Old Lineages in a New Ecosystem: Diversification of Arcellinid Amoebae (Amoebozoa) and Peatland Mosses

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Abstract

Arcellinid testate amoebae (Amoebozoa) form a group of free-living microbial eukaryotes with one of the oldest fossil records known, yet several aspects of their evolutionary history remain poorly understood. Arcellinids occur in a range of terrestrial, freshwater and even brackish habitats; however, many arcellinid morphospecies such as Hyalosphenia papilio are particularly abundant in Sphagnum-dominated peatlands, a relatively new ecosystem that appeared during the diversification of Sphagnum species in the Miocene (5–20 Myr ago). Here, we reconstruct divergence times in arcellinid testate amoebae after selecting several fossils for clock calibrations and then infer whether or not arcellinids followed a pattern of diversification that parallels the pattern described for Sphagnum. We found that the diversification of core arcellinids occurred during the Phanerozoic, which is congruent with most arcellinid fossils but not with the oldest known amoebozoan fossil (i.e. at ca. 662 or ca. 750 Myr). Overall, Sphagnum and the Hyalospheniidae exhibit different patterns of diversification. However, an extensive molecular phylogenetic analysis of distinct clades within H. papilio species complex demonstrated a correlation between the recent diversification of H. papilio, the recent diversification of Sphagnum mosses, and the establishment of peatlands.

Introduction

The Amoebozoa is a sister clade to animals and fungi and is considered to reflect one of the most ancient lineages of eukaryotes [1,2]. Amoebozoans are unicellular eukaryotes, but a few taxa such as the model species Dictyostelium discoideum can form multicellular structures when nutrient conditions are limiting. Although the phylogenetic relationships within the Amoebozoa are far from being fully resolved, one subgroup, the Tubulinea, has been consistently recovered with strong statistical support [3–5], and includes one of the most species-rich groups of amoebozoans, namely the Arcellinida (>1000 species) [3,4,6]. Arcellinid amoebae occur in a range of terrestrial, freshwater and even brackish habitats [7,8], and are particularly diverse and abundant in Sphagnum-dominated peatlands [9–11]. Several testate amoebae within the Hyalospheniidae, such as Nebra carinata and Hyalosphenia papilio, are very abundant in Sphagnum mosses, but they can be also found in other mosses if relative constant moisture and low pH are present [8,12,13]. Both the arcellinid amoebae, which are characterized by the presence of a test (syn. shell) and the Sphagnales mosses (Plantae: Sphagnopsida) have an ancient fossil record of hundreds of millions of years [14,15]. Despite this long-lasting persistence of the Sphagnales, the radiation of the current diversity (ca. 300 species) occurred recently in the Miocene [16]. It is inferred that Sphagnum-dominated peatlands are as recent as the radiation of Sphagnum mosses.

The presence of a test in arcellinids makes them the only lineage of amoebozoans with a rich and well-preserved fossil record, which offers a rare opportunity to calibrate this part of the tree of eukaryotes. However, despite being one of the oldest lineages of eukaryotes in the fossil record (ca. 750 Myr fossils; [15]), the evolutionary history and diversification of arcellinids have not been studied in detail. It is unclear whether the high diversity of arcellinids, and in particular the Hyalospheniidae [17], reflects ancient and/or recent diversification events. Also, the taxonomic assignments and phylogenetic positions of arcellinid fossils remain largely ambiguous and are subject to much debate [1,2,18]. Although a putative arcellinid fossil of ca. 750 Myr has been previously used to reconstruct divergence times [1,2], representatives of the closest sister groups to arcellinids (Amoebidae, Hartammellidae and Poseidonida) have not been incorporated into previous analyses. Therefore, the 750 Myr old fossil has not strictly been assigned to the Arcellinida but instead to nodes within the Tubulinea. Ages much younger than the 750 Myr fossils have been recently inferred for arcellinids in analyses of amoebozoan divergence times using the closest sister groups to arcellinids (e.g., Nolandia; [19]).

