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Reticulamoeba Is a Long-Branched Granofilosean (Cercozoa) That Is Missing from Sequence Databases

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Abstract

We sequenced the 18S ribosomal RNA gene of seven isolates of the enigmatic marine amoeboflagellate Reticulamoeba Grell, which resolved into four genetically distinct Reticulamoeba lineages, two of which correspond to R. gemmipara Grell and R. minor Grell, another with a relatively large cell body forming lacunae, and another that has similarities to both R. minor and R. gemmipara but with a greater propensity to form cell clusters. These lineages together form a long-branched clade that branches within the cercozoan class Granofilosea (phylum Cercozoa), showing phylogenetic affinities with the genus Mesofila. The basic morphology of Reticulamoeba is a roundish or ovoid cell with a more or less irregular outline. Long and branched reticulopodia radiate from the cell. The reticulopodia bear granules that are bidirectionally motile. There is also a biflagellate dispersal stage. Reticulamoeba is frequently observed in coastal marine environmental samples. PCR primers specific to the Reticulamoeba clade confirm that it is a frequent member of benthic marine microbial communities, and is also found in brackish water sediments and freshwater biofilm. However, so far it has not been found in large molecular datasets such as the nucleotide database in NCBI GenBank, metagenomic datasets in Camera, and the marine microbial eukaryote sampling and sequencing consortium BioMarkS, although closely related lineages can be found in some of these datasets using a highly targeted approach. Therefore, although such datasets are very powerful tools in microbial ecology, they may, for several methodological reasons, fail to detect ecologically and evolutionary key lineages.

Introduction

The genus Reticulamoeba was created in 1994 by the distinguished protozoologist Karl Grell, in which study a single species, R. gemmipara was described [1]. This was followed in 1995 by a second species description, R. minor [2]. Both described species were isolated from the Mediterranean marine littoral zone, associated with diatoms, on which they feed. Both species are amoeba-flagellate; they have a stationary, more or less flattened amoeboid stage, of roundish to irregular outline and measuring c. 3–8 µm across. Thin reticulopodia radiate outwards from around the cell across the substrate, fusing at points to form networks that radiate out across the substrate. The area covered by the granular reticulopodia can be orders of magnitude greater than that occupied by the cell itself. Bidirectionally streaming granules (‘Körnchen’) can be seen on the reticulopodia. The reticulopodia themselves may move slowly, rearranging the size and shape of the network formed. When feeding, the reticulopodia penetrate diatom frustules rather than phagocytozing whole diatoms. Grell observed that networks from different individuals can fuse with each other, forming ‘feeding communities’, at least in R. gemmipara. He also describes a bi-flagellate stage, which is initially roundish in shape, becoming more irregular. These ‘swarmers’ or ‘zoospores’ have short anterior and long posterior flagella, and swim by active beating of the anterior flagellum, the posterior trailing behind. The flagellates can both swim and glide across a surface. They eventually settle, resorb their flagella and issue reticulopodia from around the cell, thereby transforming to the amoeboid stage. The main differences between R. gemmipara and R. minor are a) the flagellate and amoeboid stages of the latter are smaller, b) flagellate formation in R. minor occurs by fission of the amoeboid stage, resulting in two, four, or more zoospores, whereas in R. gemmipara zoospores are formed by unequal fission (budding) from the edge of the amoeboid cell.

The reticulate amoeba morphotype is generally very poorly known, and most studies concerning them fall into three main categories: 1) the original descriptions, usually without molecular data and in some cases ambiguous; 2) reports by other authors, often in passing or in a context where the prime focus is not the amoeba in question; and 3) more recent studies where a morphological description is complemented by the sequence of at least one phylogenetic marker gene and phylogenetic analysis. Since Grell’s work (category 1) there has been no definite recording of Reticulamoeba. The genus has been cited in passing a few times, e.g. [3–5], but never based on a robust identification (category 2). As of June 2012, five strains currently or previously referred to as Reticulamoeba are present in GenBank (JJP-2003 and
COHH 9, 96, 98, 99). The first corresponds to Filoveta marina [6], and all the others are very closely related to it and therefore not Reticulamoeba. In this paper we show that Reticulamoeba is in fact a granofiloscean cercozoan, and provide for the first time reliable 18S rDNA sequences for this genus, as well as describing morphological characteristics of novel lineages (category 3).

