environmental microbiology

Environmental Microbiology (2010) doi:10.1111/j.1462-2920.2010.02320.x

Solenicola setigera is the first characterized member of the abundant and cosmopolitan uncultured marine stramenopile group MAST-3

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Summary

Culture-independent molecular methods based on the amplification, cloning and sequencing of small-subunit (SSU) rRNA genes are a powerful tool to study the diversity of prokaryotic and eukaryotic microorganisms for which morphological features are not conspicuous. In recent years, molecular data from environmental surveys have revealed several clades of protists lacking cultured and/or described members. Among them are various clades of marine stramenopiles (heterokonts), which are thought to play an essential ecological role as grazers, being abundant and distributed in oceans worldwide. In this work, we show that *Solenicola setigera*, a distinctive widespread colonial marine protist, is a member of the environmental clade MArine STramenopile 3 (MAST-3). *Solenicola* is generally considered as a parasite or an epiphyte of the diatom *Leptocylindrus mediterraneus*. So far, the ultrastructural, morphological and ecological data available were insufficient to elucidate its phylogenetic position, even at the division or class level. We determined SSU rRNA gene sequences of *S. setigera* specimens sampled from different locations and seasons in the type locality, the Gulf of Lions, France. They were closely related, though not identical, which, together with morphological differences under electron microscopy, suggest the occurrence of several species. *Solenicola* sequences were well nested within the MAST-3 clade in phylogenetic trees. Since *Solenicola* is the first identified member of this abundant marine clade, we propose the name Solenicolida for the MAST-3 phylogenetic group.

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Introduction

Since the beginning of the century, molecular approaches based on the amplification, cloning and sequencing of eukaryotic small-subunit (SSU) rRNA genes from oceanic samples have revealed a wide diversity of protist lineages, particularly in the smallest planktonic fractions (Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001). A wealth of molecular diversity studies of eukaryotic plankton using general or specific primers targeting particular groups has been carried out in different oceanic regions, latitudes and depths. While many of the SSU rDNA sequences identified cluster with known eukaryotic lineages, a fraction of sequences form separate clades that do not contain cultured members or species properly described taxonomically (Moreira and López-García, 2002; Massana and Pedrós-Alió, 2008). Furthermore, some of these clades are abundant in gene libraries, suggesting that they might be important players in marine ecology. Certain lineages detected by environmental sequencing may be novel to science. This is the case of the recently described Picobiliphyta (Not *et al*., 2007; Cuvelier *et al*., 2008). However, in most situations, orphan environmental lineages are simply awaiting their assignment to already described taxa for which sequences are not yet available. Bridging the gap between environmental surveys and classical protistology is an urgent task, since it would allow coupling a vast body of classical knowledge on cell biology, lifestyle and ecology of organisms with easily accessible SSU rRNA-based monitoring. A good example is that of the marine alveolates Groups I and II, which were found to be very abundant, or even dominant, in many planktonic SSU rDNA libraries, from surface waters to the deep sea (López-García *et al*., 2001; Moon-van der Staay *et al*., 2001; Massana and Pedrós-Alió, 2008; Not *et al*., 2009; Scheckenbach *et al*., 2009). It turned out that both marine

alveolate groups corresponded to well-known parasitic lineages related to the dinoflagellates, Group I to the Duboscquellida (Harada *et al*., 2007) and Group II to the Syndiniales (Gunderson *et al*., 1999; Moreira and López-García, 2002; Guillou *et al*., 2008), which highlights the importance of parasitism in the marine ecosystem, particularly in the deep sea (Moreira and López-García, 2003; Scheckenbach *et al*., 2009).

Apart from the alveolates, other planktonic oceanic lineages detected abundantly in SSU rRNA gene libraries remain to be associated to described organisms. Among them are several clades of stramenopiles (heterokonts), whose sequences branch scattered among heterotrophic stramenopile taxa in phylogenetic trees. Heterotrophic pico- and nanoflagellates are essential players in aquatic food webs as grazers of bacteria and photosynthetic prokaryotes and picoeukaryotes (Patterson, 1989; Arndt *et al*., 2000). Unfortunately, most of them are not culturable and their small size and delicacy render morphological studies difficult. MArine STramenopiles (MAST) sequences are particularly frequent in clone libraries of surface marine picoplankton, where they typically account for approximately 20% of all clones and up to 50% of sequences from uncultured groups (Massana *et al*., 2004; Not *et al*., 2009). Massana and colleagues (2004) defined groups MAST-1 to -12 based on phylogenetic analyses of environmental sequences. Two of these lineages (MAST-3 and -4) were shown to correspond to bacterivorous heterotrophic flagellates locally abundant in a coastal Mediterranean system (Massana *et al*., 2002). MAST-3 sequences formed the largest cluster, which was also highly diverse, and appeared in open sea and coasts from all the world ocean regions studied (Massana *et al*., 2004). Their high proportions on reverse transcribed SSU rRNA libraries suggest that MAST-3 flagellates are not only very abundant but also very active (Not *et al*., 2009).

