

Brief report

Eukaryotic diversity associated with carbonates and fluid–seawater interface in Lost City hydrothermal field

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Summary

Lost City is a unique off-axis hydrothermal vent field characterized by highly alkaline and relatively low-temperature fluids that harbours huge carbonate chimneys. We have carried out a molecular survey based on 18S rDNA sequences of the eukaryotic communities associated with fluid–seawater interfaces and with carbonates from venting areas and the chimney wall. Our study reveals a variety of lineages belonging to eight major taxa: Metazoa, Fungi, Heterokonta (Stramenopiles), Alveolata, Radiolaria, Cercozoa, Heterolobosea and Euglenozoa. We detected one fungal lineage that appears to be widespread in hydrothermal systems both submarine and continental. Alveolates were the most abundant and diverse group in Lost City samples, although their distribution was very different in carbonate, where ciliates dominated, and in fluid–seawater libraries, where dinoflagellates, Group I and Group II (Syndiniales) marine alveolates were profuse. Similarly, Euglenozoa also displayed a differential distribution, kinetoplastids being present on carbonates and a novel group of diplomonads so far exclusively observed in the deep sea being dominant in fluid–seawater libraries. Protist lineages identified in this ecosystem likely correspond to grazers, decomposers and parasites, playing key roles in the food web of the Lost City ecosystem.

Introduction

Since their discovery in the late 1970s, hydrothermal vents associated with mid-oceanic ridges have fascinated scientists of various disciplines. In these ecosystems, chemolithoautotrophic prokaryotes are responsible for an important primary production that sustains dense microbial communities and, often, crowded colonies of endemic fauna. However, whereas the prokaryotic and animal components of known deep-sea hydrothermal systems have been extensively described, very few studies have been devoted to the microbial eukaryotes associated with deep-sea vents. The presence of ciliates based on microscopy observations was reported early at East Pacific Rise vents (Small and Gross, 1985). Also, a few classical protistology studies based on isolation and cultivation led to the description of a limited number of thermophilic ciliates (Baumgartner *et al.*, 2002) and a variety of psychrophilic or mesophilic protists (Atkins *et al.*, 2000). Recently, two molecular surveys based on the amplification, cloning and sequencing of 18S rDNAs were carried out in deep-sea hydrothermal sites of the East Pacific Rise and the Mid-Atlantic Ridge, revealing an astonishing diversity of eukaryotic lineages (Edgcomb *et al.*, 2002; López-García *et al.*, 2003). The first of these studies, carried out in the sulfide- and hydrocarbon-rich hydrothermal sediment of the Guaymas basin (2000 m deep) in the Gulf of California, revealed the presence of putative indigenous lineages but also of contaminating organisms coming from euphotic water layers, as a large diversity of typical photosynthetic lines was observed (Edgcomb *et al.*, 2002). The second study analysed the 18S rDNA eukaryotic diversity associated with Rainbow hydrothermal sediments (2200 m deep) and with artificial substrates having stayed for 15 days at a fluid source of the Eiffel Tour chimney in the Lucky Strike site (1600 m deep) (López-García *et al.*, 2003). Both sites are located at similar depth and relatively close in the Mid-Atlantic Ridge, but whereas Rainbow fluids are very hot (~365°C), acidic (pH ~2.8) and extremely enriched in metals and rare-earth elements (Douville *et al.*, 2002), Lucky Strike fluids are slightly less hot (generally 200–212°C, up to 333°C in black smokers) and hydrogen sulfide-rich

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(Langmuir *et al.*, 1997). By contrast to the Guaymas study, none of the Mid-Atlantic Ridge lineages detected belonged to typical photosynthetic groups, suggesting that they were actually autochthonous to the deep ocean (López-García *et al.*, 2003). In addition to these hydrothermal vent systems located in the deep sea, a third recent survey was carried out in the anoxic environment around the fumaroles of a shallow (200 m deep) submarine caldera ('Tagiri' sites) at the Kagoshima bay in Japan (Takishita *et al.*, 2005).

Lost City, a spectacular hydrothermal field located approximately 15 km off the Mid-Atlantic Ridge axis at 30°N and at a depth of 750–900 m, was discovered in 2000 (Kelley *et al.*, 2001). The site is unique because, being off-axis and located in the Mid-Atlantic Ridge slow-spreading system, its fluids have a very particular composition derived from serpentinization reactions that is characterized by abundant methane, relatively little metal content, high pH (9–11) and cooler temperatures (40–90°C) compared with mid-axis fluids. The alkaline conditions promote carbonate precipitation and the formation of large chimneys that host-specific prokaryotic communities where both anaerobic methane oxidation and methanogenesis appear to be important (Schrenk *et al.*, 2004; Kelley *et al.*, 2005). These microbial communities associated with carbonates in venting areas can be dense, containing 10^7 – 10^8 cells per gram of wet weight. However, although different animal species have been identified and the presence of foraminifera has been acknowledged based on microscopy observation (Kelley *et al.*, 2005), detailed data about protist diversity associated with Lost City vents are still missing.