We used our new sequences to investigate the diversity and ecological distribution of Reticulamoeba further, by constructing and analyzing environmental SSU rDNA clone libraries, e.g. [7] using lineage-specific primers, by searching online sequence databases (e.g. NCBI GenBank nucleotide collection), and by mining 454 Sequencing datasets for sequences related to our cultured strains. The advent of high throughput, massively parallel sequencing technologies applied to environmental samples is currently revealing an even greater diversity of protist lineages than that indicated by ‘classical’ environmental cloning methods [8–11]. Strikingly, despite screening hundreds of millions of SSU rDNA gene sequences derived from samples that our cell isolation work suggested should be relatively rich in Reticulamoeba, we did not find any sequences matching known Reticulamoeba sequence types.

### Materials and Methods

#### Sample Collection, Culture Isolation and Microscopy

Benthic samples for cell isolation and DNA extraction were taken from Port Swtan (Church Bay) Anglesey, Wales, UK (53°22’25” N, 4°33’17” W), Walney Island, Cumbria, UK (54°03’04” N, 3°11’18” W), Thurlestone Beach, Devon (50°15’ N, 3°51’ W), and Chesapeake Bay, Queenstown, Maryland (38°59’ N, 76°10’ W). Other DNA samples were obtained from colleagues from the Colne Estuary [12], coastal sediment/rock scrapings from the eastern US seaboard between North Carolina and Washington DC, and recently formed biofilms in an experimental flume system in the River Lambourn, Berkshire. DNA and cDNA samples were also obtained from the BioMarKs consortium of which DB and CB are members, collected as described in [9].

Samples for cell isolation were hydrated with dilutions of CCAP Artificial Sea Water Medium (ASW) and grown at room temperature without enrichment for a few days to a few weeks. Depending on the concentration of organisms, a 10–100 μl aliquot was then serially diluted across eight or twelve wells of 250 μl of ASW in a 96-well cell culture plate (Nuncolon), with mixed marine diatoms as food source. The plate was then incubated at room temperature for a few days to a couple of weeks. Two or three rounds of serial dilution were carried out for each isolate, using only apparently pure strains to seed the final round.

Live cultures were filmed and photographed using a Nikon Eclipse 80i microscope, with a x10 differential interference contrast water immersion lens (NA 0.6) and a Sony HDV 1080i Handycam®. Films were analysed on Final Cut Express HD 3.5.1, and digital images were exported and transferred to Adobe Photoshop for processing (Figures 1 to 3).

For DNA extraction, most of the culture medium was decanted off, and using a sterile scraper, cells were collected from the bottom of the culture dish, then concentrated by centrifugation at 214 g for 15 min at 5°C. Total DNA was extracted from the pellet following the Maximum Yield Protocol of the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, CamBio, UK).

Ethics statement: No specific permission or permits were required for the described field studies. The sites were not privately owned or protected in any way and were fully open to public access. No endangered or protected species were involved in this study.

#### Amplification and Sequencing of the SSU rDNA

PCR amplifications were done in a total volume of 30 μl with an amplification profile typically consisting of 35 cycles with 30 s. at 95°C, 30 s. at 60°C, and 90 s. at 72°C, followed by 5 min. at 72°C for the final extension. PCR products were run on 1.5% TAE agarose gels. Bands of the appropriate lengths were excised, and cleaned following the protocol of the QiAvgick™ Gel Extraction Kit (Qiagen). PCR amplicons were then cloned into Stratagene Cloning Kit (Stratagene). White colonies were screened using the primers M13for (5’-CTG TGT AAA ACG ACG GCC AGC AGT-3’) and M13rev (5’-CAC AGG AAA CAG CTA TGA CCA-3’). Positive PCR products were cleaned using a polyethylene glycol (PEG) protocol: for 20 μl PCR reactions, 20 μl of a 20% PEG/2.5 M NaCl mixture was added to each tube. The tubes were mixed by vortexing and incubated for 30 min at 37°C, then centrifuged at 3000 rpm for 30 min to pellet the PCR products. Supernatant was discarded by pulse-spinning the inverted tubes at 600 rpm. The pellet was then washed with ice-cold 75% ethanol, spun for ten minutes at 3000 rpm, again inverted and pulse-spin to remove the supernatant. The ethanol wash was repeated; the PCR pellet was re-suspended in de-ionised water, and stored at −20°C. Sequencing was performed with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and analysed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland).