In contrast to other small pelagic protists, *Solenicola setigera* is known since the earliest studies on marine plankton (Gran, 1904). They are small, 4–7 μm in diameter, but they form colonies on siliceous tubes that facilitate the identification during routine phytoplankton analysis. Pavillard (1916) described *S. setigera* from the coastal NW Mediterranean Sea and the siliceous tube was identified as the diatom *Leptocylindrus* (= *Dactyliosolen*) *mediterraneus* (Peragallo) Hasle (Hasle, 1975; Hasle and Syvertsen, 1997). The consortium of *Solenicola–Leptocylindrus* is ubiquitous in the world's oceans from equatorial to Arctic and Antarctic waters, even under the ice (Fryxell, 1989; Gómez, 2007). Despite the widespread distribution of the *Solenicola–Leptocylindrus* consortia, both the actual nature of their constituents and the phylogenetic position of *Solenicola* have been the subject of controversy. *Solenicola* has been interpreted as a parasite or epiphyte of the chain-forming diatom *L. mediterraneus* (Taylor, 1982), which remains the most extended view. However, *Solenicola* has also been interpreted as a stage in the life cycle of the diatom (Margalef, 1969) and, finally, as a colonial nanoflagellate grazer able to build and control the growth of its own siliceous substrate (Gómez, 2007). Furthermore, observations of North Atlantic *Solenicola* colonies by epifluorescence and scanning and transmission electron microscopies revealed the presence of *Synechococcus*-like cyanobacteria, as well as other picoeukaryotes attached to the mucous surface and in vacuoles. This led to the conclusion that this consortium was a three-partner association with a centric chain-forming diatom, *Solenicola* and a nitrogen-fixing *Synechococcus* sp., the association with the latter being mutually beneficial (Buck and Bentham, 1998). In any case, although the morphology of *Solenicola* was studied in some detail by light and electron microscopy, these observations were insufficient to elucidate its phylogenetic position even at the class or division level. Even the occurrence of the flagellum remained doubtful (Patterson and Zölffel, 1991). Thus, *S. setigera*, initially considered a member of the Xanthophyceae, was considered in subsequent revisions a heterotrophic protist *incertae sedis* (Patterson and Zölffel, 1991).

In this study, we obtained SSU rRNA gene sequences for three independent colonies of *S. setigera* collected in the Mediterranean Sea at two different locations (Marseille and Banyuls sur Mer) and seasons. Phylogenetic analysis shows that *Solenicola* sequences were closely related, branching together within the stramenopile clade MAST-3. We tentatively propose the name of Solenicolida for the MAST-3 cluster in reference to *Solenicola*, the described genus that is thus first ascribed to this clade of environmental sequences.

Results and discussion

With the aim of establishing the phylogenetic position of the *incertae sedis* colonial protist *S. setigera*, we collected individual colonies in the Mediterranean Sea. We then studied their morphology by optical and scanning electron microscopy to confirm their identity with the described *Solenicola* species and determined their SSU rRNA gene sequence in order to carry out detailed molecular phylogenetic analyses, as described in the following.

Light microscopy observations and collection of Solenicola *colonies*

Colonies of *S. setigera* were sporadically found along the entire year in its type locality, the Gulf of Lions, NW Mediterranean Sea. For a same sampling effort, the highest abundances were observed in November–December and

