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Parvularia atlantis Gen. et Sp. Nov., a Nucleariid Filose Amoeba (Holomycota, Opisthokonta)

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Abstract

The opisthokonts constitute a eukaryotic supergroup divided into two main clades: the holozoans, which include animals and their unicellular relatives, and the holomycotans, which include fungi, opisthosporidians and nucleariids. Nucleariids are phagotrophic filose amoebae that phenotypically resemble more their distant holozoan cousins than their holomycotan phylogenetic relatives. Despite their evolutionary interest, the diversity and internal phylogenetic relationships within the nucleariids remain poorly studied. Here we formally describe and characterize by molecular phylogeny and microscopy observations *Parvularia atlantis* gen. et sp. nov. (formerly *Nuclearia* sp. ATCC 50694), and compare its features with those of other nucleariid genera. *Parvularia* is an amoebal genus characterized by radiating knobbed and branching filopodia. It exhibits prominent vacuoles observable under light microscopy, a cyst-like stage, and completely lacks cilia. *P. atlantis* possesses one or two nuclei with a central nucleolus, and mitochondria with flat or discoid cristae. These morphological features, although typical of nucleariids, represent a combination of characters different to those of any other described *Nuclearia* species. Likewise, 18S rRNA-based phylogenetic analyses show that *P. atlantis* represents a distinct lineage within the nucleariids.

Keywords

Protist evolution; nucleariid amoeba; protist diversity; filosea; taxonomy; phylogeny

FROM a taxonomic point of view, Opisthokonta is considered one of the largest eukaryotic supergroups (Adl et al. 2012). It is divided into two clades: the Holozoa, which contains animals and their unicellular relatives (Lang et al. 2002), and the Holomycota (Liu et al.

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2009), also known as Nucletmycea (Brown et al. 2009). Holomycota contains Fungi (Moore 1980, Spatafora et al. 2016), the parasitic Opisthosporidia (Karpov et al. 2014) and the freeliving nucleariid amoebae (Cavalier-Smith 1993). The nucleariid amoebae comprise only two genera confirmed by molecular phylogeny. One is *Nuclearia* Cienkowski, 1865 (Patterson 1984), which includes species with typical spherical cell morphology that feed on filamentous cyanobacteria in freshwater environments. Half a dozen *Nuclearia* species have been properly described, and are relatively easy to find and grow in culture conditions (e.g. Yoshida et al. 2009). Recently, several additional *Nuclearia* strains have been isolated and thoroughly studied both from morphological and molecular phylogeny standpoints. These strains harbor endo- and/or ectosymbionts, which pose interesting ecological and evolutionary questions (Dirren and Posch 2016). The second described genus, *Fonticula*, is sister to *Nuclearia* and contains only one species, *Fonticula alba* (Worley et al. 1979). This species presents an aggregative behavior during its life cycle in which amoebae crawl together to become a multicellular fruiting body; accordingly, *F. alba* was first considered to be a slime mold. In addition to these two genera, another genus of filose amoebae *Vampyrellidium* Zopf, 1885 (Surek and Melkonian 1980; Patterson et al. 1987) has been morphologically associated with nucleariids. *Vampyrellidium* has only two species described. However, these species are not available in culture and we lack sequenced molecular markers for them. In addition to a phagocytic feeding mode, *Vampyrellidium* amoebae are able to penetrate the cell wall of algae using a specialized flattened pseudopodium. Other genera morphologically associated to nucleariids (no sequences available) are the scale-bearing *Pinaciophora* Greef, 1869, *Pompholyxophrys* Archer 1869 and *Lithocolla* Shulze 1874 (Mikrjukov 1999).

Morphologically, all nucleariid genera lacking scales (*Nuclearia*, *Fonticula* and *Vampyrellidium*) consist of non-flagellated amoebae, with radiating thin hyaline pseudopods (i.e., branching filopodia). Cell shape is typically spherical when floating and flattened when adhering to the substrate although these amoebae are extremely plastic and can adopt elongated forms. The cytoplasm contains one or more prominent nuclei, dictyosomes, mitochondria with flat cristae and at least one big digestive vacuole that can occupy most of the cell body. Cysts have also been observed in some *Nuclearia* species (*N. pattersoni*, *N. simplex* and *N. rubra*) (Yoshida et al. 2009) and in *F. alba*, but not in *Vampyrellidium perforans*. Nucleariids differ widely from other holomycotan lineages, such as the parasitic Opisthosporidia (Karpov et al. 2014), the ancestor of which was most likely able to penetrate cell walls of various eukaryotic species to phagocytize them; or the ancestrally osmotrophic fungi, which feed by secreting extracellular enzymes to process complex food sources and absorb simpler molecules (Richards and Talbot 2013).

The systematics of the nucleariid filose amoebae is still not clear. In addition, other amoebae have been proposed to be candidate “nucleariids” based solely on morphological characters (Mikrjukov 1999; Patterson et al. 2000). Molecular phylogenetic analyses place the nucleariid amoebae in a pivotal position as an early-branching lineage close to the root of the Opisthokonta, which makes this group of primal importance to understand the early evolution of this eukaryotic supergroup. Therefore it is essential to have first a robust reference phylogenetic tree with the widest possible representation of the diversity of the lineages involved. The starting point of this process is accurately describing species and

placing them in a molecular phylogenetic framework. In this study, we describe the isolate called *Nuclearia* sp. ATCC 50694, which we classify into the new genus and species *Parvularia atlantis* gen. et sp. nov. Together with several environmental sequences, this genus represents the sister lineage to *Nuclearia*.

