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Manuscript in Molecular description and phylogeny of *Pythium mediterraneum, a novel species isolated* from South Mediterranean wetlands (Case of the freshwater Oubeira Lake, Northeastern Algeria)

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ABSTRACT

Oubeira Lake is a South-Mediterranean subtropical wetland; it is perceived to be the largest Ramsar site of El Kala National Park, Northeastern Algeria. A previous inventory of native *Pythiaceae* microbiota predominantly occurring in Oubeira inland freshwaters, has revealed a large distribution of the genus *Pythium.* these saprotrophic populations play a vital role in the aquatic habitat autopurification. Morphological and molecular data characterizing the isolates taken from water, sediments and associate lake materials in decomposition, permitted to establish *DeNovo* assembly and intrageneric phylogeny based on rDNA ITS sequences. The latter were enough significant to distinguish a segregate Keratonophilic *Pythium* taxon which perfectly clusters within clade E and occupies an ancestral evolutionary range in *P. segnitium* subclade, with a strong likelihood support. The elements of this subclade were first described by Paul Bernard and his collaborators since the earlier 2000s until 2016, in Algeria and similar warm areas, while the studied taxon is not previously described elsewhere; we herein, suggest the name of *P. mediterraneum* for the novel species.

KEYWORDS: Molecular analysis, New taxon, Oubeira Lake, rDNA ITS data, Phylogeny, *Pythium mediterranem*.

INTRODUCTION

Fugi-like or algae-like waterborne Oomycota lineage comprises at least 160 recognized species of the dominant genus *Pythium*, colonizing marine and freshwater biotopes [7]. They evolved from heterotrophic flagellates and photosynthetic algae, both categories belong to the heterokonts [8]; some of them left the zoosporic aquatic behavior and have been adapted to the terrestrial conditions to become amphibians and widespread inhabitants of soils and humus [4]. More evolved lineages, represent harmful pathogens of plant, fish, crustacean, protist and even other *Pythium* hosts [22].

Since Dreschler [3], Van Der Plaats Nectric [20] and Middelton [12], the reproduction organs shape and size has been retained as one of the taxonomic criteria to the genus level. *Pythium* is recently subdivided on the basis of molecular data, into 10 phylogenetic major clades numbered from A to J, if clade K were excepted as a separate genus recently named *Phytopythium*. This classification is strongly supported by morphological characteristics showing an evolution history of the most primitive species with globose internally proliferating sporangia of the subclades E and G, to perfect globose sporangia within subclades J, I and F, then contiguous

grouped in subclades D and C to filamentous inflated and perfect filamentous sporangia forming subclades A and B; the latter represent the most evolved lineages known as *Vanterpolii* subclade B1 [9].

During the last two decays, over 30 new species of the dominant genus *Pythium* essentially from subtropical, tropical and temperate areas as Canary islands, Spain, Portugal, Argentine, western Africa, India, southern Turkey and Tunisia, etc. by Paul Bernard and his collaborators [5], whereas his earlier studies were carried out in the Algerian South Mediterranean warm ecosystems [15,16] he namely identified there, *Pythium toruloides*, *Pythium ramificatum*, *Pythium ornamentatum*, *P.capillosum var helicoides*, *Pythium dreschleri*, *Pythium crytogynum*, *Pythium polycarpum*, almost limited to a few natural and semi-artificial hydrosystems Western Algeria [10].

Pythium recognition was at the time, traditional and slightly limited to morphological dichotomy keys, since no molecular techniques were yet available. By the advent of molecular technologies based on Polymerase Chain Reaction (PCR) for the ribosomal gene amplification is used for the genetic identification of many organisms because they comprise both highly conserved sequences during evolution and highly variable sequences among species. The ribosomal nuclear DNA consists of transcribed and non-transcribed regions. The ITS1 (internal transcribed spacer 1), and ITS2 (internal transcribed spacer 2) are non-conserved regions and can be amplified with the PCR method using universal primers ITS1 and ITS4 [1]. The sequences of these regions are used to identify different species within the genus. Many *Pythim* species could be molecularly described by Paul Bernard in Algeria, using molecular rDNA ITS fingerprints, at the image of *Pythium segnitium* [18] which represents one of the most important references in our current study realized in Oubeira. The studied geographic area is typical to South-Mediterranean subtropical wetlands and a Ramsar site of the National Park of El Kala, extreme Northeastern Algeria, where an initial survey of autochthonous *Pythiaceae* microbiota predominantly occurring in Oubeira lake inland freshwaters was carried out during the period 2011-2013; it revealed a novel species highly similar to *P. segnitium* and subjected to the current study.