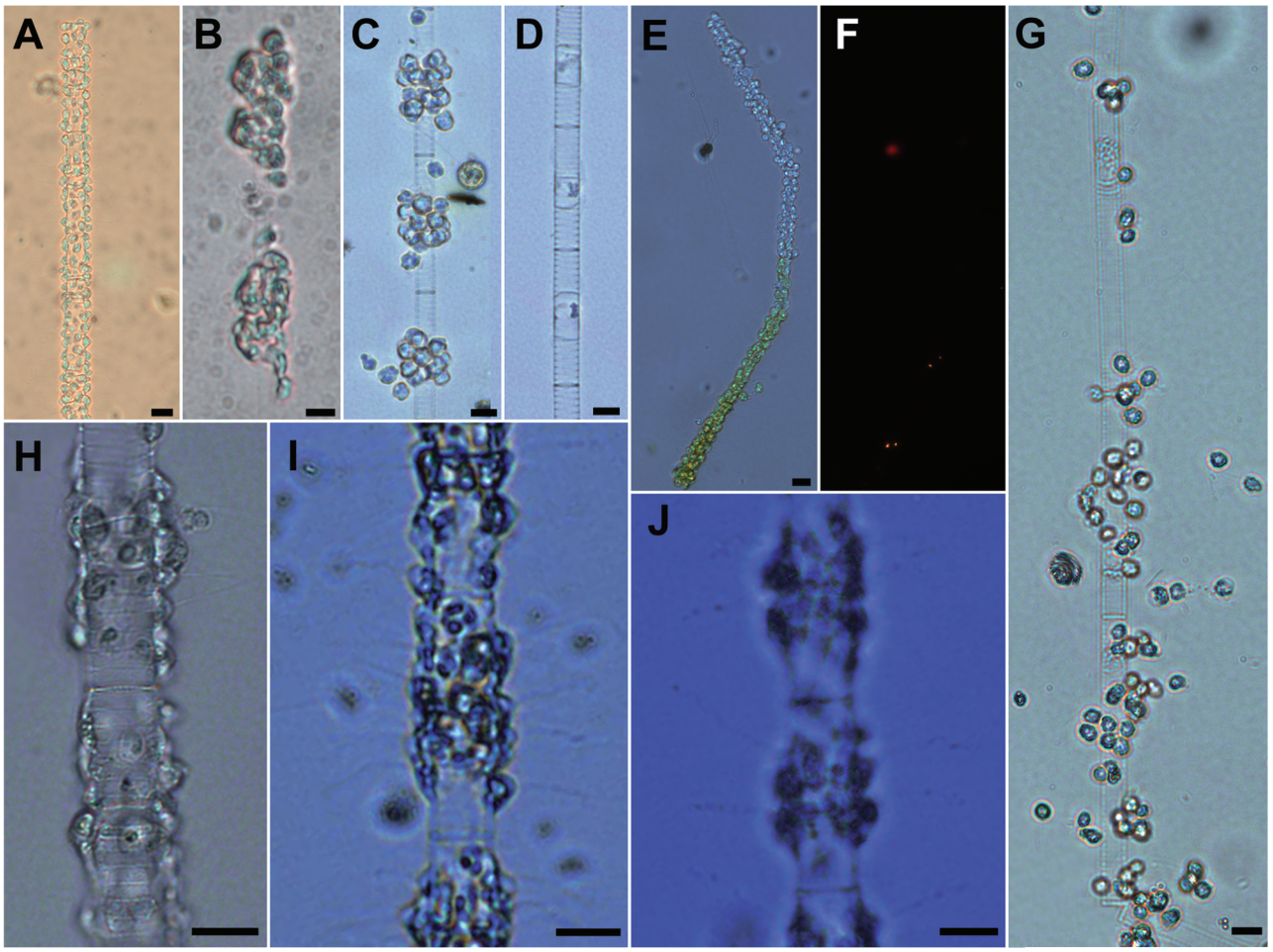

Fig. 1. Light micrographs of live colonies of *Solenicola setigera*.
A–D. The colonies collected for SSU rDNA analysis were (A) *S. setigera* FG11 from Marseille, winter, (B) *S. setigera* FG174 from Marseille, spring, and (C and D) *S. setigera* FG711 from Banyuls, spring. (D) Siliceous tube after the detachment of *Solenicola*.
E and F. Colony partially covered of an unusual greenish pigmentation (see also Video S2). (F) Note the spots of orange-yellow fluorescence under green light excitation, tentatively ingested cyanobacteria or photosynthetic picoeukaryotes. For comparison, the bigger red spot corresponds to a diatom (*Chaetoceros*). *Solenicola* cells did not show chlorophyll *a* autofluorescence when excited by either blue or green light.
G. Individuals of *Solenicola* detaching from the siliceous tube after the manipulation.
H–J. Several colonies showing the disposition of the flagella.
Scale bars 10 μm.

late spring. At Marseille, 85, 120 and 20 colonies were observed in November, December 2007 and January 2008 respectively. The species reappeared in May (10 colonies), June (40 colonies) and September 2008 (10 colonies). At Banyuls sur Mer, 60 and 150 colonies were observed, respectively, in November and December of 2008. The species reappeared in May (5 colonies) and June 2009 (50 colonies). We collected several colonies for further observation by electron microscopy and amplification of SSU rDNA, the sequence of which was successfully determined (see below) for the three following colonies: FG11, collected from the coast of Marseille on 22 December 2007 (Fig. 1A), FG174, collected on 16 June 2008 in the same locality (Fig. 1B), and FG711 from coastal waters of Banyuls sur Mer on 15 June 2009 (Fig. 1C and D).

The individuals of *Solenicola* formed clusters on the surface of siliceous tubes usually interpreted as frustules of chain-forming diatoms (Fig. 1A–J). *Solenicola* cells could entirely cover the siliceous tube (Fig. 1A and E) or be forming clusters in localized areas along the tube (Fig. 1C, I and J). The individual *Solenicola* cells were 4–7 μm long and, under optical microscopy, globular or tear-shaped. When visible, cells possessed a single flagellum of variable length, sometimes up to 24 μm long. In living colonies, the flagella beat perpendicularly to the

siliceous tube with an undulating movement (Fig. 1H–J, see Video S1 or http://www.youtube.com/watch?v=6COYUXjrBWI). This provoked the motility of the entire colony in the direction of one of the extremes of the siliceous tube, but also enhanced the traffic of small particles towards the colony. Single individuals may detach from the colony (see Videos S1 and S2).