Material and Methods

Culturing *Parvularia atlantis*

The original strain was obtained from the American Type Culture Collection (ATCC, Teddington, UK) under the name *Nuclearia* sp. ATCC 50694, deposited by TK Sawyer and originally isolated in 1997 from a freshwater sample in Atlanta, GA, USA (see comments on the taxonomic description section for a list of papers where this species was used under this undetermined name). The culture grows in liquid ATCC Medium 802 (Sonneborn's *Paramecium* medium) bacterized with *Klebsiella pneumoniae subsp. pneumoniae* (ATTC 700831). Cultures were grown and monitored at room temperature during several weeks, usually up to a month, before transferring them to fresh medium.

Molecular phylogenetic analyses

The sequence of the *Parvularia atlantis* small subunit ribosomal gene (18S rRNA gene) was obtained after RNA extraction using TRizol reagent™ (Invitrogen, Carlsbad, CA, USA) and retrotranscription with Superscript® II (Invitrogen) using the 18S rRNA reverse primer Euk-B (5′-GATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988). Two overlapping fragments of the 18S rRNA gene were subsequently amplified by PCR using the primer combination A (5′-AACCTGGTTGATCCTGCCAGT) with 1055R (5′-CGGCCATGCACCACC); and 528F (5′-CGGTAATTCCAGCTCC) with B (5′-TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988; Carr et al. 2008). The amplified DNA fragments were cloned (TOPO TA, Invitrogen) and Sanger sequenced (Genomics facility UPF, Barcelona, Spain). We obtained a consensus 18S rRNA gene sequence of 1,675 bp deposited in GenBank with accession number KY113120. Next, we compared its sequence identity to other nucleariid representative sequences: *Fonticula alba* FJ816018, *Nuclearia pattersoni* AY364635 and *Nuclearia thermophila* AB433328, using BLASTn pairwise alignments. We included this sequence in an 18S rRNA dataset containing sequences from known nucleariid species and all available environmental nucleariid sequences identified in GenBank. To retrieve the latter, we carried out BLAST+ (Camacho et al. 2009) searches with the four mentioned nucleariid sequences as queries and retrieved the 200 first hits for each one. We merged all the hits and extracted all unique sequences to build a phylogenetic tree together with representative sequences of opisthokonts and other eukaryote groups (i.e. Apusomonadida, Breviatea, Amoebozoa, SAR, Archaeplastida and Excavata) to verify the ascription of these sequences to the nucleariids. Once we identified all nucleariid sequences, we discarded most outgroup and identical nucleariid clone sequences. We then defined operational taxonomic units (OTUs) applying a 98% identity threshold and retained the longest sequence for each OTU. We also included four environmental sequences from a recent molecular survey of freshwater systems (Simon et al. 2015), and 85 marine environmental sequences with nucleariid affinity from the BioMarks project (del Campo et al. 2015). Our final 18S rRNA dataset comprised a total of 131

sequences (see Table S1). We aligned them using MAFFT (Katoh et al. 2002) with the E-INS-i algorithm and obtained a multiple sequence alignment of 2,348 sites. After manually trimming sequence ends, indels and spuriously aligned sites we ended up with a total of 1,595 sites. We inferred phylogenetic trees from this alignment using both Bayesian Inference (BI) and Maximum Likelihood (ML) methods. We performed BI analysis with MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003) using the GTR model of nucleotide substitution, taking into account rate variation amongst sites with a gamma distribution (approximated by four categories) plus an invariable sites category. Four chains ran for 4,400,000 generations and were analyzed after a burn-in of 25%. For ML analyses, we used RAxML v8 (Stamatakis 2014) with the same model parameters as in the Bayesian analysis to search for the best tree out of a thousand, and to perform 1000 non-parametric bootstraps. All trees were visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and modified with Inkscape vector graphics software (inkscape.org). The trimmed alignment and phylogenetic trees can be found in https://figshare.com/s/1b8a7f7806816eb0e1c1.

Light Microscopy

Differential interference contrast images (DIC) were taken in an Axioplan 2 microscope with DIC objective Plan-NEOFLUAR 100x/ 1.3 oil (Zeiss, Goettingen, Germany) and captured with the software AxioVision v4.8.2. Phase contrast (PH) images were taken in an Axio Observer.Z1 microscope with 63x objective Plan-Apochromat 63x/1.4 oil (Zeiss) and captured with ZEN software v2012. Images obtained were edited with Fiji v1.47 (Schindelin et al. 2012). Time-lapses at low magnification (400x) were done on a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Movies can be found in https://figshare.com/s/1b8a7f7806816eb0e1c1.

Transmission electron microscopy

An actively growing culture (15 days old) was centrifuged at 10,000 *g* during 5 minutes at 10 ºC. Lower speeds for extended times did not produce concentrated pellets suitable for posterior observation. Samples were cryoimmobilized using a Leica EMPACT High-Pressure Freezer (Leica Microsystems, Vienna, Austria). Planchettes containing the frozen samples were transferred to cryotubes containing 2% OsO_4 (EMS, Hatfield, PA, USA) 0.1% uranyl acetate (EMS) in acetone under liquid nitrogen and freeze-substitution was performed at -90 °C for 85 h in a EM AFS (Leica Microsystems). Samples were warmed up to 4 °C with a rate of 5 °C/h, kept at 4 °C for 2 h, and then they were transferred to room temperature and kept for 2 h in darkness. They were rinsed with acetone, then infiltrated and embedded in Epon- 812 resin (EMS). Sample sections of 60 nm in thickness were obtained using a UCT ultramicrotome (Leica Microsystems), and then stained with methanolic 2% uranyl acetate and lead citrate. Sections were observed in a Tecnai Spirit microscope (EM) (FEI, Eindhoven, The Netherlands) equipped with a LaB6 cathode. Images were acquired at 120 kV with a 1376 x 1024 pixel CCD camera (FEI).