In this work, we report the first 18S rDNA survey of eukaryotic diversity from Lost City carbonates and the fluids percolating through them. Our analysis reveals a relatively wide diversity of lineages, none of which is typically exclusively photosynthetic, including members of eight different taxonomic groups: Metazoa, Fungi, Heterokonta (Stramenopiles), Alveolata, Radiolaria, Cercozoa, Heterolobosea and Euglenozoa. Comparison with the eukaryotic diversity in deep-sea plankton, and the Guaymas and mid-axis Atlantic deep-sea vents suggests that most of the lineages associated to these deep-sea vents are those thriving in deep-sea plankton, in the case of fluid–seawater interfaces, and lineages typically associated to anoxic settings.

Eukaryotic diversity in Lost City

The samples that we used in our study were collected with the deep-sea submersibles 'MIR' during the 50th cruise of R/V 'Akademik Mstislav Keldysh' (see Experimental procedures in Appendix S1 in *Supplementary material*). Two types of samples from the highest pylon in Lost City vent

field were selected: venting fluids as they mixed with the surrounding seawater (samples LC22 and LC23) and carbonates (samples LC103 and LC104) (Fig. 1, and Table S1 in *Supplementary material*). The temperature of the fluids sampled fluctuated, ranging from 40°C to 75°C, and their pH varied between 9 and 9.8. Fluids were enriched in Mg^{2+} and SO_4^{2-} compared with other hydrothermal fluids (Lein *et al.*, 2004). After preliminary microscopy observations, we applied molecular methods to study the eukaryotic diversity associated to these samples. Once DNA was extracted, we amplified near full-length 18S rDNA by PCR, cloned the amplified products and sequenced partially inserts of expected size (see *Supplementary material*). A total of 266 good-quality partial sequences (~800 pb) were analysed initially (71 sequences from LC22; 85 sequences from LC23; 65 sequences from LC103; 45 sequences from LC104), from which a total of 47 clones (at least one from each of the clusters of sequences with > 98% identity detected) were selected as representatives to be completely sequenced. Complete sequences were incorporated to an alignment of ~3000 eukaryotic 18S rDNA together with their closest homologues identified by BLAST in GenBank (Table S2 in *Supplementary material*). Subsequently, we constructed three data sets that included Lost City sequences and a choice of representative sequences covering the taxonomic diversity of the groups identified in order to carry out detailed phylogenetic analysis by maximum likelihood and Bayesian analyses. They corresponded to animal and fungal sequences within the opisthokonts (Fig. 2), a variety of alveolates and one heterokont sequence, and diverse protist groups spanning the rest of the eukaryotic diversity found (Fig. 3).

Opisthokont sequences

We identified a number of phylotypes belonging to the Metazoa and the Fungi in Lost City samples. Metazoan sequences were often abundant in our libraries (Table S2 in *Supplementary material*), but they tend to be over-represented in 18S rDNA libraries due to a higher contribution of template DNA by pluricellular organisms. Within the Metazoa, copepods, which were found only in the fluid–seawater samples, and abundantly observed in the water column above the vent, were the most diverse (Fig. 2). Copepods appear to be abundant in other deep-sea hydrothermal areas, suggesting that they may be attracted by the rich primary production in these ecosystems (Humes, 1987; Heptner and Ivanenko, 2002). Sequences of members of the Porifera, Nematoda and Polychaeta were exclusively detected in carbonates, which would be in accordance with their benthic lifestyle. Indeed, these animals were observed and sampled for independent taxonomic analyses (A. Vereshchaka,