The aim of the present study is to reconstruct the evolutionary history of arcellinids, with special emphasis on the diversification
of the Hyalospheniidae. After careful examination of arcellinid fossils, we reconstructed divergence times in these testate amoebae using a robust phylogeny inferred from multiple genetic markers. Moreover, we compared the diversification pattern recovered within the Hyalospheniidae, particularly in species very abundant in Sphagnum-dominated peatlands such as Hyalosphenia papilio, with the pattern of diversification in Sphagnum mosses. A mitochondrial cytochrome c oxidase subunit 1 (COI) dataset, including all publicly available and new sequences, was used to reconstruct the diversification pattern within the Hyalospheniidae. The 99% sequence similarity threshold and the Generalized Mixed Yule Coalescent (GMYC) model [20,21] were used to identify evolutionarily independent units (i.e., putative species) for different morphospecies within the Hyalospheniidae.

Materials and Methods

2.1 COI amplification and sequencing

Mitochondrial cytochrome c oxidase subunit I gene (COI) fragments were obtained from a total of eighteen single cells of the morphospecies Arcella sp (Figure S1A), Hyalosphenia papilio, Nebela collaris, N. flabellatum, N. margarita (Figure S1C–E), and one unidentified Nebela morphospecies (Figure S1B). The single cell specimens were isolated, amplified by semi-nested PCR, and sequenced as described in Heger and colleagues [22]. Single cell specimens were isolated either from Sphagnum moss or litter samples (Table S1). The specific and universal primers used in this study are listed in Table S2. COI sequences were deposited in GenBank with the accession numbers KJ544147-KJ544164. Five of the single cells that we sequenced were also characterized with light microscopy (Figure S1).

2.2 Dataset constructions

Reconstruction of divergence times in arcellinids was carried out using two datasets. The first matrix consisted of five concatenated proteins (i.e. actin, alpha-tubulin, beta-tubulin, cEF2 and 14-3-3) plus small subunit (SSU) rDNA sequences and corresponds with the alignment recently published by Lahr and colleagues [4]. This alignment contains a total of 53 tubulinean taxa (including 29 arcellinids). In order to distinguish the signal from these two sources, two sub-matrices were analyzed: one consisted of the five proteins for 30 taxa (including 19 arcellinids) and the other one consisted of SSU rDNA sequences for 49 taxa (including 24 arcellinids).

The second matrix consisted of COI sequences which were obtained from three different sources: (i) 16 Hyalospheniidae isolates and two new sequences from Arcella sp. (Table S1); (ii) 85 additional isolates within the Hyalospheniidae from Kosakyan and colleagues [23,24]; and (iii) 298 isolates of the morphospecies Hyalosphenia papilio from Heger and colleagues [22]. These 401 COI sequences (Table S1 and Table S3) were manually aligned into the Kosakyan and colleagues' [24] alignment with the program Bioedit 7.0.9 [25]. When several sequences were identical or differed only by the presence of unknown nucleic acid residues (N), only the longest sequence was kept. The final alignment comprised 125 COI sequences.

2.3 Phylogenetic reconstructions of the COI dataset

Molecular phylogenetic analyses were performed on the 125-taxon alignment. Tree reconstructions were conducted using Maximum Likelihood (ML) analyses using Treefinder [26] and Bayesian inference methods using MrBayes v. 3.1.2 [27] as described in Heger and colleagues [22]. The Modeltest program [28] identified the general-time-reversible model with invariant sites and gamma distribution (GTR+I+G) as the most appropriate model of sequence evolution. Inferred trees were rooted with the two new Anilla COI sequences, an inferred sister group of the Hyalospheniidae [29]. ML analyses were run for 300 replicates. Trees were viewed using FigTree (program distributed as part of BEAST: http://tree.bio.ed.ac.uk/software/figtree/).