MATERIALS AND METHODS

Spatiotemporal context:

During the period 2011-2013, we an inventory of waterborne fungi-like microbial populations has been carried out for the first time, in Oubeira lake (36°.81842N, 8°.44179E), a perennial freshwater hydrosystem of nearly 2260 ha (figure 1b), and Ramsar site since 1983 [2]. It gathers an outstanding mosaic of indigenous aquatic microbial biodiversity attracted and stabilised in subtropical climate conditions (figure 1a).

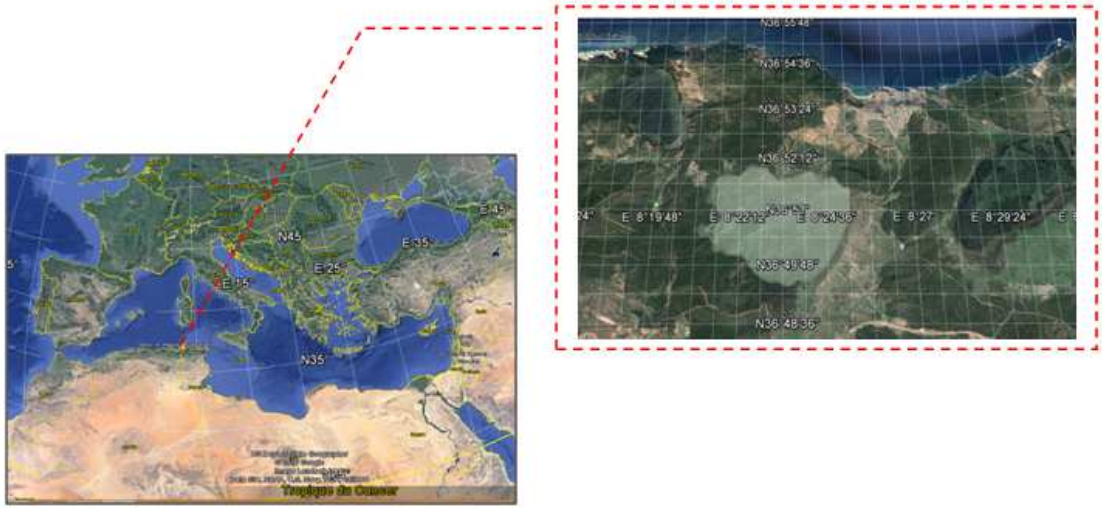

Fig. 1: Satellite localisation of Oubeira Lake (extreme Northeastern Algeria). ©2016Google, Map Data ©2016 AND Image Landsat ©SPOT IMAGE, date: 10.04.2013).

The latest statistics talk about more than 80,000 migrant birds flying over the region; more than 7.181 water birds have been recently registered in the lake while the number did not exceed 4000 individuals for the last few years.

Sampling and isolates manipulation:

Organic materials like feathers falls submerged in the lake waters and softened by the action of microbial degradation were sampled in order to isolate keratinophilic oomycetes. Decaying leaves, dead insects and sediments were also sampled during this study. They were taken from the shores at five different water input

points called “wadis”, distant from each other for about 100 to 500 meters. Microbiologic labour consisted in shredding solid samples into about 25 mm^2 pieces of dead organic materials, then plating them on modified and V8 medium, to which we added Ampicillin, Nystatin, Pimaricyn, Rifampicyn and Pentachloronitrobenzene.

Morphology:

The inverted (Wilovert) photonic microscope of the type Hund, was used to observe mature sporangia at a low magnification (x20), after 10 days incubation at room temperature.

Mycelia conservation:

The isolates were conserved in sterilised water until we could explore them. We selected cultivable isolates of whitish mycelium with soft texture and typical growth. The mycelium was collected in sterile 1.5 ml Eppendorf, and placed at -20° C for at least 12 hours, up to complete freezing.