The specimens of *Solenicola* that we observed were colourless. No trace of chlorophyll *a* autofluorescence was observed by epifluorescence microscopy when the cells were excited by blue or green light. On rare occasions, one colony showed a partial yellow-greenish pigmentation (Fig. 1E, see Video S2 or http://www.youtube.com/watch?v=-B8m8YNIRas). However, the pigmentation did not correspond to chlorophyll *a* as tested by epifluorescence microscopy (Fig. 1F). The only signal of chlorophyll *a* corresponded to scattered spots of small circular spots of orange (phycoerythrin-derived pigments of cyanobacteria) or larger spots of red fluorescence (photosynthetic picoeukaryotes) that might correspond to recently captured preys by *Solenicola*.

During manipulation, the siliceous tubes often broke apart (Fig. 1E). *Solenicola* cells could suddenly detach from the siliceous tube. The detached cells did not swim, but remained grouped and motionless (Fig. 1G, see Video S3 or http://www.youtube.com/watch?v=q7mysOtm-eE).

Scanning electron microscopy of Solenicola

Five colonies of *Solenicola* collected in December 2008 from the port of Banyuls sur Mer, France, were fixed in glutaraldehyde and examined using scanning electron microscopy (Fig. 2). Two of the siliceous tubes colonized by *Solenicola* were fully covered with the nanoflagellate cells (Fig. 2A–J), while the other three colonies showed clusters of *Solenicola* restricted to localized areas of the tubes (Fig. 2K–AA). In the former, the cells bore a visible flagellum, whereas in the second case, the cells that remained attached to the tubes appeared to have lost their flagella, as attested by remaining flagellar insertion regions (e.g. Fig. 2L).

When visible, each cell showed a single smooth flagellum with a pointed end (Fig. 2C). The diameter was approximately 0.18 μm and the length was variable (up to 24 μm long) (Fig. 2F). The flagellum emerged from a ring-like basis (0.5 μm in diameter) that was apparently composed of 10 circularly arrayed globules (Fig. 2B, G and H). Although *Solenicola* cells were generally rounded or tear shaped (most cells shown in Fig. 2A and E), some cells, which were made often visible after the detachment of the rest of cells covering them, were rather pleomorphic, showing lobose pseudopodial and/or filopodial extensions that were intimately attached to their siliceous substrate (Fig. 2K, Q and W). Some of these pseudopodial extensions often showed protruding bulges in the distal ends (Fig. 2N). The morphology and structural details of the siliceous tubes colonized by *Solenicola* varied depending on the colonies (Fig. 2R–AA), which may suggest the occurrence of different closely related *Solenicola* species or strains with different substrate specificity.

Interestingly, one of the examined colonies showed particles attached to the flagella with a hexagonal contour of 0.27 μm in diameter, tentatively large icosahedral viruses (Fig. 2D). In a second colony, the polyhedral particles were attached to the cell body of *Solenicola* (Fig. 2I and J). Although rare, descriptions of large double-stranded viruses infecting stramenopiles exist, for instance in the pelagophyte *Aureococcus* (Gastrich *et al*., 1998) or the bicosoecid *Cafeteria* (Massana *et al*., 2007), suggesting that viruses exert a control on their populations.

Molecular phylogeny of Solenicola *from individual filaments*

We obtained nearly complete SSU rDNA sequences from the three *Solenicola* colonies shown in Fig. 1A–D using PCR amplification with eukaryotic universal primers. All the sequences obtained from the three SSU rDNA libraries (FG11, FG174 and FG712) were identical between them in each case and corresponded to stramenopile sequences. The two closest BLAST hits to our stramenopile sequences were the environmental clones NPK2-2 and BL0000921-38, both sharing 91% identity with our sequences. We concluded that the stramenopile sequences did correspond to *Solenicola* and we included them in a large alignment of eukaryotic sequences to determine its phylogenetic position. Preliminary phylogenetic analyses with SSU rDNA sequences from a variety of eukaryotic phyla revealed that *Solenicola* belonged to the stramenopiles (not shown). We then carried out more detailed phylogenetic analyses with representatives of the different stramenopile families. They showed that *Solenicola* branched with maximum support (100% bootstrap value – BV) within the environmental group MAST-3 (Fig. 3), defined by Massana and colleagues (2004). Our three *Solenicola* sequences were closely related but not identical (maximum divergence was 3% between the sequence of the FG712 colony and the other two sequences). This genetic diversity might indicate the occurrence of different species. The closest relative to *Solenicola* was the environmental clone NPK2-2, obtained from Artic waters at 2 m depth (Luo *et al*., 2009) and then several other environmental sequences from diverse oceanic regions, including the Mediterranean Sea (BL0000921-38 and ME1-28), Sargasso Sea (SMC27C17) and the Framvaren Fjiord in Norway (NIF_1E11, NIF_3D5 and NIF_3F1). All of them grouped with *Solenicola* with strong support (BV of 99%).