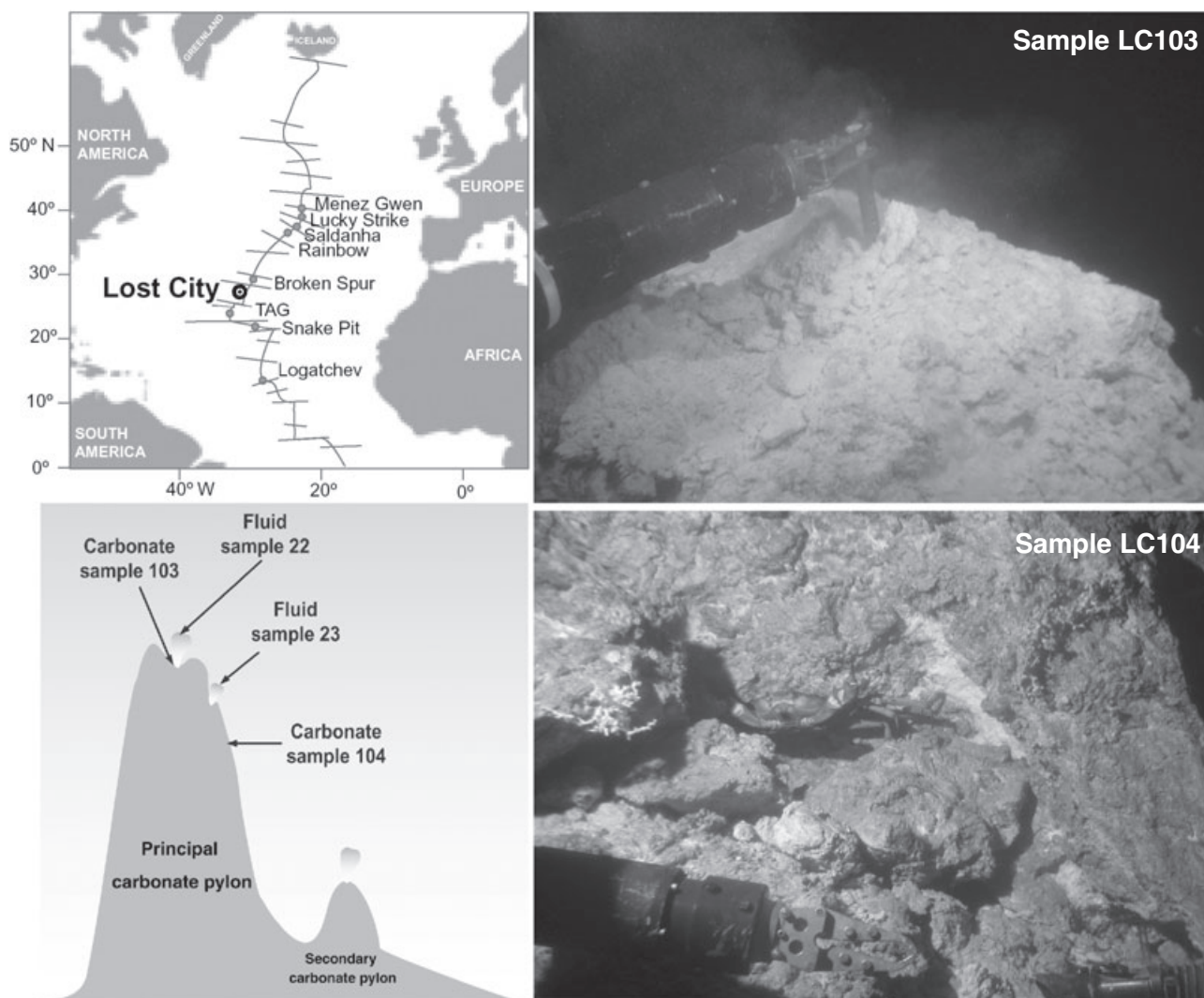


Fig. 1. Lost City sampling sites. The map indicates the position of Lost City relative to other Mid-Atlantic Ridge deep-sea vent sites. The schematic drawing on the left (down) shows the relative position of the sites from which samples were taken. Panels on the right show the MIR arm coring the top of the main pylon to collect sample LC103 and the lateral carbonate wall (some hydroids and a crab are visible on it) from which sample LC104 was collected.

unpublished). Sequences belonging to the Cnidaria were detected in both carbonates and seawater (Fig. 2). We only identified two fungal phylotypes, one within the Ascomycota, and the other within the Basidiomycota. Interestingly, the basidiomycete phylotype (LC23-5EP-14), which was relatively abundant in one fluid–seawater sample (Table S2 in *Supplementary material*), formed a cluster with other environmental sequences relatively far from cultivated species that were retrieved from other hydrothermal settings, either deep-sea vents (A1-E024 from Guaymas basin and AT9-6 from fluid–seawater at Lucky Strike) or cool terrestrial geothermal systems. Thus, PAT6-EK5-11 was retrieved from near-neutral pH, saline mud volcanoes in the Paterno area close to the Etna in Sicily, Italy, and BOH3 clones from carbonate-rich mud volca-

noes in Bohemia, Czech Republic (J. Berthon, D. Moreira and P. López-García, unpublished). The other clone in this group, BAQA52 was obtained in a study of brackish sediment in Berkeley Aquatic Park (Dawson and Pace, 2002). Therefore, this group may represent a group of anaerobic fungi that is ubiquitous in hydrothermal environments.