2.4 Species delimitation

We identified genetic lineages with the Hyalospheniidae based on a 99% sequence similarity threshold, as suggested by Heger and colleagues [22]. Sequence divergences were calculated using the Kimura 2-parameter [30] in R, version 3.0.1 [31], using the package 'ape', version 3.0-1. Subsequently, we used the Generalized Mixed Yule Coalescent (GMYC) model as a complementary species criterion [20,21]. The GMYC method is based on the shift in the branching rate of the phylogenetic tree [20,32,33]. GMYC analyses were performed as described by Heger and colleagues [22]. The performance of the single threshold [20] and multiple threshold model [33] did not differ significantly, as revealed by the Chi-squared comparison test (chi square = 4.419, d.f. = 3, P = 0.219; Figure 1).

2.5 Reconstruction of divergence times and fossil assignment

Divergence times were reconstructed using BEAST v1.6 [34]. BEAST analyses on the five proteins plus SSU rDNA used the WAG+G and GTR+G models of substitution, respectively. For the COI dataset, the GTR+G model was used following the Kosakyan and colleagues [24] analyses. Uncorrelated lognormal (UCLN) model allowing different rates in different branches of the tree was applied. All analyses were run for 20×10⁶ generations, of which 5 million were discarded as burn-in. All fossils were constrained applying a uniform distribution. Different combinations of fossils were explored as described below.

Fossil calibration was applied to five nodes where 10 fossil records could be assigned. These 10 fossil records correspond to two arcellinids, two Arcella, two Centropyxis, one hyalospheniid, two Diffluga and one Lesquereusia; all were selected from a wide variety of publications (Table 1, Table S4). An age of 770 Myr from the oldest arcellinid fossils (770-742 Myr) [15] was used on the root as a maximum bound (node A; Figure 2), which allows considering the second oldest record (662 Myr, [35]). This node includes non-arcellinid taxa but phylogenetic analyses have revealed that arcellinid testate amoebae are paraphyletic within the Tubulinea [4]; therefore, we used it only as a maximum age constraint. A conservative age for the Arcella fossils was used for its crown node (node D; Figure 2) at a minimum bound of 100 Myr (mid Cretaceous; [36]) and a maximum bound of 5% older (105 Myr). Because the phylogenetic position of Centropyxis is not resolved [4], its fossil age (220 Myr, Carnian within the Triassic, [37]) was assigned to the first well-resolved node containing Centropyxis as a minimum age (node B; Figure 2) and a maximum bound equal to the root age (770 Myr). The Hyalospheniidae fossil (node C; Figure 2) was used as a conservative minimum bound (100 Myr, Albian–Cenomanian, within the Cretaceous; [38]) and a maximum bound equal to the root age (770 Myr) was applied in order to explore the uncertainty of the age of this clade. Two taxa represented in our dataset, Diffluga and Lesquereusia, could be used for the assignment of two fossils from the lower Albian (Cretaceous; [39,40]). Diffluga is a paraphyletic group [41], which leads to uncertainty. With this in mind, we explored the effect on the initial calibration scheme of constraining the split of Diffluga-Lesquereusia (node E) to a conservative minimum age of 100 Myr and a maximum age equal to the root (770 Myr). In addition, to
evaluate the accuracy of nodes D and E, the *Difflugia-Lesquereusia* calibration (node E >100<150 Myr) was combined with a relaxation of *Arcella* upper bound (node D) to an age equal to the root. All these calibrations were combined into three different analyses to check congruency among them (Table 2).

In order to evaluate three recently published fossil records, we also explored alternative fossil age assignments of 299 Myr for (i) *Arcella* (node D), (ii) *Centropyxis* (node B) and (iii) *Difflugia and Lesquereusia* (node E); we examined each of them independently following two recent reports [42]. Because this new calibration is three times older than the initial calibrations for *Arcella* and *Difflugia*, an upper bound of 5% older (314 Myr) was applied in both analyses. In light of the fact that preliminary analyses recovered the maximum bound for the root (ca. 770 Myr), we allowed a relaxation of the root age to <3000 Myr.