Molecular processing:

DNA was extracted using GenElute™ plant Genomic DNA Miniprep. Kit of Sigma Aldrich, and finally stored at -20 °C according to Manufacturer's specifications. The ITS1 and ITS2 and their flanking regions of the ribosomal DNA was amplified using the primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3 ') and ITS-4 (5 'TCC TCC GCT TAT TGA TAT GC 3') (White et al 1990). Amplification was accomplished by using the following program: step (1) initial denaturing for 3 min at 95 °C; step (2) denaturing for 30 sec at 95 °C; step (3) annealing for 30 sec at 55 °C; step (4) extension for 1 min at 72 °C; step (5) final extension for 5 min at 72 °C. The steps 2-4 were repeated 35 times [23]. The PCR product was quantified by electrophoresis on Agarose Gel, then finally cleaned-up using Thermo-Fisher-Scientific kit, by adding to 5µl of it, a mix of 0.5µl Exonuclease 1 and 1µl Fast-up alkaline phosphate according to manufaturer's specifications.

Phylogenic analyses:

The sequences were, checked, analyzed in Chromas Lite v. 211 without editing except at few evident points, then assembled and trimmed at extremities to around 690 bp. within Geneious v.8.1. Multiple alignment was performed using Mafft 7 online aligner, by choosing Q-INS-i strategy setting and 1PAM=2k for scoring matrix [6]. Then, we chose Maximum Parsimony with default parameters, to obtain the best tree inferred from 10 parsimonious produced trees. Bootstrap statistics and branch lengths were computed with Mega 6 using Tamura-Nei Model [21]. Maximum Likelihood was additionally performed by RAxML and Mr. Bayes methods, using TOPALi (v2.5) online software, with standard parameters for DNA data, and 500 bootstrap replicates carried out.

RESULTS AND DISCUSSION

DeNovo assembly:

Using Geneious program, *DeNovo* assembly permitted to select a cluster of 8 sequences among 66 *Pythium* isolates (table 1).

Table 1: Selected isolates from different substrates sampled in the Oubeira Lake. Accession numbers from Genebank were accorded to rDNA ITS1 and 2 amplified regions of the extracted genomic DNA.

Isolate code	*Genus*	*ITS Accession number from GB*	*Substrate*
LK67	*Pythium*	*KU588242*	*Birds feathers*
LK68	*Pythium*	*KU588226*	*Dead plant materials*
LK69	*Pythium*	*KU588244*	*Birds feathers*
LK70	*Pythium*	*KU588240*	*Birds feathers*
LK-18	*Pythium*	*KU588231*	*Birds feather*
LK-19	*Pythium*	*KU588261*	*Sediments*
LK-20	*Pythium*	*KU588257*	*Sediments*
LK-21	*Pythium*	*KU588220*	*Dead plant materials*

Basic Local Alignment Search Tool (BLAST) on NCBI data base:

BLAST query of the ITS rDNA data is 87% identity with *Pythium segnitium*. As shown in the figure 2.The query permitted to select 11 similar Voucher specimens from CBS database (table 2), including two taxa *P. Paroecandrum* and *P. sylvaticum* from clade F, as an outgroup.

Table 2: Closest indexed *Pythiaceae* references from NCBI database; references are all recently published.

Indexed species	Phylogeny	ITS numbers	Accession	Ressources	Location
Pythium rostratifringens	*Clade E*	*KF806440*		*Moschard,M. et al. 2013*	*France*
Pythium rostratum	*Clade E*	*HQ643767*		*Robideau,G.P. et al. 2011*	-
Pythium kannovanense	*Clade E*	*KP723168*		*Robideau,G.P. et al. 2011*	-
Pythium middeltonii	*Clade E*	*HQ643694*		*Robideau,G.P. et al. 2011*	-
Pythium multisporum	*Clade E*	*HQ643700*		*Robideau,G.P. et al. 2011*	-
Pythium rhizosaccharum	*Clade E*	*HQ643759*		*Robideau,G.P. et al. 2011*	-
Pythium segnitium	*Clade E*	*KF831233*		*Paul B. et al.2002*	*Algeria*
PythiumPB2013m	*Clade E*	*KF802196*		*Paul B. 2013*	*India*
Pythium PB2013d	*Clade E*	*KF831232*		*Paul B. 2013*	*India*
Pythium parocaendrum	*Clade F*	*HQ643734*		*Robideau,G.P. et al. 2011*	-
Pythium sylvaticum	*CladeF*	*HQ643845*		*Robideau,G.P. et al. 2011*	-

Phylogenetic position of the novel species inside clade E:

The studied isolate shows a maximum of 89% identity to 6 recently deposited taxa in Genbank by Paul Bernard, Lefort and their collaborators; they are *Pythium FL2016g*, *Pythium FL2016b*, *Pythium PB2013d* and *Pythium PB2013m* and *Pythium CAL* 2011f, yet unformally described, as unpublished.