Heterokont and alveolate diversity

We identified only one heterokont (stramenopile) sequence, which was closely related to the group of environmental sequences MAST-9, represented by the clone BL010625-32 (Fig. 3). This group appears to occur mostly in hydrothermal vents and has been proposed to be adapted to anoxic or microoxic habitats (Massana *et al.*,

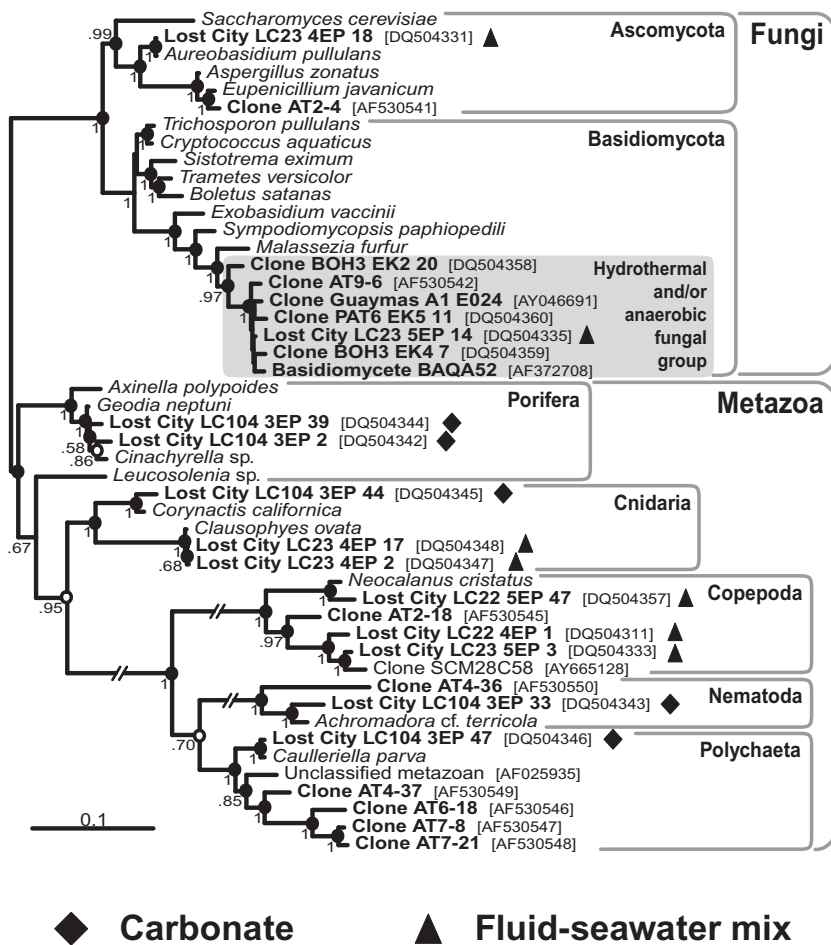


Fig. 2. Bayesian phylogenetic tree of 18S rDNA metazoan and fungal sequences retrieved from Lost City hydrothermal field. Phylotypes shown in bold have been retrieved from hydrothermal sites. Bayesian posterior probabilities are indicated at nodes. Maximum likelihood bootstrap values higher than 50% are shown with empty and filled circles to indicate values between 50% and 75%, and between 75% and 100% respectively.

2004). It is distantly related to the osmotrophic labyrinthulids and thraustochytrids and is probably formed by heterotrophic organisms.

By contrast to this limited diversity of heterokonts, we retrieved a wide variety of alveolates (Fig. 3). Ciliates were the most abundant in our clone libraries, especially in the carbonates (Table S2 in *Supplementary material* and Fig. 4). Lost City sequences were affiliated to three ciliate classes, Hypotricha (LC103-4EP-1), Spirotrichea (LC23-5EP-24, LC22-5EP-7) and Oligohymenophorea (LC22-5EP-48, LC22-5EP-20, LC103-5EP-27), and likely correspond to grazing organisms. One possible exception could be LC103-5EP-27, closely related to members of the genus *Cohnilembus*, which contains also commensal species in the gut of various invertebrates (Lynn and Strüder-Kypke, 2005). Different dinoflagellate sequences were also detected, but in this case, all of them were identified in the seawater above the carbonate chimneys, in accordance with their planktonic way of life. Finally and, as in the case of dinoflagellates only in the seawater–fluid mix, various phylotypes related to the marine alveolates Groups I and II and an alveolate sequence of unclear affinity (LC22-4EP-19) were obtained. Group II alveolates

correspond indeed to the Syndiniales, a group of small parasites traditionally classed within the dinoflagellates that appears to be highly diverse and ubiquitous in the water column and around hydrothermal vents (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Moreira and López-García, 2003). Lost City sequences branched within two distinct clusters in the Syndiniales (Fig. 3). One of them corresponded to the Syndiniaceae, grouping *Hematodinium* and *Syndinium* species, which generally infect invertebrates, including copepods, whereas the other group, the Amoebophryaceae, including *Amoebophrya* species, are generally endoparasites of other protists, such as dinoflagellates, ciliates or radiolaria. Alveolate Group I was described on the basis of environmental sequences exclusively. It encompasses a large genetic diversity and appears also ubiquitous in the oceanic water column. Unfortunately, to date, there is no information about their morphology or lifestyle.