Scanning electron microscopy

As above, we used an actively growing culture (15 days old). The bottom of the culture flask was scrapped softly in order to obtain both adherent and floating cells. Cells were fixed by adding directly 250 µl of OsO_4 4% (Sigma-Aldrich, Saint Louis, MO, USA). The sample

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remained 90 minutes in the dark, and the solution was passed through a Whatman Nucleopore filter membrane (0.8 μm pore-diameter filter, previously treated with Poly-LLysine (Sigma-Aldrich)) to capture the fixed cells. The filter was submerged overnight at 4 ºC in phosphate buffer saline (PBS, 0.01 M phosphate buffer saline, NaCl 0.138M, KCl 0.0027M at pH 7.4, Sigma-Aldrich). Then the sample was dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95% (twice) and 100% (twice) for 15 minutes each), critical point dried with CO_2 (Critical Point Dryer, Bal-Tec CPD 030) and sputtercoated with gold. Finally, the sample was examined under a Hitachi S-3500 N microscope (Hitachi, Tokyo, Japan) operating at 5 kV.

Fluorescent cell staining

The bottom of a 15-days old culture was carefully scraped and 2 ml were transferred to a glass bottom dish (LabClinics, Barcelona, Spain) for confocal microscopy observation. The culture was left overnight in order to let *Parvularia atlantis* cells properly adhere to the bottom of the dish. Then the medium was removed by pipetting carefully and cells were fixed with 6% acetone and 4% formaldehyde for 5 minutes. Cells were rinsed with PBS for 5 minutes prior to the addition of a solution containing Alexa Fluor®phalloidin 488 (Thermo Scientific, Waltham, MA, USA) and DRAQ5 (Thermo Scientific) in the following proportions: 1/40 and 1/100. Cells were incubated with the dyes for 30 minutes in dark conditions. After two PBS washes, the samples were mounted with Dako medium (Agilent Technologies, Santa Clara, CA, USA) for microscopy observation on a Leica TC5 SP5 confocal microscope (Leica microsystems, Germany).

Results

Morphological observations

Parvularia atlantis gen. et sp. nov. (formerly *Nuclearia* sp. ATCC 50694) presents two different cellular forms in our culture conditions: amoeba and cyst-like. These forms are not easy to distinguish using low magnification (200x and 400x). In order to reveal particular cellular structures of each form we imaged *P. atlantis* at 630x and 1000x magnification using light microscopy, with phase contrast (PH) and differential interference contrast (DIC) techniques. We also carried out transmission and scanning electron microscopies (TEM and SEM).

Parvularia atlantis cells are filose amoebae with a quasi-spherical cell body (Fig. 1A). Cell diameter (without pseudopodia) varies between 3.36 and 5.92 μm with a mean of 4.34 μm; n = 114 cells. Filopodia radiate from the cell body in all directions, often branching but never anastomosing. Although density, length and direction can be variable, short filopodia are abundant and long ones are scarce and several times longer than the cell body (Fig. 1B, C, S1A). Phalloidin staining, applied for the first time in nucleariids, clearly showed the actin-based cytoskeleton extending inside these long fine filamentous pseudopods in fixed *P. atlantis* cells (Fig. 1D, S2). Cells can be floating or attached to the substrate either by the whole cell body (Fig. 1B) or only by the filopodia (Fig. 1C). When amoebae cells are fully attached, they can adopt elongated crawling forms (Fig. 1B, S1B). Light microscopy images show that *P.atlantis* amoeboid cells can have two nuclei (Fig. 1E). The nuclei contain a

clearly visible central nucleolus (Fig. 1F). Furthermore, amoebae present a big vacuole (Fig. 1G, S1B) and several smaller vesicles. *P. atlantis* cells feed on rod-shaped bacteria. Amoebae cells often appear surrounded by many bacterial cells (Fig. 1H, Movie S1), with a kind of halo between the amoebae and the bacteria.

Cyst-like forms are also frequent in *P. atlantis* cultures. This cyst-like form is spherical as seen both in PH and DIC, with a thicker silhouette and a bright side in opposition to an intense shadow, respectively, and lacks filopodia. In consequence, cysts do not attach but float or rest on the substrate (Fig. 1I, S1C, D). The cyst cell is less translucent than in amoebae preventing a good observation of its content, although sometimes vesicles can be seen in DIC and PH. Time-lapse videos (Movie S2) show that while amoebae actively crawl on the surface feeding on bacteria, cyst-like forms remain still, without active movement. These videos also show a transition from a cyst-like to the active amoeba form (Movie S3). Transmission electron microscopy (TEM) images from centrifuged cyst-like forms always show an extracellular envelope; i.e., a cell wall separated from the main cell body (Fig 2). The limited quality of our TEM observations revealed few details of the *P. atlantis* ultrastructure. Basically, all cells have a big vacuole and some smaller vesicles (Fig. 2A) and the few mitochondria observed in detail had flat or discoidal cristae (Fig. 2C). Also, TEM observations provided snapshots of cell division, probably closed since nuclear membranes are always intact (Fig. 2B, D-F). Interestingly, the cell in Fig. 2D shows the cleavage furrow, and Fig. 2F and 2E show two and three cells within the same extracellular envelope. No other ultrastructural cell features, such dictyosomes or microtubules, were observed.