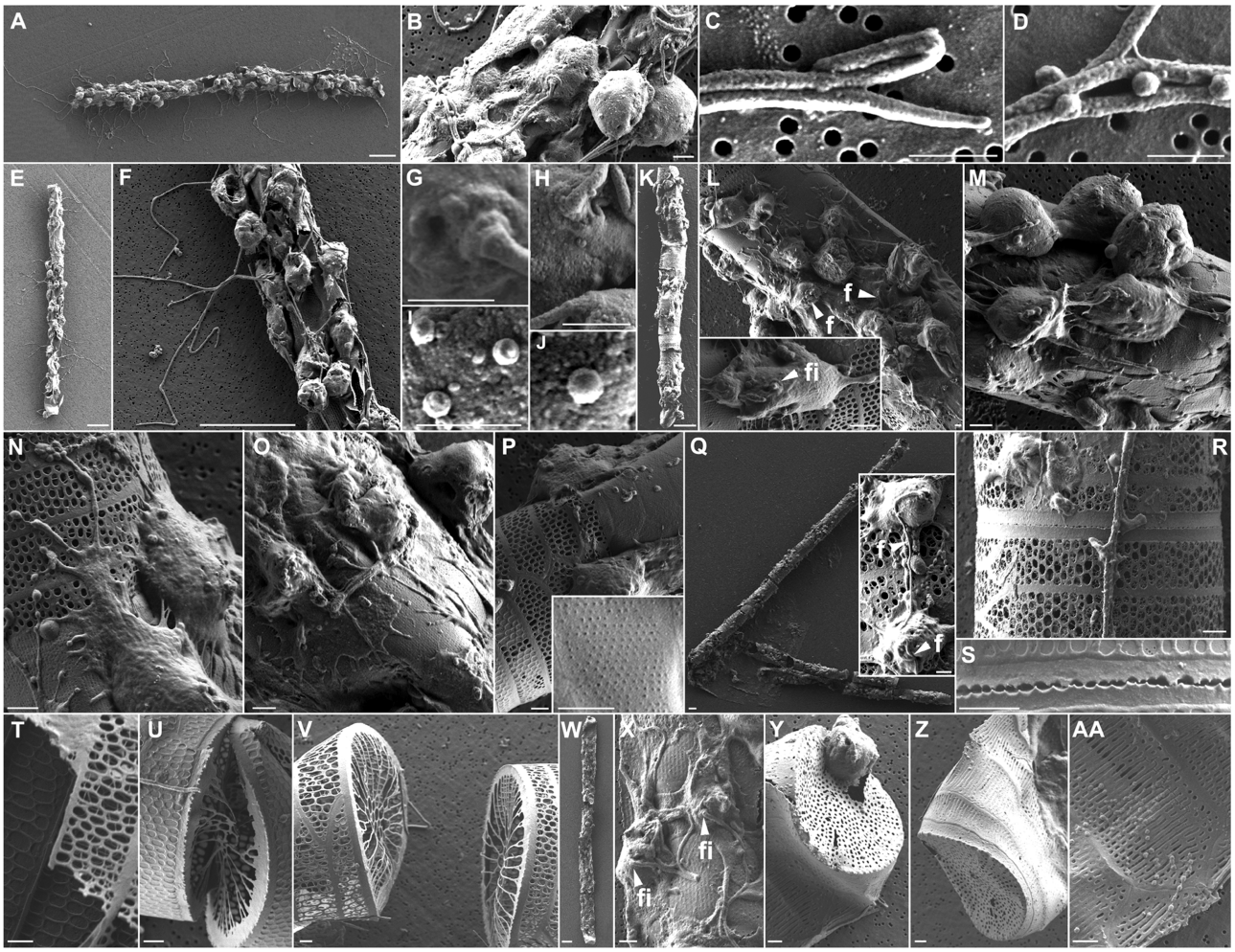

Fig. 2. Scanning electron micrographs of five colonies of *Solenicola* collected from the port of Banyuls sur Mer, France.
A–D. Siliceous tube fully covered of *Solenicola* cells. (B) Note the basis of the flagellum. (C) Pointed distal end of the smooth flagellum. (D) Tentative viral particles attached to the flagellum.
E–J. Another siliceous tube fully covered of *Solenicola* cells. (G and H) Note the flagellar insertion zone. (I and J) Attached polyhedral particles, tentatively viruses, attached to the cell body of *Solenicola*.
K–P. Another siliceous tube incompletely covered by *Solenicola* cells. (L) The arrowheads point the flagella or flagellar insertions. (N) Note the pseudopodial extensions of cells adhering to the tube. (O) See the splashed surface of *Solenicola* cells adhering to the siliceous tube. (P) Details of the siliceous tube ornamentation.
Q–V. Another colony of *Solenicola* on a siliceous tube. (R–V) Details of the siliceous structure of the tubes.
W–AA. A different colony of *Solenicola*. (X) *Solenicola* cells with extending pseudopodia and flagella. (Y–AA) Structural details of the siliceous tube.
f, flagellum; fi, flagellar insertion. Scale bar 1 μm in all micrographs, except 10 μm in (A), (E), (F), (K), (Q), (W).