For the COI marker analysis, the alignment comprises one sequence from each evolutionary independent lineage detected with the GMYC model. The Hyalospheniidae crown age was calibrated using an age range reconstructed from the result of the three main analyses of arcellinids (Table 2; Figure 3). These results were used as minimum and maximum bounds applying a uniform distribution. The root between *Arcella* and the Hyalospheniidae was constrained to a reasonable maximum bound of 300 Myr (five times older than its fossil record). Inclusion of *Arcella* as the outgroup in the COI analysis allowed us to evaluate the *Arcella - Hyalospheniidae* split with results from the concatenated dataset.

### 2.6 Diversification rates and saturation analyses

A model fit to speciation with one, two or three diversification rates was tested with LASER [43]. This program compares birth-death models with various rates of diversification against the null expectation of a constant rate. LASER was used to identify when a given shift on diversification rate occurred in our GMYC species chronograms. One hundred trees were randomly taken from the GMYC Highest Posterior Density trees (HPD) and analysed with LASER. The critical Delta AICc value was estimated from simulations under rate-constant model. Lineage-Through-Time (LTT) plots were drawn for these chronograms. The overall diversification rate assuming an extinction between 0 and 0.9 was estimated with Magallon and Sanderson [44] equation 7 as implemented in R version 3.0.1 [31]. The program DAMBE [45] was used to analyze nucleotide saturation in our dataset. No saturation was observed for the COI fossil dataset (data not shown).

### Results

#### 3.1 New sequences obtained in this study

Eighteen new COI sequences were obtained for the present study (i.e., two *Arcella* sp. and 21 isolates within the Hyalospheniidae; Table S1). The obtained COI fragment lengths ranged from 338 bp to 631 bp. The two *Arcella* sp. sequences represented the first arcellinid COI sequences that did not belong to the *Hyalospheniidae*. Both *Arcella* sp. sequences were identical even though they were isolated from different regions.

#### 3.2 COI phylogeny and species delimitation

Bayesian and Maximum Likelihood analyses were congruent with each other, with only a few exceptions. Our phylogenetic tree revealed six main clades (Figure 1), as reported in Kosakyan and colleagues [24]. Bayesian analyses provided the highest posterior probabilities (PP) for these six lineages (i.e., 1.00 PP), and Maximum Likelihood analyses provided strong to moderate statistical support, except for clade 4, which is not supported (Figure 1). Besides these six clades, a few species had an uncertain affiliation, such as the newly sequenced *Nebola* sp. (Figure S1B). Based on comparative morphology, a total of 26 morphospecies were identified. Sequences from the same morphospecies formed a monophyletic group, except the sequences from the *N. tincta* complex (Figure 1). The number of non-identical sequences per morphospecies varied from one to seventy one (i.e., *Hyalosphenia papilio*). Based on a 99% sequence similarity threshold, forty-nine lineages were identified. Similarly, the General Mixed Yule-coalescent (GMYC), based either on the single or multiple threshold model, revealed a total of forty-seven entities. Within the *H. papilio* morphospecies, which has been intensively sampled, both the 99% sequence similarity threshold and the GMYC model

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**Table 1. Reference of relevant fossil records and calibrations used in this study.**

| Taxa          | Node | Period                        | Calibration (Myr) | Reference |
|---------------|------|-------------------------------|-------------------|-----------|
| Arcellinida    | A    | Neoproterozoic, Cryogenian    | ca. 662           | [35]      |
| Arcellinida    | A    | Neoproterozoic                | <770-742          | [15]      |
| Centropyxis    | B    | Carnian (Triassic)           | >220              | [37]      |
| Hyalospheniida | C    | Albian- Cenomanian (Cretaceous) | >100          | [38]      |
| Arcella        | D    | Mid Cretaceous                | >100              | [36]      |
| Difflugia      | E    | Lower Albian (Cretaceous)     | >100              | [40]      |
| Lesquereusia,  | E    | Late Albian (Cretaceous)      | >100              | [39]      |
| Difflugia      | E    | Early Permian                 | >299              | [49]      |
| Arcella,       | B, D,E| Late Carboniferous–Early Permian | >299            | [42]      |