The first sequences were obtained from isolate 7 (R. gemmipara from Walney Island). Most of the usual combinations of universal or cercozoan-specific primers regularly used before, e.g. [13] didn’t lead to any good amplicons. Any strong band of the expected size proved to be from the diatoms on which the Reticulamoeba cells were feeding. We then focused on trying to amplify shorter fragments only, using a combination of cercozoan-specific and/or anti-diatom primers (Table S1). For three of the resulting PCR products, a faint band longer than the expected size was observed: n3NDf –1256R, s12aSF – s2bN, 1259F –s2bN. Direct sequencing of these bands proved impossible, so we cloned them and finally obtained the first real Reticulamoeba sequences. Two reverse primers specific for that isolate (V4r-d5a and V4r-d5b) were designed in the V4 region to amplify the missing first third of the gene with a nested PCR approach using forward primers sA1n and sA3n, respectively. Having sequenced the complete SSU rDNA of that first Reticulamoeba isolate made it possible to design an updated version of cercozoan-specific reverse primer 1256R, taking into account Reticulamoeba-specific substitutions (1256R-d5), as well as a new granofiloscean-specific forward primer (sA4-gran), to amplify the first two thirds of the SSU rDNA gene from all other isolates. This revealed the presence of four distinct SSU rDNA types in our isolates. Based on these four sequence types, two pairs of Reticulamoeba-specific forward (V2f-d5 and C3f-d5) and reverse (V5r-d5a and V5r-d5b) primers were designed to construct Reticulamoeba clone libraries from environmental DNAs using a nested PCR approach. The resulting fragment (C3f-d5 to V5r-d5b) is about 800 bp. The two forward primers were also used to obtain the missing last third of the gene from one isolate of each identified species, again using a nested PCR approach, together with universal reverse primer sB1n and an updated version of primer sB2N (sB2-d5), respectively.

New sequences were deposited in GenBank with Accession numbers KC109661-KC109732.

#### Construction of the Datasets and Phylogenetic Analyses

BLAST searches [14] were performed using our new Reticulamoeba SSU rDNA sequences and revealed that exact matches were
not present in the GenBank database. The closest sequences belonged to members of the phylum Cerozoa (Rhizaria). This was confirmed by preliminary trees including a wide range of eukaryotes, showing that in spite of their sequence divergence, our Reticulamoeba isolates clearly belong to the cercozoan subphylum Filosa. Two datasets were constructed for phylogenetic analyses. The first one, Figure 4 is restricted to Reticulamoeba sequences only so that the more variable regions of the gene could be included to illustrate the levels of inter- versus intra-specific sequence heterogeneity in that genus. It corresponds to a fragment of the SSU rDNA from forward primer C3f-d5 to reverse primer s1256R-d5 (1067 unambiguously aligned positions). This dataset includes all clone sequences we obtained from our Reticulamoeba isolates, plus the sequences obtained in our environmental libraries. The second (Figure 5; 1505 unambiguously aligned positions) includes the complete SSU rDNA sequences from five
Reticulamoeba isolates, corresponding to four distinct SSU rDNA types, with two distinct isolates of type *R. gemmipara*. Sequences of representatives of all main cercozoan lineages were included, to determine the exact affiliation of *Reticulamoeba* within that phylum.