The 20 sequences cited in the tables above, allowed to construct the phylogenic tree (figure 02).

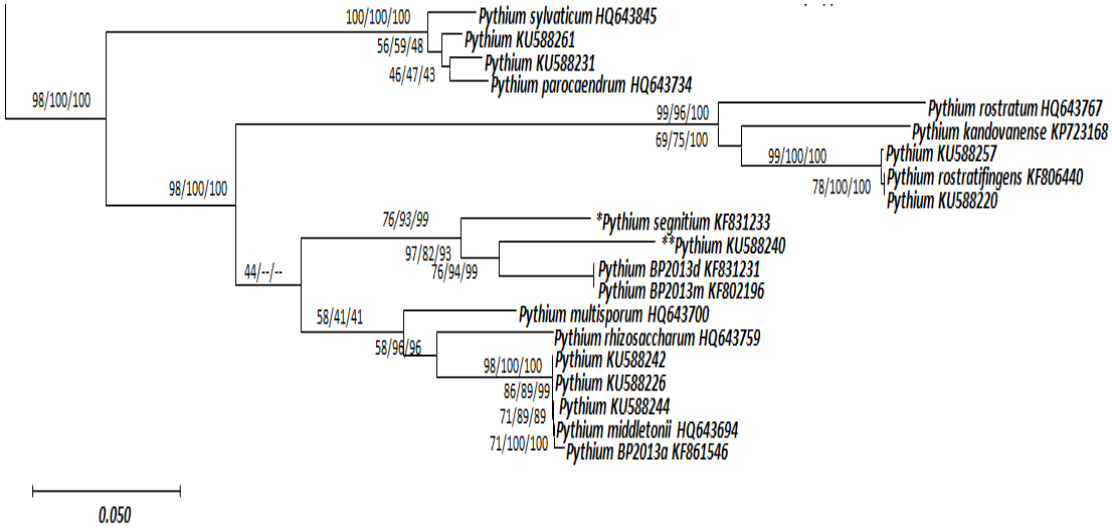

Fig. 02: The Phylogeny reconstructed using the Maximum Parsimony, RaxML and Mr. Bayes softwares. The respective percentages of replicate trees in which the associated sequences clustered together are shown on the branches.

We distinguish three subclades, namely *P. Rostrifengens*, *P. Middeltonii and P. Segnitium*; the latter includes the studied sequence holding in Genbank, the ITS accession number ***KU588240***, with very strong support provided by Maximum parsimony, Maximum likelihood and Mr. Bayes methods.

Molecular Description:

Clustalw alignment permitted to compare the new taxon ITS sequence with its three closest relatives (figure 03).