Molecular phylogeny

We determined the *P. atlantis* 18S rRNA gene sequence, which was 92%, 89% and 86% identical to *Nuclearia pattersoni* (AY364635), *N. thermophila* (AB433328) and *Fonticula alba* (FJ816018), respectively. Logically, pair-wise identity between *N. pattersoni* and *N. thermophila* was the highest: 95%. The similarity between *F. alba* and *N. pattersoni* and *N. thermophila* was much lower, 90% and 91%, respectively. The multiple sequence alignment showed that all available *Nuclearia* 18 rRNA sequences exhibit two exclusive insertions in the variable regions V4 and V7, absent in the sequences from *P. atlantis*, *F. alba* and the rest of opisthokont lineages (Fig. S3).

Then, we inferred the phylogenetic position of *P. atlantis* by reconstructing an 18S rRNA gene tree (Fig. 3). After mining different databases (see methods), we retrieved all publicly available nucleariid 18S rRNA gene sequences, including both cultured species and environmental sequences, in order to use the broadest possible molecular diversity. Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of the gathered dataset resulted in the same backbone for the opisthokont phylogenetic tree. The statistical support for the monophyly of the nucleariids was high in BI analysis (0.96 posterior probability (pp)) but weak in ML trees (only 23% bootstrap support (bs)); see Fig. 3, S4, S5. Yet, this topology agreed with previous phylogenomic analyses where *Nuclearia* spp. and *F. alba* (Liu et al. 2009) on the one hand, and *F. alba* and *P. atlantis* ("*Nuclearia* sp. ATCC 50694") on the other hand (Torruella et al. 2015), branched together. In our phylogenetic analyses (Fig. 3), *F. alba* was sister to the marine environmental clade called MAFO (del Campo et al. 2015).

However, the low statistical support for this relationship (0.62 pp and 14% bs) did not allow to reliably conclude the monophyly of the two groups. The *Nuclearia* clade, with a better statistical support (0.98 pp and 61% bs), contained all the *Nuclearia* species for which the 18S rRNA gene sequence is available plus some related environmental sequences. In addition, a group of four environmental nucleariid sequences (env NUC-1) branched as sister of *Nuclearia* spp. (Fig.3, S3, S4). The *Parvularia* clade, previously referred to as FRESHOP (see del Campo and Ruiz-Trillo 2013), contains not only the species described in this study but also five environmental sequences forming a clear monophyletic group with high statistical support (1.0 pp and 100% bs). In our tree, the *Parvularia* clade branched within a group of environmental nucleariid sequences (env NUC- 2), albeit with very low statistical support (0.81 pp and 16% bs).

Discussion

Our 18S rRNA gene phylogenetic analysis clearly show that *Parvularia atlantis* gen. et sp. nov. represents a distinct phylogenetic lineage from the other described nucleariids (Fig. 3). This is further supported by the fact that the *Parvularia* 18S rRNA gene sequence shares similar low pairwise identities with *Fonticula* and *Nuclearia* spp. and does not show the V4 and V7 insertions present in *Nuclearia* sequences (Fig. S3). All these results concur to support the erection of the new genus *Parvularia*. This is important, because the characterization of this new genus enriches the limited diversity known for the group and helps clarifying the taxonomy and phylogeny of the nucleariids. *P. atlantis* was originally deposited without formal description as *Nuclearia* sp. ATCC 50694, then sequenced and tentatively named as such (de Mendoza et al. 2013; Suga et al. 2014; Torruella et al. 2015). The misidentification of opisthokont amoebae as *Nuclearia* sp., followed by their reclassification after molecular phylogenetic analysis, is not infrequent. For instance, the filasterean *Capsaspora owczarzaki* was initially described as *Nuclearia* sp. (Zettler et al. 2001) until it was properly placed as a unicellular holozoan relative of animals (Hertel, et al. 2002; Ruiz-Trillo et al. 2004). Regarding the diversity of nucleariids, our data show that, in addition to *Fonticula*, *Nuclearia* and *Parvularia,* there are other nucleariid groups currently composed only by environmental sequences, such as the marine MAFO (del Campo et al. 2015) and the freshwater env-NUC1 and env-NUC2 (Fig. S4, S5). These environmental groups likely correspond to undescribed genera, highlighting a wider diversity than previously thought for the group. Determining the proper phylogenetic boundaries of nucleariids, also requires the molecular study of other filose amoebae of uncertain affinities, e.g., genera such *Vampyrellidium*, *Pinaciophora*, *Pompholyxophrys* or *Lithocolla* (Mirkjukov 1999, Patterson, et al. 2000). As mentioned before, without molecular phylogenetic data, some genera of filose amoebae have been originally assigned to nucleariids based on morphology (e.g., Cavalier-Smith and Chao 2012) but subsequently shown to be members of other eukaryotic supergroups, such the rhizarian vampyrellids (Hess et al. 2012); not to confuse with *Vampyrellidium* Zopf, 1885 (Surek and Melkonian 1980). Finally, our 18S rRNA phylogenetic results show limited phylogenetic signal (Fig. 3, S4, S5), so in order to confirm the monophyly of nucleariids and to properly solve their internal evolutionary relationships, more markers and more isolates should be included in phylogenetic analyses.