All phylogenetic analyses were performed using the GTR model of substitution [15,16], taking into account a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. All necessary parameters were estimated from the datasets. For each dataset, a maximum likelihood (ML) tree [17] was determined with the program RaxML [18], using 250 inferences from distinct maximum parsimony starting trees. The reliability of internal branches was assessed with the bootstrap method [19]; 200 non-parametric bootstrap replicates with 10 inferences for each from distinct maximum parsimony starting trees (option –b –# 200 –u 10). In addition, Bayesian analyses were performed with MrBayes version 3.1 [20,21]. For each dataset, two runs of four simultaneous chains were run for 2,500,000 generations (heat parameters set to default), and trees were sampled every 100 generations. For each run 25,000 trees were sampled, 5,000 of which were discarded as the burn-in. Posterior

Figure 3. Amoeboid form of *Reticulamoeba* Isolate 4 (Lineage 2). Flagellate forms not shown but very similar to *R. minor* and *R. gemmipara*. The cells of this isolate were always strongly associated with diatoms, as in these photos. Scale bar = 10 µm.
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probabilities of the branching pattern were estimated from the 40,000 remaining trees and mapped onto the maximum likelihood tree when present. In all cases, the posterior probability 50% majority-rule consensus tree was fully compatible with the corresponding ML tree. The dataset for Figure S1 was analysed using the RaxML BlackBox (v. 7.3.1) hosted on the Cipres Science Gateway (www.phylo.org/portal2/; [22]) only.

Sequence Dataset Mining
The following datasets were blastn-searched for SSU rRNA gene sequences related to the Reticulamoeba genotypes as determined above: 1) NCBI GenBank nr/nt, 2) ‘All Metagenomic 454 Reads’ in the CAMERA database (http://camera.calit2.net/; [23,24]), 3) NCBI Environmental Sample Nucleotides (env_nt) via CAMERA, and 4) BioMarKs V4 SSU rDNA sequences generated using eukaryote-wide primers as described in [9]. For blastn searches against the NCBI and CAMERA databases, seed sequences were generated for each of the SSU-types recovered from the cultured strains by roughly dividing the longest SSU read into quarters and then generating three more fragments of similar size overlapping the boundaries of the original quarters. A fragment of c. 350 bp was also generated spanning the most variable V4 region and more conserved flanking regions with a strong signal for the Reticulamoeba clade.

Figure 4. Maximum Likelihood (RAxML) SSU rDNA phylogeny of Reticulamoeba isolates 1–7 and the six main lineages identified in this study. 58 sequences, 1067 positions. ML bootstrap values shown when >70%.

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Results

Morphology and SSU rDNA Phylogeny

We isolated seven strains of Reticulamoeba, and obtained the SSU rDNA sequence for each. Three of the isolates (Isolates 1–3; Figure 1) corresponded to Grell’s description of R. minor, and two (Isolates 6 & 7; Figure 2) to R. gemmipara. Isolate 4 (Figure 3) was intermediate between these two species in amoeboid cell morphology (although the mode of fission was not seen), and Isolate 5 (which died quickly in culture and is therefore not illustrated) had an amoeboid phase that itself was reticulate, i.e. several lacunae formed within the cell body, so that the cell was composed of a small network of cytoplasmic strands 1–3 μm wide; the overall size of the cell was larger than the other isolates. Isolates 1, 4, and 6 were from Church Bay samples; 2, 5, and 7 from Walsney Island, and 3 from Thurlestone beach. Different isolates within a Lineage were morphologically and behaviourally indistinguishable; the choice of images in Figs. 1–3 is based on their suitability for illustration.

Because all isolates had relatively high levels of intra-genomic SSU rDNA sequence diversity, the PCR products were cloned and the resulting sequences analysed phylogenetically. Figure 4 shows that the sequences recovered from Isolates 1–3 form a clade (Lineage 1 (= R. minor)) in which there is no apparent phylogenetic distinction between the three isolates. Cloned sequences from Isolates 4 and 5 (sister Lineages 2 and 3, which together are sister to Lineage 1) each also form clades, though will lower internal sequence diversity than Lineage 1. Isolates 6 and 7 (Lineage 4 (= R. gemmipara)) showed a similar molecular diversity pattern to Lineages 2 and 3. It is possible that the apparently high intra-genomic SSU diversity and mixed genotypes across Isolates 1–3 could be explained by there being more than one Reticulamoeba lineage in the sequenced isolates. However, we think this unlikely as this pattern only relates to these three lineages (there is no mixing of these sequence types with any other lineages or between any of the other lineages), and distinctive sequence signatures in particularly variable regions along the SSU of isolates 1–3 show a mosaic distribution across the three isolates suggesting a partly reticulate evolutionary history of these lineages. The simplest explanation is that these three isolates are a single evolutionary unit and species: R. minor.