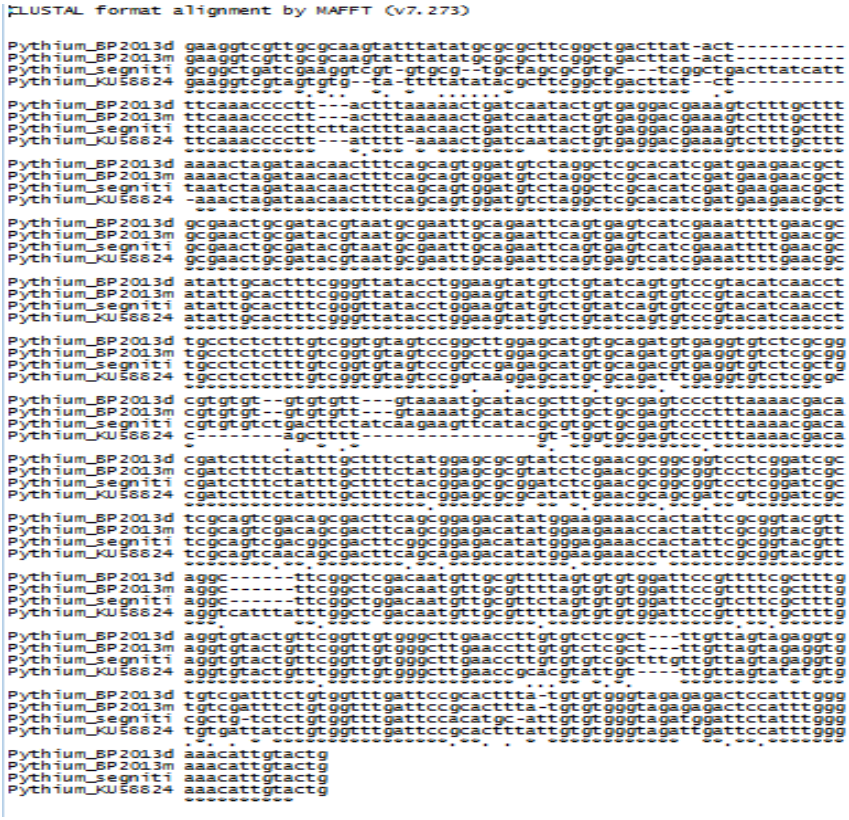

Fig. 03: Clustalw alignement of *P. KU588240* with its references *P. segnitium*, *P. PB2013d* and *P. PB2013m*.

The clustalw alignement obtained by online Mafft aligner of the taxon KU588240 with its closest relatives along 733bp, shows a minimum 9.68% of divergence distributed as 5.6% substitutions, 3.27% deletions and 0.81% insertions.

Pythium mediterraneum sp. nov. Etymology:

'mediterreneum' refers to the climate level where the new species and its related taxa, are usually found.

Morphology:

The mycelium is typical to Pythium cultures on V8 agar (figure 4), with radial centrifuge growth and whitish aggregates resembling leaves blight in the field, start to be formed on the mycelium after 10 days incubation at room temperature, in total obscurity. They represent abundant mature sporangial aggregates, which appear in figure 6, under the photon microscope.

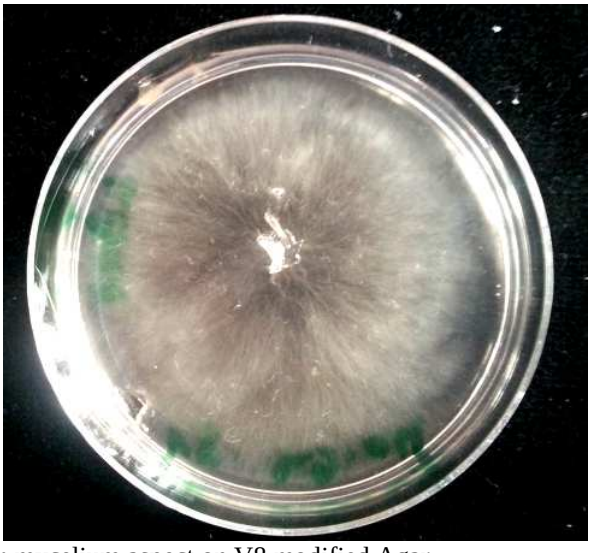

Fig. 4: *Pythium mediterraneum* mycelium aspect on V8 modified Agar.

The studied isolate is morphologically close to *P. Segnitium* subclade from clade E, with ovoid to elongate internally proliferating asexual sporangia (figure 05).