Phenotypic traits used to classify nucleariids may be inadequately defined or differently interpreted by distinct researchers. Recently, Dirren and Posch (Dirren and Posch 2016) noted that all morphological characters typically associated with the genus *Nuclearia* can be observed or not depending on the culture and observation conditions. For example, the cell size can vary during long culture periods. Morphologically, *P. atlantis* clearly resembles a nucleariid, but differs from all previously characterized nucleariid species in several of the features traditionally used for *Nuclearia* classification (Patterson 1984; Yoshida et al. 2009). One of the most obvious differences refers to the size range: *Vampyrellidium perforans* ranges from 4 to 25 μm, the smaller *F. alba* from 7 to 13 μm, and *Nuclearia* spp. from a few to a hundred micrometres. In fact, the smallest described *Nuclearia* species (for which sequence data are not available) is *N. leuckarti* (cell diameter around 10 μm or less) (Yoshida et al. 2009). However, *P. atlantis* never reaches cell sizes over 6 microns, even in elongated forms (Fig. 1B, S1B). In addition, *P. atlantis* presents a cyst-like form, and cells can contain two nuclei, features that are not found in *N. leuckarti*. Other *Nuclearia* species for which we also lack sequence data, such as *N. polypodia*, *N. flavescens*, *N. radians*, *N. flavocapsulata* and *N. rubra,* are all described as uninucleate, and only the latter was described as able to form cysts. Even the genus *Vampyrellidium* (Surek and Melkonian 1980) seems to be morphologically more similar to *Nuclearia* species than to *Parvularia*, being uninucleate, around 20 μm in cell diameter and unable to encyst. Therefore, *P. atlantis* is molecularly and morphologically divergent from any nucleariid species known so far. Associating unambiguous morphological features to species confidently placed in molecular phylogenetic trees is crucial to understand macroevolution. Phenotypic characters shared by *P. atlantis* and other nucleariids can be used to reconstruct ancestral character states for this group. For example, *P. atlantis* shares many features with other nucleariids. All of them are plastic amoebae with branching filopodia that can have spherical to flattened cell shapes (Surek and Melkonian 1980; Patterson 1984; Brown et al. 2009; Yoshida et al. 2009). The prominent nucleus with a conspicuous central nucleolus is also a common feature in *Vampyrellidium perforans* (Fig. 16 in Surek and Melkonian 1980), *Fonticula alba* (Fig. 2B in Brown et al. 2009) and most *Nuclearia* species (Yoshida et al. 2011). *P. atlantis* amoeba stage can be binucleated as also described in *F. alba*, *N. delicatula*, *Nuclearia* sp. strain A5 and *N. thermophila* (Dirren and Posch 2016). The presence of cysts or cyst-like forms is also recurrent in nucleariids, and assumed to be ancestral in *Nuclearia* spp. (Dirren and Posch 2016). Since *F. alba* and *P. atlantis* also produce cysts or cyst-like structures (Fig. 1I, S1C, S1D), we can assume that this capacity is ancestral to the whole nucleariid group. The ontology of these cysts has never been studied in detail. Whether they represent a resting or dormant form under adverse conditions, a digestive form that does not need filopodia to prey, or a sexual (i.e., sporocyst) or division stage of the life cycle, is unknown. In *P. atlantis,* light microscopy observations suggest that cyst-like forms are passively motile. Unfortunately, the cell body is opaque, such that inner activity cannot be monitored (e.g., live staining molecules such phalloidin do not penetrate). On the other hand, TEM images show that cyst-like forms, with a dense cell envelope (i.e., cell wall), can be in process of division (Fig. 2B, D, F). It is worth mentioning that despite the lack of molecular data for *Nuclearia* spp., it seems unlikely that any of these coats could contain chitin as in other holomycotan lineages (James and Berbee 2012), since the necessary chitin synthase classes were not found in genomic data of *F. alba* and transcriptomic data of *P. atlantis* (Torruella et

al. 2015). Both the cell wall composition and cyst-like state physiology should be further explored in nucleariids. Another interesting external character in nucleariids is the secretion of extracellular material embedding the trophic cells. In *F. alba*, this extracellular material is used to build the elevated stalk that constitutes the multicellular fruiting body (Deasey and Olive 1981), and in *Nuclearia* spp. it has been recently proposed to host symbiotic bacteria that may protect the amoeba from cyanobacterial toxins (Dirren and Posch 2016). In *P. atlantis,* the presence of this extracellular coat is not clear. Amoebae in culture conditions are often surrounded by a translucent perimeter space surrounded by bacteria (Fig. 1H). Although observations under higher magnification have not clearly revealed a mucus sheath as seen in other nucleariids, it can be hypothesized that *P. atlantis* produces hyaline extracellular material under particular conditions.

In summary, we can conclude that the last common ancestor of nucleariid-like genera was an unciliated amoeboid cell with thin branching filopodia, binucleated and encysted at some point of its life cycle. To further validate such evolutionary scenario, further cultivation, description and molecular phylogenetic identification of new nucleariids will be needed. Even without culturing, cell isolation followed by transcriptome or genome sequencing by the use of single cell ‘omics’ techniques will increase the data needed to perform thorough comparative studies between extant living species. Linking phenotypes and genotypes of protists representing a good taxonomic sampling will help unveiling the deep history of opisthokont evolution.