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| Difflugia      | E    | Early Permian                 | >299              | [49]      |
| Arcella,       | B, D,E| Late Carboniferous–Early Permian | >299            | [42]      |
revealed an important number of putative species (13 and 12, respectively). Overall, the 99% sequence similarity threshold and the GMYC model provide convincing support for the identification of Evolutionarily Independent Units within the Hyalospheniidae [22,46] (Figure 3).

3.3 Fossil calibrations in arcellinid testate amoebae

The validity of the fossil records was evaluated by reconstructing divergence times with different combinations of fossils. The reconstructed age of the Hyalospheniidae is robust across different calibration schemes (Table 2). The original scheme of four fossils gives an age of 132 (100–198) Myr for the Hyalospheniidae. For Arcellina, Centropyxis, and Diffugia-Lesquerelisia, two alternative fossil ages were used. The use of two different combinations of the upper bounds of the fifth fossil Diffugia-Lesquerelisia (node E; 100 Myr) with Arcellina fossil (node D; 100 Myr) retrieved similar ages overall to the four fossils scheme (Table 2): 141 (100–216) and 145 (100–228) Myr for the Hyalospheniidae. The original scheme using 220 Myr for Centropyxis also recovered similar ages to the alternative scheme using 299 Myr (Table 2): 132 (100–198) vs 146 (100–225) Myr for the Hyalospheniidae, respectively; 483 (334–703) vs. 573 (440–757) Myr for the root, respectively. In contrast, the use of an alternative age of 299 Myr vs 100 Myr for Arcella and for Diffugia-Lesquerelisia gave unrealistic ages given the oldest undisputed fossil
records of eukaryotes: root 1098 (685–1550) Myr and 1213 (814–1700) Myr (Table 2 and Table S5). Therefore, the 299 Myr fossil record of *Arcella* and *Diffugia-Lesquereusia* was not taken into account. Overall, the original scheme of four fossils gives slightly narrower confidence intervals and the incorporation of the 299 Myr fossils for *Centropyxis*, *Arcella* and *Diffugia-Lesquereusia* clade retrieved older ages.

### 3.4 Reconstruction of divergence times in Arcellinida

Our reconstruction of divergence times using five calibration points recovered an age of arcellinids *sensu stricto* (i.e., core Arcellinida) of 341–409 (245–529) Myr (Table 2). The age of the root, in this study considered as Arcellinida *sensu lato* was reconstructed at 483–573 (334–757) Myr. An age similar to the oldest fossils attributed to arcellinids of 770–742 Myr [15] or alternatively 662 Myr [35] was only reached when using unrealistic fossil record ages like 299 Myr for *Arcella*, which recovered an age of over 1000 Myr for the root (Table S5). By contrast, the age reconstructed for the Hyalospheniidae of 132–146 (100–225) Myr from multiple fossil combinations predates its oldest fossil record (ca. 100 Myr; Albian–Cenomanian, [38]) that was used as minimum bound in the reconstructions.

Results from the concatenated dataset were in agreement with both the five-protein dataset alone and SSU rDNA dataset alone. The root of the tree was 483 (334–705) Myr for the concatenated dataset based on the original calibration scheme (Table 2 and Table S5), 475 (289–705) Myr for the five-protein dataset and 429 (299–643) Myr for the SSU rDNA dataset. When considering the confidence intervals, the SSU rDNA sequences retrieved slightly younger ages (Table S5), 475 (289–705) Myr for the five-protein dataset and 429 (299–417) Myr for the SSU rDNA dataset based on the original calibration scheme (Table 2 and Table S5). Therefore, the 299 Myr fossil record of arcellinids split 60 Myr ago.