The morphology of Reticulamoeba strongly suggests a cercozoan affinity, specifically with Granofiloidea, which share its granular filopodia-like amoeba-flagellate characters, although the granules on other Granofiloidea move very much less than in Reticulamoeba, or not at all [6]. We were surprised therefore when DNA extracted from Reticulamoeba did not amplify with any of the several primer sets known to amplify most filozoan Cercozoa (see Methods). The difficulty of obtaining SSU rDNA sequences from Reticulamoeba, requiring several new primer sets and ‘walking’ along the SSU rRNA gene was explained by the highly divergent nature of the sequences, represented by the long branch leading to the Reticulamoeba radiation in Figure 5. This tree does, however, confirm that Reticulamoeba branches within Granofiloidea, most consistently as sister to the freshwater genus Mesofila. The affinity is concordant with the morphology and lifestyle of Mesofila, from which it differs in three main respects: 1) the filopodial granules of Mesofila are stationary, and 3) habitat. However, like Reticulamoeba, Mesofila readily forms gliding/swimming flagellate forms, to a much more noticeable extent than most other naked Granofiloidea.

SSU rDNA of Reticulamoeba: Sequence Divergence and Intra-specific Heterogeneity

The Reticulamoeba SSU rDNA sequences obtained in this study proved unusual in many respects. Even though they possess most typical cercozoan- and filozoan-specific sequence signatures, they appear to be very divergent compared to the sequences of other Filosa, with very many specific substitutions distributed across the whole length of the gene. Secondly, as suggested by the size of all amplicons, they are indeed longer than the average size of the gene in most eukaryotes, with specific insertions in many variable regions. Finally, there proved to be a surprisingly high level of intra-genomic heterogeneity between different copies of the SSU rDNA gene within all sequenced Reticulamoeba isolates. This heterogeneity is significantly higher than any we are aware of in other amoeboid organisms, including both size and primary sequence variation. Several different sequence patterns can be observed in all variable regions of the gene, and the different clone sequences we obtained for each isolate appear to correspond to random combinations of these patterns. This is the reason why cloning proved to be necessary for all amplicons we sequenced.

Importantly, the observed sequence heterogeneity within isolates (and between isolates sharing the same morphology) is limited to the most variable regions of the SSU rDNA. Even though surprisingly high, it remains significantly lower than the observed sequence heterogeneity between isolates exhibiting different morphologies. Therefore we could readily assign each of our isolates to a well-defined SSU rDNA type, and these correlated perfectly with a morphological type, and assumedly by extension to different species. By contrast with intra-specific heterogeneity, inter-specific differences extend to less variable regions of the gene, but are very conserved within species. This is illustrated by both tree figures. In figure 4, we can see that the various clone sequences from isolates of a same morphological type are intertwined and exhibit the same levels of sequence heterogeneity. In figure 5, we can see that once the most variable regions of the gene, and the different clone sequences we obtained for each isolate appear to correspond to random combinations of these patterns. This is the reason why cloning proved to be necessary for all amplicons we sequenced.

Diversity and Ecology of Reticulamoeba

Although Reticulamoeba cells can be difficult to see in crude environmental cultures, we found them frequently when screening littoral benthic, particularly sandy or silty samples. Therefore we hypothesized that this genus is much more abundant than suggested by its minimal (and entirely specimen-derived: see below) representation in Genbank. To investigate this further, we designed Reticulamoeba-specific primers (see Methods) to screen environmental DNA extractions (each representing c. 0.5 - 1 g sediment) from eleven samples taken along an estuarine gradient, six newly-formed freshwater (river) biofilm samples, nine coastal sediment and rock scrapings from various sites on the US east coast.
cost between North Carolina and Washington DC, and Bio-
Markks coastal (offshore) sediment samples (nine DNA and nine
cDNA, paired from the same set of sites) from Roscoff (France),
Oslo (Norway), Barcelona (Spain), Varna (Bulgaria), and Naples
(Italy). Of the estuarine samples, sample 6 (a midpoint estuary site;
[12]) gave a positive PCR result, as did two of the two biofilm
samples, one Maryland coastal sediment, and one BioMarkks
sediment DNA sample. In contrast seven out of nine BioMarkks
cDNA samples amplified. The branching position of the cloned
sequences is shown on Figure 4. The library sequences exclusively
grouped with the Reticulamoeba sequences derived from cultures,
including two novel sister lineages (5 and 6; Figure 4) recovered
only from the BioMarkks cDNA samples, which are sister to the
cultured and other environmental sequences (as confirmed by
Figure S1). Lineage 6 had a shorter amplicon by c. 40 bp than
lineage 5. Interestingly, we detected lineage 4 twice independently
in freshwater river biofilm samples. This is intriguing since
Reticulamoeba has never been seen or otherwise recorded from
freshwater (as distinct from brackish water).