Taxonomical description

Based on the presented data we formally describe the *Nuclearia* sp. ATCC 50694 strain as a new genus and species, *Parvularia atlantis* López-Escardó & Torruella, gen. et sp. nov. within Nucleariidae, Holomycota, Opisthokonta. *Parvularia* López-Escardó & Torruella, gen. nov. Fresh-water free-living nucleariid: small spherical to elongated filose amoeba with cyst-like forms. Phylogenetically distant from *Fonticula* and *Nuclearia* genera, 18S rRNA gene sequence having less than 95% pair-wise identity with *Fonticula* and *Nuclearia* sequences and lacking the V4 and V7 insertions characteristic of *Nuclearia* spp. **Etymology:** *Parvularia*, from Latin *Parvus* (small); related to *Nuclearia*. **Type species:** *P. atlantis* López-Escardó & Torruella sp. nov. **Zoobank registration:** urn:lsid:zoobank.org:act:D177D3E9-09A8-46ED-91C2- BE1A33881F7C

Parvularia atlantis López-Escardó & Torruella sp. nov.

Spherical cell, diameter between 3 to 5 μm excluding the pseudopodia. Fine hyaline pseudopods radiate from any part of the cell body in variable density, length and direction, usually branching, never anastomosing. It feeds upon rod-shaped bacterial cells, not being able to feed on large cells such filamentous cyanobacteria or algae as *Nuclearia* spp. do. Slow motile trophic cells are uni- or binucleated with a big vacuole occupying most of the cell volume and smaller vesicles. Nuclei with a clearly visible central nucleolus. Cyst-like form has spherical shape embedded within a more or less opaque cell wall. Mitochondria with flat cristae.

Etymology: *atlantis*, from Atlanta (Georgia, USA), name of the original isolation site. **Type:** Fig. 1 López-Escardó et al., this publication. *Nuclearia* sp. ATCC 50694 strain was informally deposited in culture collection on 1997 by TK Sawyer. As reported by the culture collection: *it was isolated from a liquid freshwater sample obtained by Barry Fields in the CDC, Atlanta*, 339 Georgia, USA. **Comments:** This organism was originally submitted as *Nuclearia* sp. to the American Type Culture Collection, so this undetermined name was tentatively used in the RNAseq BioSample SRS725006 and in previous articles (de Mendoza et al. 2013; Suga et al. 2014; Torruella et al. 2015). The authors have tried to contact the people who originally isolated it without success; therefore more details on its ecological origin could not be clarified. **Gene sequence data:** The nearly complete 18S rRNA sequence has been deposited in GenBank under Accession Number KY113120.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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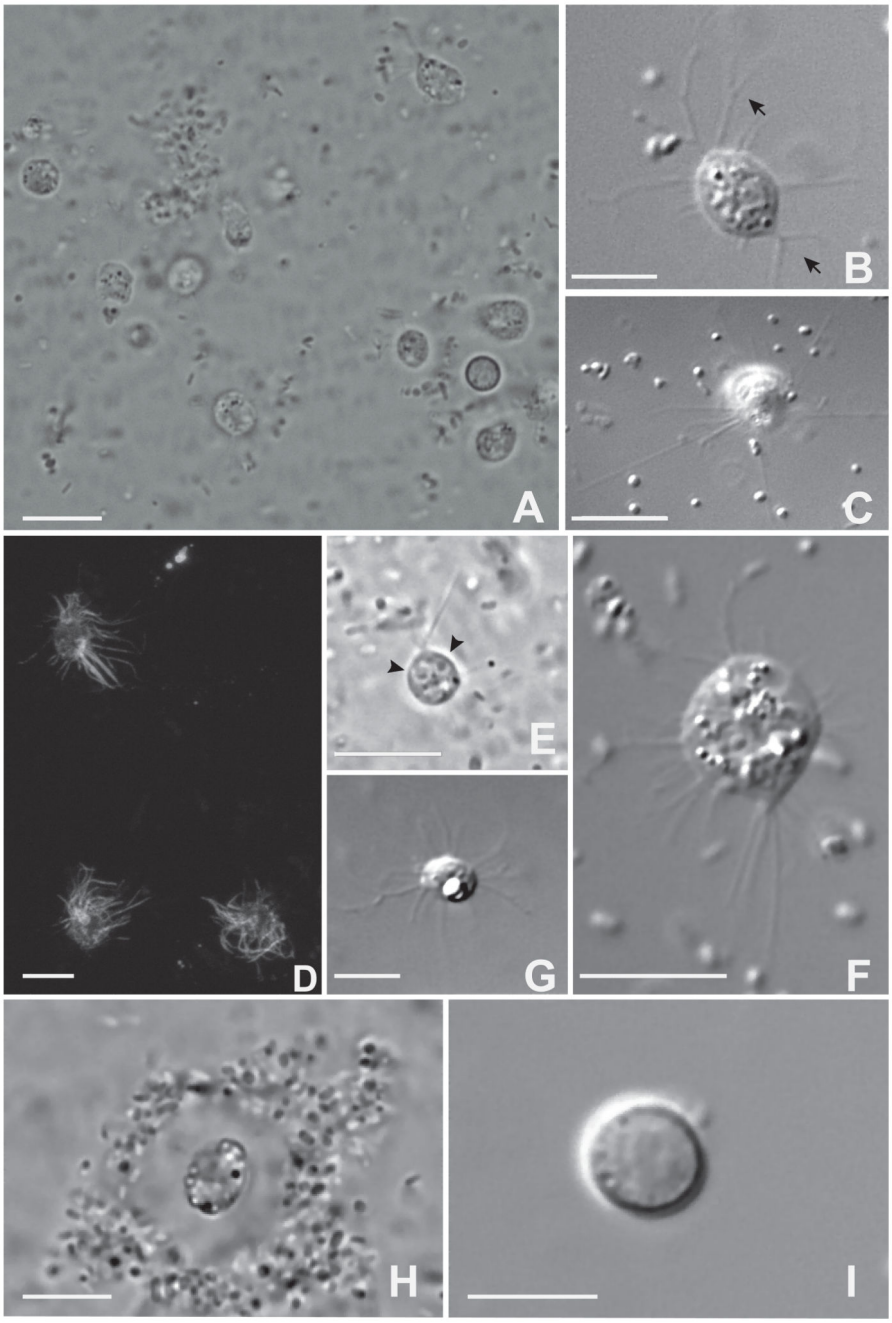