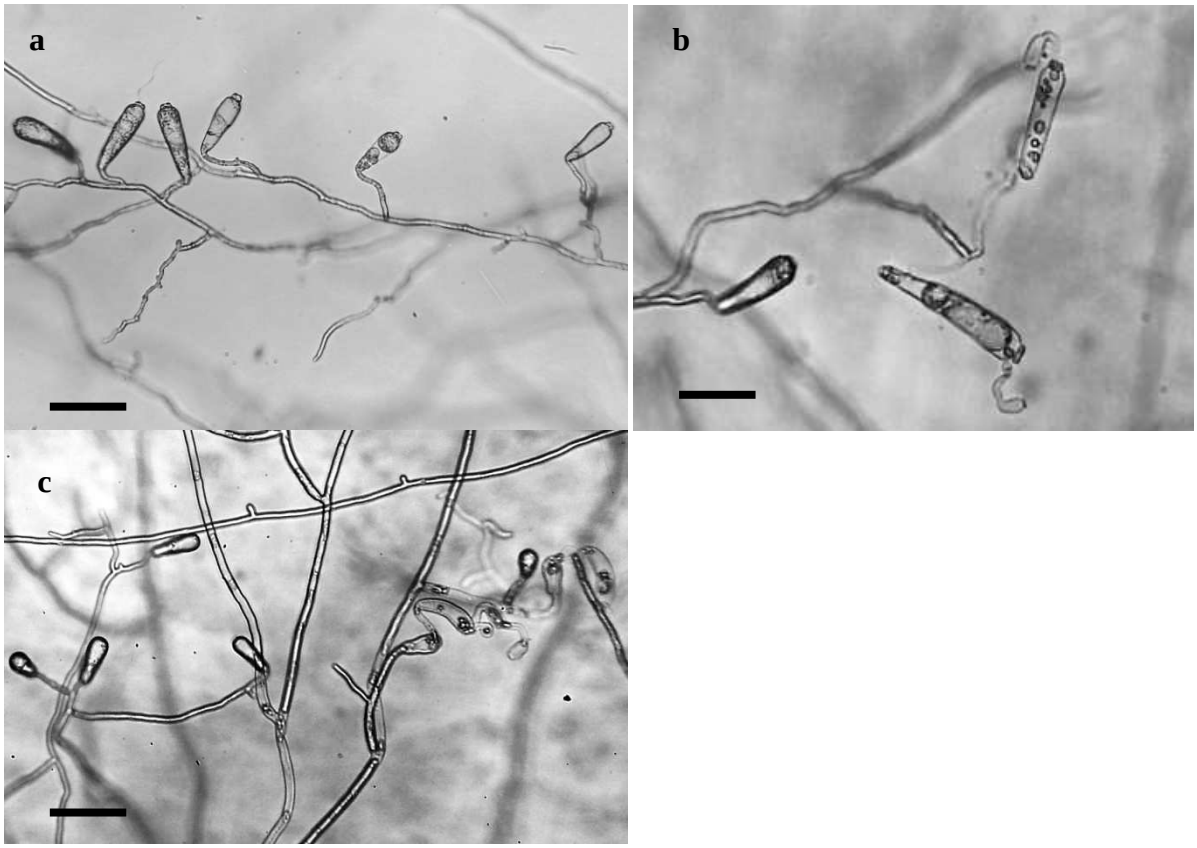

Fig. 05: *Pythium mediterraneum* microscopic description. (a) Elongated piriform asexual, internally

proliferating sporangia (b) at maturity a unique apical orifice generates juvenile sporangia developed continually in chain arrangement (c) more than two successive sporangia borne in a chain/ (a) Scale bar =50µm; (b) Scale bar =25µm; (c) Scale bar =50µm.

The morphological characteristics show an evolved aspect within *Clade* E lower globose and often internally proliferating sporangia (Marano et al., 2014). They all are likely to be ancestral [22]. In the same subclade we can cite a morphologically distinct species with elongated sporangia, such as *P. longandrum* described by Paul [17].

Conclusion:

The chosen geographic area of our study seems to be a real museum for many ascendant taxa of the lowest *Pythium* species in the phylogenic tree regarding the literature. The studied taxon was isolated from decomposing feathers plumed in the lake waters during 2013 winter season, and is supposed to be indigenous of the studied region since all of its relatives are originated from the Mediterranean periphery or similar warm areas. Thus, we suggest the name *P. mediterraneum* to be added to the *Pythium segnitium*-like database.

Based on global and raw phylogenic data, *Pythium* species of clade E, are the most primitives among the *Pythiaceae* population, in Oubeira lake, with a weak distribution of around 12%. The phylogeny revealed an ancestral range within *Clade* E primitive members so characterized by globose and often internally proliferating sporangia. They all are likely to be ancestral [22], but furthermore, the earliest true Pythim individuals in the Oubeira lake.

Typical morphology characterizes P. mediterraneum, at different sporangial growth stages is particularly specific to the member of clade E, with globose elongated sponagia, internally proliferating.

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