### 3.5 Diversification of Hyalospheniidae from COI marker

A constant diversification was observed in the Lineage-Through-Time (LTT) plots of the Hyalospheniidae from the crown age until ca. 10 Myr from (Figure 4). Analyses of 100 trees randomly taken from the GMYC Highest Posterior Density trees (HPD) recovered a mean shift time leading to a rate increase 7 Myr ago (95% CI = 4.9–10.3) followed by a rate decrease 2.5 Myr ago (95% CI = 2.1–10.3). Forty-five out of 100 trees showed a Delta AIrc higher than the critical Delta AIrc value (4.15) for the yule-3-rates against the best rate constant birth-death model. The overall diversification rate from the GMYC trees varied between 0.023–0.012 (extinction 0 and 0.9, respectively).

The analysis using the GMYC model demonstrated a high proportion of evolutionarily independent units older than 7 Myr. However, evenly distributed cladogenesis in the last 25 Myr was only seen for three morphospecies complexes: *Quadrulella symetrica*, *Nebella tincta sensu lato* and *Hyalosphenia papilio*. The new samples sequenced in this study recovered two new cladogenetic events of <7 Myr for *N. tincta* (New 295 = KJ544161) and *H. papilio* (New 1108 = KJ544152 and New 1109 = KJ544153) and a new cladogenesis event of >90 Myr for *Nebela* sp. (New 1181 = KJ544164). However, inclusion of more samples not always means ancient cladogenesis (e.g., nine sequences of *N. marginata* recovered only one cladogenesis event of <1 Myr; results not shown).

### Discussion

#### 4.1 A temporal scale for arcellinid diversification

Despite being the lineage of amoebozoans with the richest fossil record, the assignment of the oldest arcellinid fossils to a phylogenetic node is controversial [1,2,19]. In this study, we found that the oldest amoebozoan fossil (770–742 Myr; [15]) cannot be assigned with certainty to the Arcellinida *sensu stricto* (i.e.,

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**Table 2. Divergence times from 5 proteins +SSU rDNA dataset under different calibration schemes.**

|                    | Root   | Arcellinid core | Hyalospheniidae | Arcella-Hyalospheniidae |
|--------------------|--------|-----------------|-----------------|------------------------|
| Four fossils (A, B, C, D) | 483(334–703) | 341(245–486) | 132(100–198) | 273(220–386) |
| Five fossil (A, B, C, D), *Diffugia* (E) >100<770 | 534(381–749) | 378(270–527) | 141(100–216) | 305(220–424) |
| Five fossil (A, B, C, D, *Diffugia* (E) >100<105 and *Arcella* (D) >100<770 | 568(408–766) | 400(286–571) | 145(100–228) | 326(229–459) |
| Four fossils (A, C, D) *Centropyxis* (B) >299 <770 | 573(440–757) | 409(324–529) | 146(100–225) | 332(299–417) |

Divergence times for major clades are indicated in Myr (million years). Capital letters represent fossil calibrations (upper and lower bounds) for the corresponding node (see Figure 2): A) root <770 Myr, B) *Centropyxis* clade >220<770 Myr, C) *Hyalospheniidae* >100<770 Myr, D) *Arcella* >100<105 Myr, unless otherwise specified and E) corresponds to the node *Diffugia-Lesquereusia*. doi:10.1371/journal.pone.0095238.t002
the core Arcellinida). However, the paraphyly of arcellinids allows
us to consider an assignment of the oldest fossil to deeper nodes.
Therefore, when we took into account the paraphyly of the
arcellinid testate amoebae, as recently suggested by Lahr et al.
(2013), we were able to reconcile conclusions based on the fossil
record and molecular clock analyses.