Other protist lineages

In addition to metazoa, fungi, heterokonts and alveolates, we retrieved sequences belonging to four protist phyla,

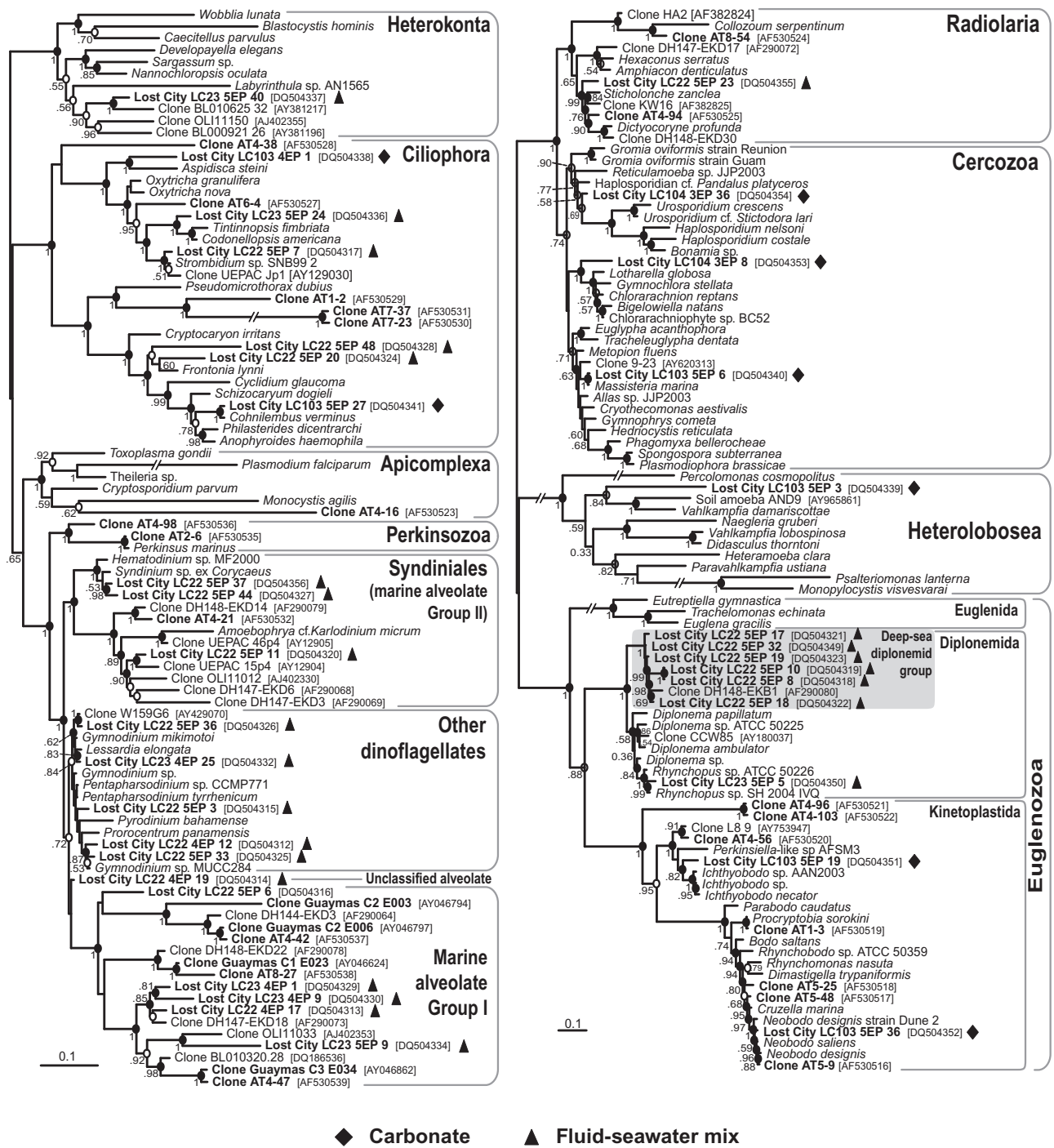


Fig. 3. Bayesian phylogenetic trees of 18S rDNA heterokont and alveolate sequences (left) and of Radiolaria, Cercozoa, Heterolobosea and Euglenozoa (right) retrieved from Lost City hydrothermal field. Phylotypes shown in bold have been retrieved from hydrothermal sites. Bayesian posterior probabilities are indicated at nodes. Maximum likelihood bootstrap values higher than 50% are shown with empty and filled circles to indicate values between 50% and 75%, and between 75% and 100% respectively.

