 | 228±52.5 | 25±4.8 | 53±2.5 | 107±5.1 | 8–13 | This study |
| | fixed C&L[a] | (182–369; 46) | (55–94; 46) | (154–338; 46) | (11–33; 46) | (48–61; 46) | (97–121; 46) | (n=16)[b] | (Pl/Po/R[c]) |
| | Champy's-DaFano's | 277±46.7 | 111±19.9 | 250±49.7 | 29±6.3 | 57±4.3 | 115±8.5 | ND | This study |
| | fixed C&L[a] | (198–358; 31) | (72–171; 31) | (182–336; 16) | (17–44; 16) | (49–67; 29) | (103–128; 8) | | (Roscoff, France) |

[a]Chatton-Lwoff silver stain; [b]The numbers of contractile vacuoles counted in fixed cells can only be considered a minimal estimate; [c]Plymouth, England/Portsmouth, England/ Roscoff, France. ND: Not Determined

**Table 1.** Morphometric characterization of *Durchoniella* species from the intestine of the cirratulid polychaete *Cirriformia tentaculata* in the present study compared to those presented by Léger and Duboscq (1904) and de Puytorac (1954). Measurements of lengths and widths are given in micrometres with mean ± standard deviation followed by range and sample size, where known.

| ***Durchoniella* species** | **Treatment** | **Somatic** | | **Macronuclear** | | **Kineties** | | **No. contractile vacuoles** | **Source (location)** |
|---|---|---|---|---|---|---|---|---|---|
| | | **Length** | **Width** | **Length** | **Width** | **No. Inferior** | **No. Total** | | |
| ***D. brasili*** | Living | 80-450 | ND | ND | ND | 70 | ND | 4-5 | Léger & Duboscq (1904) (Calvados, France) |
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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | fixed C&L[a] | | | | | | | | |
| ***D. legeri duboscqui*** | Living | 180–350 | 75–90 | ND | ND | ND | 138–140 | 6–14 | de Puytorac (1954) (Luc-sur-Mer, France) |
| | Living | 314±52.5 (230–400; 21) | 119±36.4 (68–183; 21) | 314±20.8 (277–340; 6) | 75±19.8 (49–97; 6) | ND | ND | 6–15 (n=15) | This study (Roscoff, France) |
| | Glutaraldehyde fixed C&L[a] | 256±53.2 (182–369; 46) | 74±7.4 (55–94; 46) | 228±52.5 (154–338; 46) | 25±4.8 (11–33; 46) | 53±2.5 (48–61; 46) | 107±5.1 (97–121; 46) | 8–13 (n=16)[b] | This study (Pl/Po/R[c]) |
| | Champy's-DaFano's fixed C&L[a] | 277±46.7 (198–358; 31) | 111±19.9 (72–171; 31) | 250±49.7 (182–336; 16) | 29±6.3 (17–44; 16) | 57±4.3 (49–67; 29) | 115±8.5 (103–128; 8) | ND | This study (Roscoff, France) |

[a]Chatton-Lwoff silver stain; [b]The numbers of contractile vacuoles counted in fixed cells can only be considered a minimal estimate; [c]Plymouth, England/Portsmouth, England/ Roscoff, France. ND: Not Determined