Fig. 1.

Morphology of *Parvularia atlantis* gen. et sp. nov. (A) living culture observed by light microscopy (phase contrast, 630x), the arrow indicates the cyst-like form, the other cells are amoebae. (B) Adherent filose amoeba, arrows indicate branching filopodia (DIC, 1000x). (C) Filopodia of *P. atlantis* cells attached to the slide bottom (DIC, 1000x). (D) Composite of 4 images (630x magnification) taken at different focus distances in order to represent together the different cell planes of filose amoebae stained with Phalloidin Alexa Fluor® 488 (related to Fig. S1 in supplementary material). (E) Amoeba with two nuclei

(arrowheads), both nucleolus can be observed as well, being darker than the rest of nuclear material (phase contrast, 1000x). (F) Amoeba with visible nucleus and a clear nucleolus in the middle (DIC, 1000x). (G) Amoeba with a big vacuole inside (DIC, 1000x). (H) Amoeba surrounded by bacterial cells leaving and space between them and the amoeba (phase contrast, 1000x) (related to Movie S3). (I) Cyst-like form (DIC, 1000x). Scale bar = (A) 10 μm; (B, G) 3 μm; (C, D, E, F, H, I) 5 μm.

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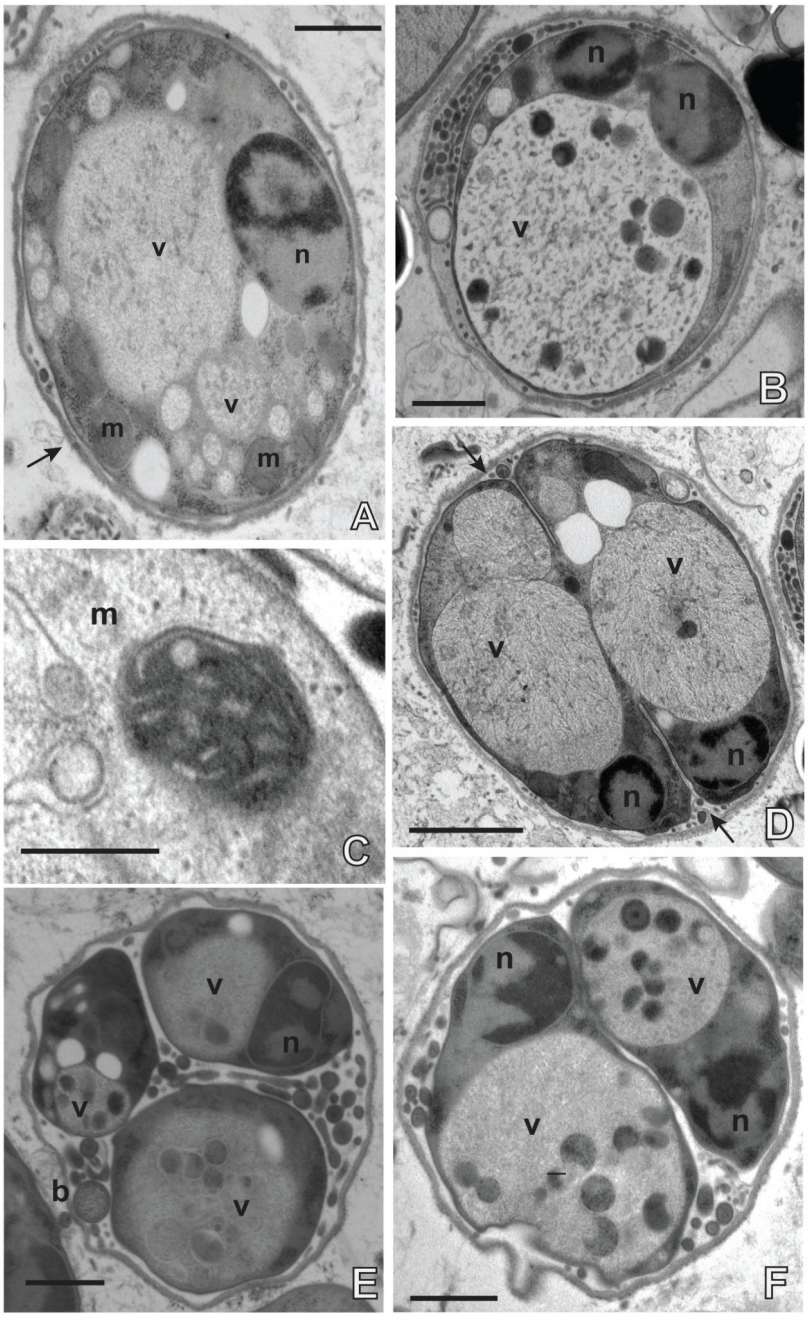

Fig. 2.