To see whether there were other, partial, sequences closest to *Solenicola*, we additionally constructed a phylogenetic tree including all MAST-3 partial sequences available in the database. None of the partial MAST-3 sequences available in GenBank was closer to *Solenicola* than the aforementioned sequences (data not shown). The *Solenicola* clade was sister of a second group of sequences, all of them classified as MAST-3 according to Massana and colleagues (2004). The complete clade MAST-3 was strongly supported (BV of 100%), which prompted us to propose the name of Solenicolida for the whole cluster according to the name of its first morphologically identified genus. The clade Solenicolida emerged with a number of other environmental (MAST-12) or characterized heterotrophic (Opalinata, Placididea and Bicoecia) stramenopile groups in a well supported super-group (BV of 96%). The relative branching order of these groups was not well resolved (BV < 80%).

Solenicolida, a widespread and diverse group of nanoflagellates

Our molecular phylogenetic analyses of SSU rDNA sequences determined from individual colonies show that

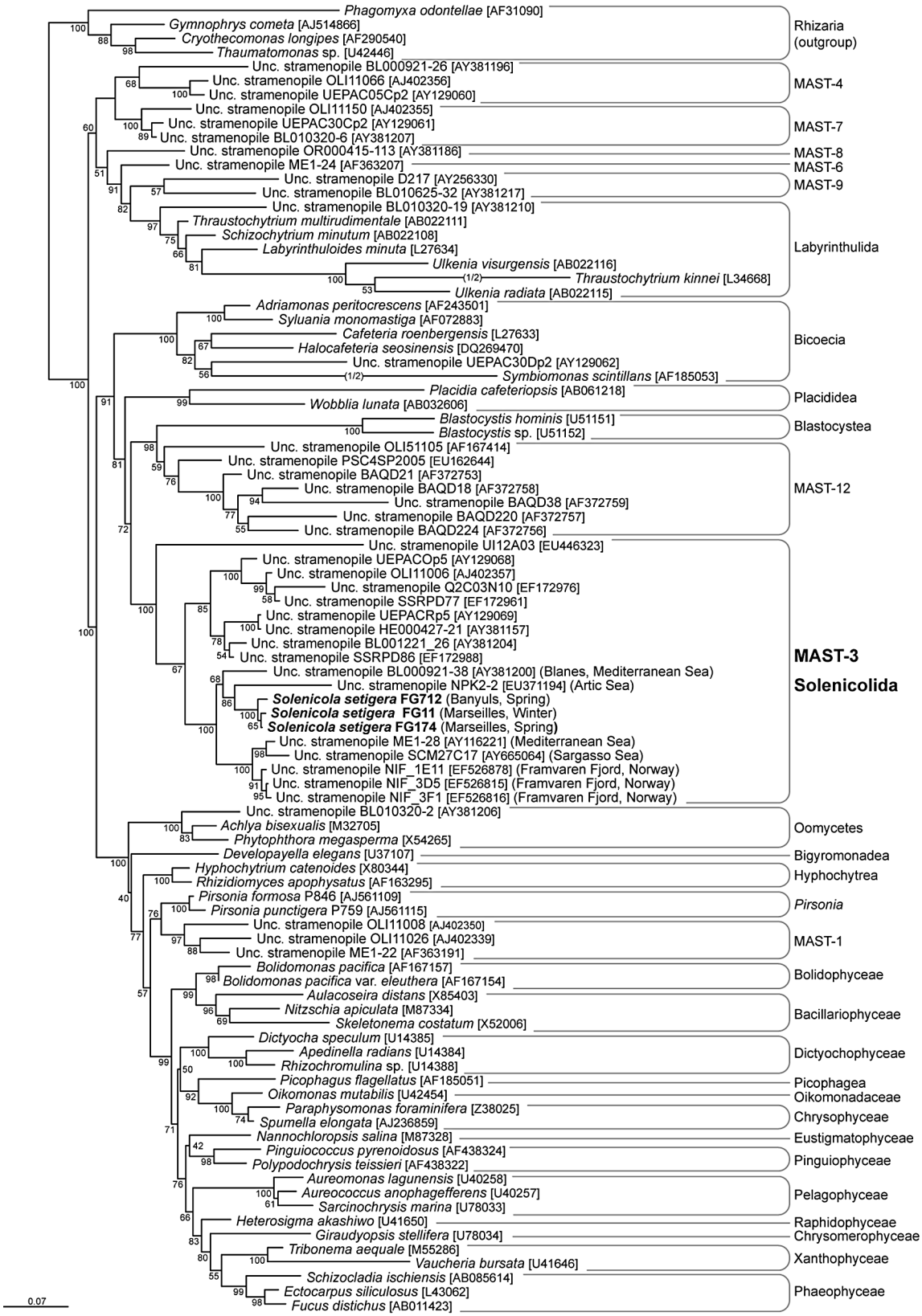

Fig. 3. Maximum likelihood phylogenetic tree of stramenopile SSU rDNA sequences rooted using four rhizarian sequences as outgroup. The tree was constructed using 95 taxa and 1325 conserved aligned positions; sequence accession numbers are provided between brackets. Numbers at nodes are bootstrap values. Sequences obtained in this work are indicated in bold.