Next Generation Sequencing Database Mining
Our environmental SSU clone libraries suggested that Reticu-
loamoeba is more abundant and diverse than existing sequence data
implied. To investigate whether this was reflected by its
representation in massively high throughput next generation
sequencing (NGS: 454, Illumina) datasets, we used full and partial
SSU rDNA sequence ‘seeds’ to look for representatives/relatives of
all six lineages in three main types of dataset: NCBI GenBank
nucleotide database, 454 amplicon libraries generating using
eukaryote-wide SSU primers, and NGS and Sanger-sequenced
cDNA samples amplified. All sequence types in Figure 6 (from the
BioMarkks (V4 region) including sequences from BioMarkks.
The BioMarkks (V4 region) sequences were generated using
eukaryote-wide primers, and are labelled ‘BioMarkks: ...’. The two such lineages shown were the only
sequences in the whole of the BioMarkks data that were related to
Reticulamoeba. 738 positions used for analysis.
doi:10.1371/journal.pone.0049090.g006

Figure 6. Maximum Likelihood (RAxML) SSU rDNA tree
including sequences from BioMarkks. The BioMarkks (V4 region)
sequences were generated using eukaryote-wide primers, and are
labelled ‘BioMarkks: ...’. The two such lineages shown were the only
sequences in the whole of the BioMarkks data that were related to
Reticulamoeba. 738 positions used for analysis.
doi:10.1371/journal.pone.0049090.g006

Discussion
We have frequently seen in crude environmental cultures and
occasionally isolated Reticulamoeba-like amoeboidflagellates that
resisted amplification with primer sets that easily amplify most
Cercozoa and, particularly Granifilosea. Therefore the difficulty
of PCR-amplifying those strains that differed from other
granifilosean in having bi-directionally streaming granules on their
reticulopodia was a puzzle that was only resolved with intensive
PCR attempts using a variety of primers and targeting short
amplicons, and eventually largely sequencing by ‘walking’ along the
SSU rRNA molecule. This resulting in associating a SSU
sequence with Grell's hitherto elusive Reticulamoeba, and showing
that it groups robustly within Granifilosea (although the intra-
granifilosean relationship requires confirmation with other genes as
the SSU branch is very long and therefore likely prone to
phylogenetic long branch attraction effects).

Our observations by microscopy and using lineage-specific primers
strongly indicate that Reticulamoeba lineages are much more
densely and widely distributed than currently available databases
suggest. This is almost certainly because the SSU rDNA of
Reticulamoeba is relatively difficult to amplify and is therefore biased
against by more general PCR primers, for example those designed
to detect a broad eukaryotic diversity. A robust confirmation of
this is that none of the four unambiguously identified Reticulamoeba
lineages (1 to 4) was recovered from the V4 BioMarkks data
generated with eukaryote-wide primers, even though these have
been shown to be broadly inclusive, e.g. [11]. However, one novel
lineage (5) was detected in the general eukaryote V4 reads from
two BioMarkks samples. By contrast, lineage-specific PCR probing
of the same nucleic acid samples revealed five of the six lineages,
the sixth being newly found by this specific PCR approach.

It is striking that Reticulamoeba lineages were detected in the
BioMarkks samples with a strong bias towards the RNA-derived
cDNA) samples. All sequence types in Figure 6 (from the
some novel granifilosean sequences, one very divergent one 83%
similar to Mesofila, and others 93% similar to op32 (novel clade
Gran-3; Bass et al. 2009) and 96% identity to eb6 (novel clade
Gran-1).