Ultrastructure of *Parvularia atlantis* gen. et sp. nov cyst-like forms. (A) Cyst-like cell with the extracellular coat (arrow). (B) Cell with two nuclei. (B, D, F) Cell division in cystlike forms. (C) Mitochondrion with flat or discoid cristae. (D) Cell undergoing cytokinesis. (F) Two cells within the same extracellular coat. (E) Three cells under the same extracellular coat. Mitochondrion (m), nucleus (n), vacuole (v). Scale bar = (A, B, D, E, F) 500 nm; (C) 200 nm.

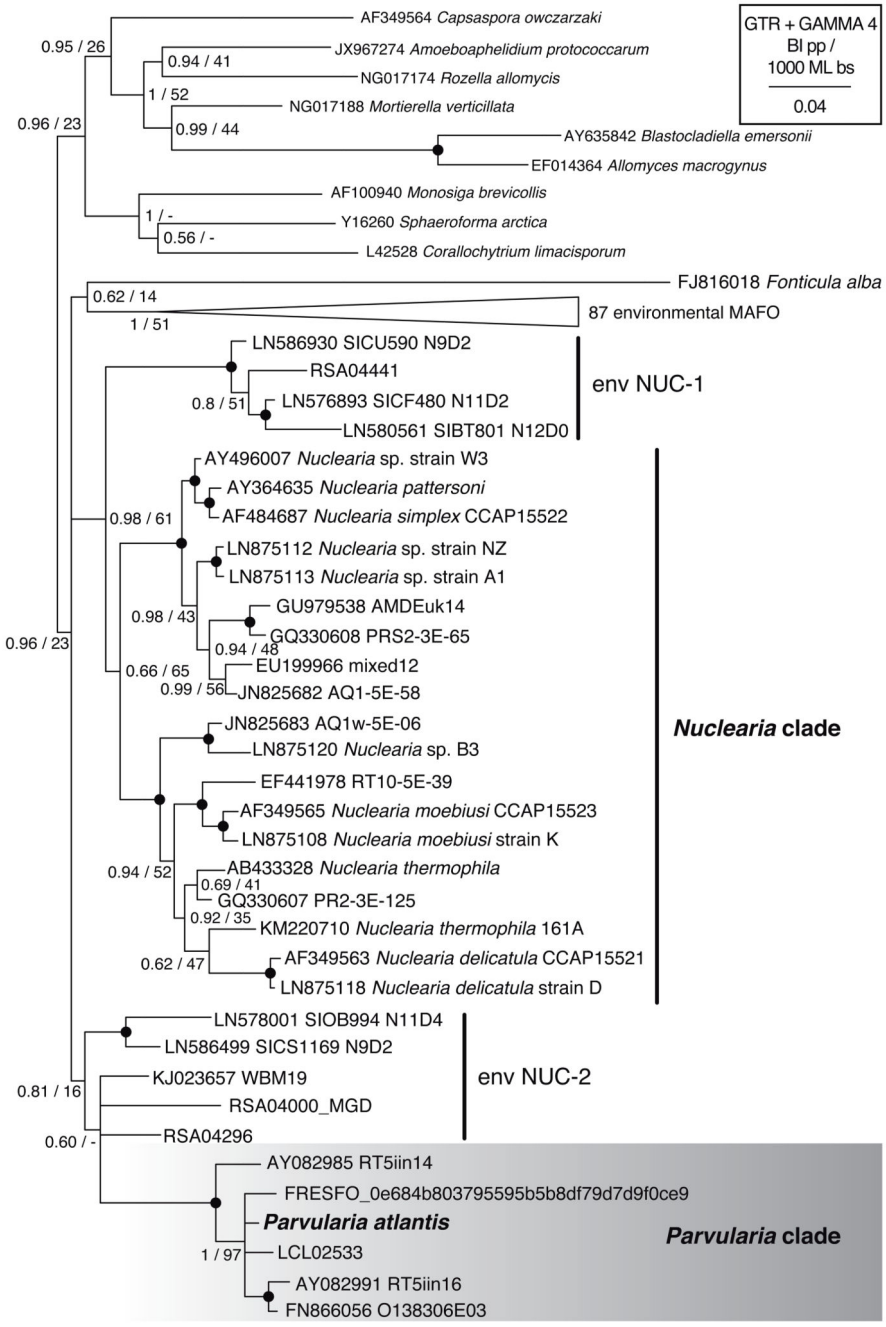

Fig. 3.

18S rRNA gene-based Bayesian phylogenetic tree showing the position of *Parvularia atlantis* gen. et sp. nov. The tree contains a representation of opisthokonts as outgroups and all nucleariid OTUs retrieved from GenBank. The tree was inferred using 1,595 conserved nucleotide positions. Statistical supports are shown only at deep nodes; Bayesian posterior probabilities (pp) are displayed on the left and Maximum Likelihood bootstrap (bs) values on the right. Black dots represent supports higher than 0.8 pp and 70% bs.