Radiolaria, Cercozoa, Heterolobosea and Euglenozoa (Fig. 3). The clone LC22-5EP-23 from the fluid-seawater mix branched among the Radiolaria, but its affiliation within this phylum remains uncertain. Three Lost City carbonate sequences belonged to the Cercozoa, one of

the most diverse and species-rich groups of flagellated protists (Bass and Cavalier-Smith, 2004). LC104-3EP-36 emerged as a very short branch with moderate support at the base of a group including the described species of Haplosporidia, a long-branching group composed exclu-

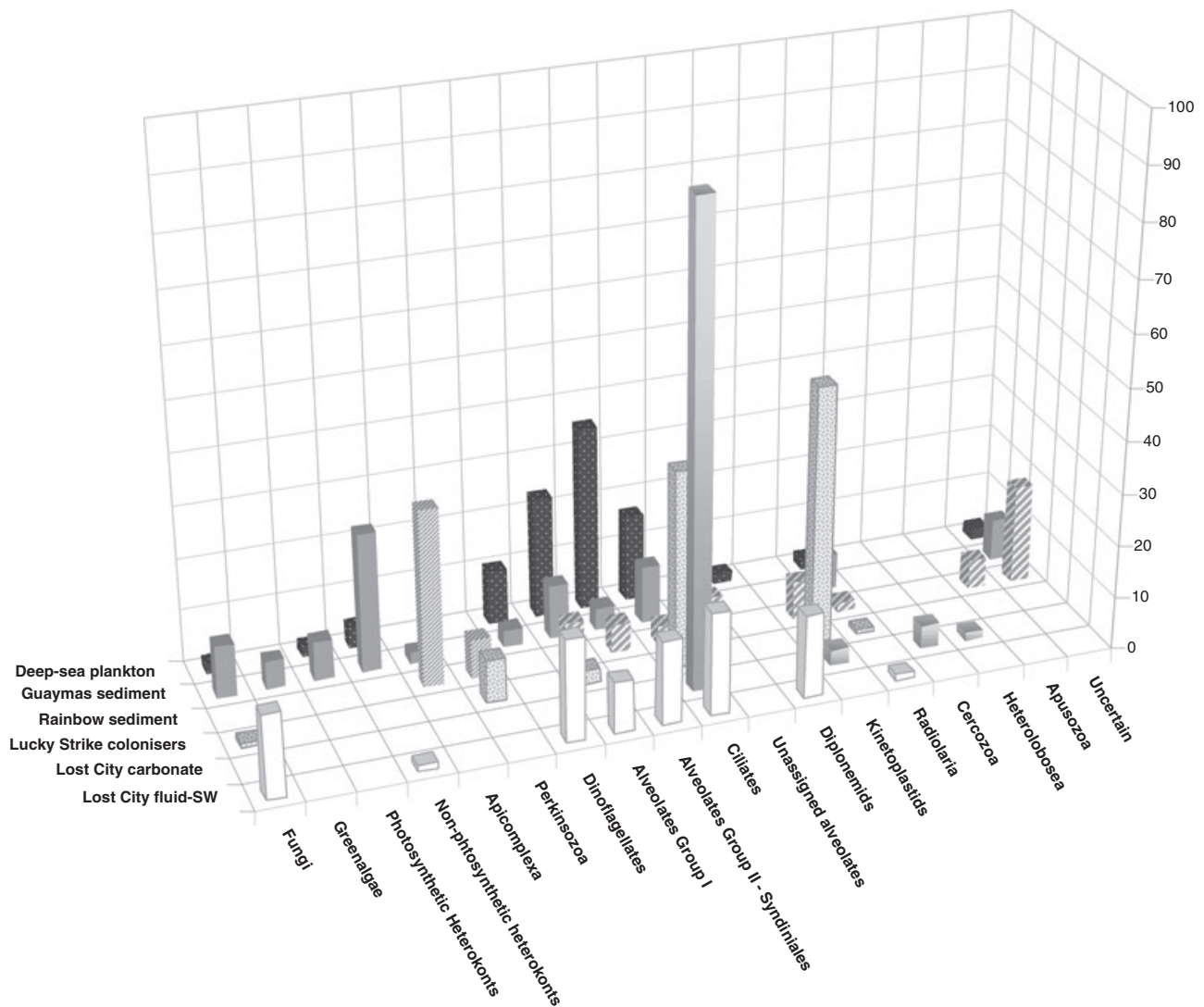


Fig. 4. Relative abundance of protist clones retrieved from Lost City carbonate and hydrothermal fluid-bearing seawater (fluid-SW) libraries compared with that found in deep-sea plankton and other oceanic deep-sea hydrothermal systems. Deep-sea plankton data correspond to the average of clones obtained at the Antarctic Polar Front at 500, 2000 and 3000 m depth (López-García *et al.*, 2001).