The CAMERA All Metagenomic 454 Reads (227.3 M
sequences) blastn search also returned only high identity matches
in conserved regions of the SSU. Only returned sequences with
>95% identity to the query sequences were investigated further.
These included matches to other Cercozoa including the
granifilosean sequence s6 (novel clade Gran-2; [6]) and the
endomyxan sm5 (novel clade Endo-5).
easiest groups to detect in similar marine metagenomic datasets. The planktonic marine stramenopile groups in the BioMarKs member of MAST-3 [9], which is the most highly represented of none of the fragments of any of the six Reticulamoeba depth of sequencing that might offset the likelihood of extreme biases [10]. Next generation sequencing technologies now offer a represent the composition of the communities from which they metatranscriptomes) sequence libraries can theoretically better PCR amplification step and shotgun sequenced RNA-derived metagenomes, i.e. shotgun-sequenced genomic DNA without a community diversity.

Metagenomic (hereafter for simplicity used to refer to both true metagenomes, i.e. shotgun-sequenced genomic DNA without a PCR amplification step and shotgun sequenced RNA-derived metatranscriptomes) sequence libraries can theoretically better represent the composition of the communities from which they were constructed because they avoid PCR and its attendance biases [10]. Next generation sequencing technologies now offer a depth of sequencing that might offset the likelihood of extreme taxonomic undersampling because of the size and complexity of the whole genomes comprising microbial communities. However, none of the fragments of any of the six Reticulamoeba lineages, nor ‘full length’ SSU rDNA sequences matched any metagenomic sequence in the largest, most comprehensive metadataset hosted by CAMERA. This could be partly explained by the fact that, although it has a swimming flagellate stage, Reticulamoeba is far more obviously benthic than planktonic, and the marine datasets in CAMERA are very strongly planktonic. To gauge the sensitivity of this method of recovering SSU tags from metagenomic datasets we created an equivalent set of V4 blastn seeds from the three sequences of Solenicola setigera [28] from NCBI. Solenicola is a member of MAST-3 [9], which is the most highly represented of the planktonic marine stramenopile groups in the BioMarKs eukaryote-wide data. This suggests that it should be among the easiest groups to detect in similar marine metagenomic datasets. Indeed, we recovered many identical and highly similar V4 reads (>95% sequence identity across highly variable regions) from at least 36 individual metagenomic samples in CAMERA, by blastn-searching the three Solenicola V4s against the All Metagenomic 454 Reads. This shows (as do other studies such as Not et al. 2009) that it is a reasonable proposition to search for SSU tags in metagenomic datasets such as those hosted by CAMERA, and that Reticulamoeba is not represented because the samples do not cover its main habitat and/or it is insufficiently abundant in the samples to be detected in this way.

It remains a striking fact, therefore, that although there is good evidence for Reticulamoeba being a frequent and diverse element of marine benthic (at least) protist communities, no direct evidence can be found for it in any existing sequence dataset, even though these have been constructed using a diverse range of techniques (well representing all that are currently available) and harnessing the power of massively parallel next generation sequencing technologies. Culture-based investigations have recently revealed other elusive, long-branched Cercozoa [e.g. Staino maris, Helkesimastix, Cholamonas, Gutulinosus; [29–31]] that require intensive and case-specific attention to yield genetic data that can be used to detect their presence in nature. How many more such lineages are there, and how diverse and abundant are they relative to those more easily detected by environmental screening techniques?

Supporting Information

Figure S1 Maximum Likelihood (RaxML) SSU rDNA phylogeny of Reticulamoeba clade within Cercozoa. 37 sequences, 1656 positions. Showing the relative positions of Reticulamoeba isolates and novel environmental sequences from BioMarKs data.

Table S1 Primers used in this study.

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Author Contributions

Conceived and designed the experiments: DB CB. Performed the experiments: DB AY CB. Analyzed the data: CB DB. Contributed reagents/materials/analysis tools: DB. Wrote the paper: DB CB. Contributed to preparation and analysis of Biosamples total database: SS. Coordinated overall Biomarks sampling and sequence generation: SR.

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