Solenicola is the first documented member of a highly diverse and widespread group of stramenopiles or heterokonts, the MAST-3 (Massana *et al*., 2004). In addition to be largely detected in environmental SSU rDNA libraries from surface plankton of many coastal and open ocean regions, the even higher relative proportions of MAST-3 sequences in cDNA libraries constructed from SSU rRNAs suggest that organisms of this group are also more active than other abundant planktonic protists (Not *et al*., 2009). These observations indicate that MAST-3/Solenicolida species play an important ecological role in the marine ecosystem, most likely as nanoflagellate grazers. However, given the wide genetic diversity of this clade within the stramenopiles, the extrapolation of a grazer mode of life to the whole group remains hypothetical even if, in addition to *Solenicola*, other MAST-3 lineages appear bacterivorous (Massana *et al*., 2002). Nevertheless, the majority of groups forming the large stramenopile clade hosting MAST-3, including the environmental cluster MAST-12, opalinids, Placididea and bicosoecids (Fig. 3), are composed exclusively of grazers, which reinforces the grazer hypothesis. The only known exception corresponds to the parasitic opalinids (e.g. *Blastocystis*), since fluorescent *in situ* hybridization with specific probes also suggests that members of the MAST-12 are heterotrophic nanoflagellates (Kolodziej and Stoeck, 2007).

Similarly, the high morphological heterogeneity found in relatively close lineages to Solenicolida, such as the bicosoecids and opalinids, but also that found at the larger stramenopile scale, precludes the establishment of morphological synapomorphies (e.g. the presence of a single smooth and pointed flagellum) to the group. In fact, Heterokonta (and its rankless synonym 'stramenopiles') was initially established for all eukaryotes with biflagellated cells having typically a forward directed flagellum with tripartite rigid tubular flagellar hairs (mastigonemes) and a trailing hairless (smooth) flagellum, plus all their descendants having secondarily lost one or both flagella (see review in Cavalier-Smith and Chao, 2006). However, although stramenopiles do generally possess two flagella, some lost their posterior flagellum (*Siluania*, *Symbiomonas*, *Paramonas*) (Guillou *et al*., 1999) or even both flagella (e.g. the pelagophyte *Aureococcus,* the thraustrochytrids, the diatoms or the brown algae, although retaining flagellated gametes) (Sieburth *et al*., 1988; Cavalier-Smith and Chao, 2006). Other stramenopiles lack tripartite tubular hairs such as *Caecitellus*, *Halocafeteria* (Park *et al*., 2006) and *Rictus lutensis*, a recently described divergent bicosoecid (Yubuki *et al*., 2010). *Solenicola* belongs to atypical heterokonts in the sense that its cells have a single smooth flagellum with a pointed distal end (Fig. 2). From the optical microscopy observations, the hairless *Solenicola* flagellum seems to have at least two functions, maintaining the entire colony in suspension and creating a feeding current towards the mucous surface of the colony. In this sense, *Solenicola* appears to use a trophic strategy found in some bicosoecids, as well as many other protists. Whether this feeding strategy and the presence of one single hairless flagellum is a general feature of the whole Solenicolida clade will require the morphological identification of additional members of the group.

Concluding remarks

Heterotrophic pico- and nanoeukaryotes, generally small flagellates, play key roles within the microbial food web as grazers of bacteria and photosynthetic prokaryotes and picoeukaryotes. Despite their ecological importance, our knowledge on the diversity of the marine pico- and nanoheterotrophic protists is scarce. SSU rDNA-based analyses show that small marine protists are highly diverse but, most frequently, their morphology and ecological role is undocumented. Linking molecular, morphological and ecological information is nonetheless essential to elucidate eukaryotic evolution and function. In the present study, we have brought together molecular phylogenetic data and morphological and ecological information for a cosmopolitan protist from surface oceans, *S. setigera*. *Solenicola* becomes the first identified member of the widely distributed, abundant and active marine stramenopile group MAST-3. The identification and description of other members of this group will allow defining morphological synapomorphies for the group and better evaluating their global impact on marine food webs.

Experimental procedures

Sampling, light microscopy observations and isolation of material

Siliceous tubes harbouring cells of the colonial protist *S. setigera* were collected by slowly filtering surface seawater taken from the end of the pier (depth 3 m) of the Station Marine d'Endoume, Marseille (43°16′48″N, 5°20′57″E) from October 2007 to September 2008. A strainer with netting of 20, 40 or 60 μm mesh size was used to collect the organisms and the filtered volume varied between 10 and 100 l, according to the concentration of particles. The concentrated sample was examined in Utermöhl chambers at 100× magnification with a Nikon inverted microscope (Nikon Eclipse TE200) and was photographed at 200× or 400× magnification with a digital camera (Nikon Coolpix E995). Sampling continued from October 2008 to August 2009 in the surface waters of the port (depth of 2 m) of Banyuls sur Mer, France (42°28′50″N, 3°08′09″E) or from a coastal station offshore Banyuls (42°29′N, 3°08′E). The samples were prepared with the same procedure described above. The specimens were observed with an Olympus inverted microscope

(Olympus IX51) and photographed or filmed with an Olympus DP71 digital camera. Specimens were also examined for autofluorescence excited by blue and green light epifluorescence microscopy. In all cases, each siliceous tube with colonies of *Solenicola* was micropipetted individually with a fine capillary into a clean chamber and washed several times in serial drops of 0.2 μm filtered and sterilized seawater. Finally, the colony was deposited into a 0.2 ml Eppendorf tube filled with several drops of 100% ethanol. The sample was kept at room temperature and in darkness until the molecular analysis could be performed.