sively by parasites of invertebrates (Perkins, 1990). It may be well a basal haplosporidian, as it is close to another sequence also basal to the described haplosporidians that apparently corresponds to the haplosporidian parasite of the shrimp *Pandalus platyceros* (Reece *et al.*, 2004) (Fig. 3). If this is the case, this phylotype could correspond to a parasite as well. The clone LC104-3EP-8 occupies an interesting phylogenetic position, as it branched at the base of the Chlorarachniophyta, thus breaking the relatively long branch leading to this group. The third cercozoan clone (LC103-5EP-6) was very closely related to *Massisteria marina*, a heterotrophic flagellate that was isolated from four hydrothermal vents along the East Pacific Rise (Juan de Fuca Ridge, Guaymas basin, 21°N and 9°N) (Atkins *et al.*, 2000).

Massisteria marina appears therefore to be ubiquitous and to show some affinity for hydrothermal vents, likely because of their high primary productivity.

We also identified one sequence (LC103-5EP-3) distantly related to members of the Heterolobosea, a phylum of widely diverse flagellated free-living amoeba. Some heterolobosean amoeba are thermotolerant, such as *Naegleria* spp., and have been isolated and detected in hot springs and effluents from power plants (Sheehan *et al.*, 2003).

After the alveolates, and similarly to the fungi, the most represented phylum in our Lost City libraries was that of the Euglenozoa (Table S2 in *Supplementary material* and Fig. 4). Members of two classes of this phylum, Diplonemida and Kinetoplastida, were detected in Lost City

samples. Kinetoplastid sequences were detected only in carbonate samples, whereas diplomemid sequences were exclusively detected in the fluid–seawater interface (Fig. 3). One kinetoplastid phylotype (LC103-5EP-36) was very close to *Neobodo* species, among the free-living bodonid flagellates, whereas the other kinetoplastid sequence (LC103-5EP-19) was related to a group of the parasitic *Ichthyobodo* species. Within the diplomemids, one phylotype (LC23-5EP-5) was closely related to the heterotrophic genus *Rhynchopus*. Interestingly, all the other phylotypes detected formed a well-supported cluster with the environmental sequence DH148-EKB1 coming from 3000-m-deep plankton (López-García *et al.*, 2001). This cluster appears to form a new group within the diplomemids, which has been detected so far only in the deep ocean. On the one hand, the detection of this new group of phylotypes spans the described genetic diversity within the Diplonemida, a class still poorly known and little observed in natural samples. On the other hand, given the phylogenetic position of this group as sister to known diplomemids, it would be particularly interesting to isolate members of this cluster in order to characterize their mitochondrial DNA. Diplonemids are the sister group to the kinetoplastids, which are characterized by complex networks of massed mitochondrial DNAs (Lukes *et al.*, 2005). Very recently, the mitochondrial genome structure of *Diplonema* has been shown to be not only different from that of kinetoplastids but unique among eukaryotes as a whole (Marande *et al.*, 2005). Therefore, having cultured representatives of related organisms could help to understand the evolutionary origin of the complex mitochondrial genomes and their regulation, perhaps leading to the discovery of new mitochondrial genome organization variants in these euglenozoans.

Comparison of protist diversity in Lost City with that of deep-sea plankton and other deep-sea vents and some functional predictions

Although protist diversity data are still quite limited for the deep sea and deep-sea hydrothermal vents, it is possible to carry out preliminary comparisons between types of samples (plankton and benthos) and between sites to see whether general trends in the distribution of particular taxa occur.

Important differences in the distribution of lineages between carbonate and fluid–seawater were apparent in Lost City although, in both types of samples, regardless of metazoan sequences, the most abundant and diverse phylum was by far that of the Alveolata, followed by Euglenozoa and Fungi (Fig. 4). However, although alveolates dominated both types of samples, only ciliates were detected in carbonates, whereas more or less equivalent proportions of dinoflagellate, ciliate, Group I

and Group II (Syndiniales) phylotypes were found in the fluid–seawater interface. Similarly, in the case of Euglenozoa, diplomemids were only detected in fluid–seawater libraries, whereas kinetoplastids were exclusively identified in carbonate samples. Fungi were also exclusively detected in fluid–seawater libraries. A differential distribution of lineages was also observed for the less represented phyla. Thus, heterokont and radiolarian sequences were retrieved only from fluid–seawater samples, whereas cercozoan and heterolobosean sequences were found only in carbonates (Fig. 4). It is also worth mentioning that we did not detect any lineage of uncertain phylogenetic affiliation in Lost City, in contrast with observations from hydrothermal sediments at Guaymas and Rainbow that revealed the existence of potential new high-rank taxonomic groups (Edgcomb *et al.*, 2002; López-García *et al.*, 2003). Some lineages in those sediments can indeed represent novel eukaryotic phyla as deduced from careful analyses that ruled out the possibility of methodological artefacts linked to the presence of chimeric or fast-evolving sequences, and to the lack of adequate taxonomic representation in 18S rDNA phylogenies (Berney *et al.*, 2004; Cavalier-Smith, 2004). These observations suggest that potential novel divergent lineages are generally confined to anoxic sediments, whose study is more difficult and has been often disregarded.