Amplification, cloning and sequencing

Ethanol-fixed filaments harbouring *Solenicola* cells were centrifuged gently for 5 min at 3000 r.p.m. Ethanol was then evaporated in a vacuum desiccator and cells were resuspended directly in 25 μl of Ex TaKaRa (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France). Each PCR reaction contained thus one single *Solenicola* filament after several serial washes (see above). Although the number of cells per filament was variable and they were difficult to count, we estimate that there were around 20 cells per filament on average. PCR reactions were performed in a volume of 50 μl reaction mix containing 15 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5′-CTCAARGAYTAAGCCATGCA-3′) and EK-1520R (5′-CYGCAGGTTCACCTAC-3′). The PCR reactions were performed under the following conditions: 2 min denaturation at 94°C; 10 cycles of 'touch-down' PCR (denaturation at 94°C for 15 s; a 30 s annealing step at decreasing temperature from 65°C down to 55°C employing a 1°C decrease with each cycle, extension at 72°C for 2 min); 20 additional cycles at 55°C annealing temperature; and a final elongation step of 7 min at 72°C. A nested PCR reaction was then carried out using 2–5 μl of the first PCR reaction in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5′-GAAACTGCGAATGGCTC-3′) and EK-1498R (5′-CACCTACGGAAACCTTGTTA-3′) and similar PCR conditions as described above. Negative controls without template DNA were used at all amplification steps. Amplicons of the expected size (~1600 bp) were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *Escherichia coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. A dozen colonies were picked from each library and the clone inserts amplified using vector primers T7P and M13R. Clones containing the expected amplicon sizes were then sequenced bidirectionally using primers 82F and EK-1498R and/or vector primers (Cogenics, Meylan, France).

Phylogenetic analyses

The new sequences were aligned to a large multiple sequence alignment containing 13 000 publicly available complete or nearly complete (> 1300 bp) eukaryotic SSU rDNA sequences using the profile alignment option of MUSCLE 3.7 (Edgar, 2004). The resulting alignment (Supplementary File S1) was manually inspected using the program ED of the MUST package (Philippe, 1993). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. Preliminary phylogenetic trees with all sequences were constructed using the Neighbour Joining (NJ) method (Saitou and Nei, 1987) implemented in the MUST package (Philippe, 1993). These trees allowed identifying the closest relatives of our sequences, which were selected, together with a sample of other stramenopile species, to carry out more computationally intensive maximum likelihood (ML). ML analyses were performed with the program TREEFINDER (Jobb *et al.*, 2004) applying a $GTR + \Gamma + I$ model of nucleotide substitution, taking into account a proportion of invariable sites, and a Γ-shaped distribution of substitution rates with four rate categories (α parameter = 0.334; 45% invariable sites). Bootstrap values were calculated using 1000 pseudoreplicates with the same substitution model. Sequences were deposited in GenBank with Accession No. HM163289–HM163291.

Scanning electron microscopy

Live colonies collected from the port of Banyuls sur Mer in November and December 2008 were micropipetted individually under the inverted microscope with a fine capillary. They were preserved in sterilized and filtered seawater with unbuffered glutaraldehyde (2% final concentration). Samples were filtered through GTTP Millipore filter (0.2 μm pore size) and rinsed twice in distilled water with ethanol 30% for 5 min. Cells were then dehydrated through an ethanol series, dried under a lamp overnight and coated with carbon using a Leica EM SCD500 high vacuum film deposition system. SEM observations were performed on a Zeiss Ultra 55 FEG-SEM at IMPMC (Paris, France). The microscope was operated at 1 kV at a working distance between 3.3 and 3.8 mm. Images were acquired in secondary electron mode using an Everhart Thornley detector.

Acknowledgements

F.G. was recipient of a postdoctoral grant from the Ministerio Español de Educación y Ciencia #2007-0213. D.M. and P.L.-G. acknowledge financial support from the ANR Biodiversity programme (ANR BDIV 07 004-02 'Aquaparadox'). The Focused Ion Beam (FIB) and Scanning Electron Microscope (SEM) facility of the Institut de Minéralogie et de Physique des Milieux Condensés is supported by Région Ile-de-France Grant SESAME 2006 N°I-07-593/R, INSU-CNRS, INP-CNRS, University Pierre et Marie Curie – Paris 6, and by the French National Research Agency (ANR) Grant #ANR-07-BLAN-0124-01. BQR from UFR STEP at University Paris Diderot contributed partly to the purchase of the Leica EM SCD500 high vacuum film deposition system at IMPMC.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

File S1. Multiple alignment of the 18S rRNA sequences used in this study. The alignment is in NEXUS format.
Videos S1–S3. Videos showing living colonies of *Solenicola* under optical microscopy.

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