When comparing protist diversity among different deep-sea samples, including both plankton and a variety of deep-sea hydrothermal samples (sediments, artificial substrates, natural substrates – carbonates – and fluid–seawater mix), the most remarkable trend is the dominance of alveolate lineages in all 18S rDNA libraries from deep-sea samples (Fig. 4). Nevertheless, the proportion of the different alveolate subgroups varies depending on the type of deep-sea environment, probably reflecting a preference for particular sets of physicochemical and/or ecological conditions. For instance, Perkinsozoa, typical parasites of bivalve molluscs, are found in Mid-Atlantic Ridge samples where extensive colonies of endemic hydrothermal bivalves have been described (López-García *et al.*, 2003). The atypical case of Guaymas sediment, where the autochthonous microbial community appeared to be contaminated by organisms sedimenting from the whole water column (Edgcomb *et al.*, 2002), becomes apparent in this comparison. In addition to the abundance of different alveolate phylotypes in Guaymas sediment, there is not only a notable occurrence of photosynthetic eukaryotes (green algae and photosynthetic heterokonts) but also a high proportion of heterotrophic heterokonts (Fig. 4). The latter appear to be particularly abundant in the plankton of the photic zone (Massana *et al.*, 2004) rather than the deep sea. As a matter of fact, a comparable abundance of

heterokonts is detected in the shallow (200 m deep) Tagiri hydrothermal site (Takishita *et al.*, 2005) (Fig. S1 in *Supplementary material*), confirming the association of large heterotrophic heterokont diversity with surface oceanic waters.

Although less apparent that the dominance of alveolates in the deep sea, another observation in this comparison is the preference of some taxonomic groups for solid substrates. Despite the fact that ciliates are present in all deep-sea samples and are quite diverse, they appear particularly abundant in 18S rDNA libraries from artificial colonization substrates and carbonates. Kinetoplastids, and more particularly bodonids, seem to exhibit a similar trend, as they have not been detected in deep-sea plankton or fluid–seawater mix up to date. This trend may be related to the grazing lifestyle of free-living bodonids and many ciliates, which may feed easily on the microbial mats that colonize substrates around vents.

The wide eukaryotic diversity identified in Lost City confirms that protists are present in deep-sea hydrothermal systems and possibly play a variety of ecological functions in the trophic chain. Lost City microbial eukaryotes do not only include typical predators or grazers that likely exert a control on bacterial populations, such as the bodonids, many ciliates, radiolaria and possibly the dinoflagellates and some cercozoa, but also decomposers and parasites. Fungi are most likely decomposers, and it is possible that many Lost City fungi are anaerobic or microaerophilic, as at least one fungal phylotype branches within a group of fungi formed by environmental sequences exclusively retrieved so far from deep-sea vents, continental mud volcanoes and brackish sediments (Fig. 2). Parasitic lineages appear to be also present in Lost City, such as the Syndiniales, the haplosporidian cercozoa and, perhaps, some ciliates and basal kinetoplastids related to *Ichthyobodo*. This is in agreement with previous observations of diverse parasitic protists in association with deep-sea vent environments (Moreira and López-García, 2003). In the case of divergent lineages, such as the new deep-sea diplomonid cluster, it is not possible to ascertain their mode of life for the moment. Now that their presence has been detected, it should be possible to design specific probes to identify and quantify them by fluorescent *in situ* hybridization as well as to attempt directed isolation and cultivation to gain insights in their physiology and lifestyle.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Comparison of the relative abundance of protist phylotypes belonging to large taxonomic taxa retrieved from Lost City 18S rDNA libraries, other oceanic hydrothermal systems and deep-sea plankton. Deep-sea plankton data correspond to the average of clones obtained at the Antarctic Polar Front at 500, 2000 and 3000 m depth.

Table S1. Samples collected from Lost City hydrothermal field the 27–28 August 2005 during the 50th Cruise of R/V 'Akademik Mstislav Keldysg' analysed in this study.'

Table S2. Near full-length eukaryotic 18S rDNA sequences retrieved from Lost City fluid–seawater interface (LC22, LC23) and carbonate (LC103, LC104) samples.

Appendix S1. Experimental procedures.

This material is available as part of the online article from <http://www.blackwell-synergy.com>