Two studies have recently discussed reconstructed ages of
arcellinids (attributed to 770-742 Myr; [15]) while referring to a
different node. In the case of Parfrey et al. (2011), the node linked
arcellinids and Rhizamoeba, (not the most closely related lineages,
which are Amoebidae, Hartmanellidae and Poseidonida); in the
case of Berney and Pawlowski (2006), the node was within the
Tubulinea (i.e., the node linking Hartmanella and Echinamoeba).
The age reconstructed by Berney and Pawlowski [1] (ca. 600 Myr)
is consistent with our results for the Tubulinea [483–573 (334–757)
Myr]. In Parfrey et al. [2], the age reconstructed for the divergence
between arcellinids and Rhizamoeba is ca. 1000 Myr when using
Proterozoic fossils. However, Parfrey et al. [2] inferred ages of ca.
600 Myr when they only used more reliable (undisputed)
Phanerozoic fossils. Our results do not conflict with these two
previous studies nor with the fossil record inferred by Porter and
Knoll ([15]; 770-742 Myr) and Bosak and colleagues ([33]; 662
Myr). When considering all of these studies together, we think that
the most conservative interpretation of the oldest amoebozoan
fossils is to assign them to the Tubulinea, which comprises many
naked amoebae. This interpretation is further supported by a
recent study on divergence times for Amoebozoa where arcellinids
and the closest sister groups have been incorporated and that
recovered ca. 600 Myr for Tubulinea [19]. This leads us to infer
that either arcellinid testate amoebae appeared several times
independently during the evolution of the Tubulinea or the test
was independently lost in different lineages from a common
ancestor with a test. The first scenario would be favored when
considering the eukaryophagy hypothesis [47,48]. The high
number of eukaryophagous protist fossils in the mid-Neooproter-
ozoic may have triggered the radiation of tests in amoebozoans
and other protists [47] because tests provide physical protection
against predators.

The other amoebozoan fossils (Table 1 and Table S4) were in
agreement with the molecular clock reconstructions. After careful
examination of 24 studies, we selected 10 fossils (including the two
oldest arcellinid fossils) and assigned them to five nodes for
reconstruction of divergence times. The other 14 fossils (Table S4)
reported in these studies were not incorporated into our analyses
because either they have been previously dismissed (five fossils),
the taxonomic assignment was uncertain (two fossils), or they did not
correspond to the oldest fossil of the group (Table S4). In our
analyses, the most incongruent results were obtained when
incorporating Arcella, Diffugia and Lesqueresia fossils of 299 Myr
[42,49]. If these lineages are indeed that old, then our molecular
clock reconstructions using these fossils (>1100 Myr for split of
Rhizamoeba from arcellinids; Table S3) would be incongruent with

Figure 3. Maximum credibility tree of Hyalospheniidae obtained from COI dataset analyses. A. Reconstructed ages and 95% Highest
Posterior Density (HPD; indicated with horizontal blue bars) were retrieved using BEAST [34] with only one COI sequences per GMYC lineage (i.e.
evolutionarily independent unit). Arcellinid lineages occurring only in Sphagnum dominated peatlands are marked with green lines. Lineages that
occur in Sphagnum dominated peatlands and/or other habitats are marked with black lines. Numbers on nodes correspond to divergence times in
million years. Taxonomic assignment is indicated on the right. B. Sphagnum chronogram from eight mitochondrial, plastid, and nuclear genes. Dates
(± standard errors) from Shaw and colleagues [16]. Red dotted vertical lines indicate early split of the Sphagnopsida (65.5 Myr ago) and radiation of
ca. 300 spp of Sphagnum (13.6 Myr ago).

Figure 4. Lineage-Through-Time (LTT) plot of Hyalospheniidae. Lineage-Through-Time (LTT) was plotted using 100 trees of the
Hyalospheniidae from the COI dataset analyses. The number of lineages (x axis) is plotted against time (y axis) in such way that each increase on the
number of lineages represents a cladogenesis (a node) in one of the 100 phylogetic trees. These 100 trees were randomly taken from Highest
Posterior Density (HPD) generated in BEAST from the GMYC dataset. Diversification rate shifts leading to a rate increase (7 Myr ago) and a rate
decrease (2.5 Myr ago) as detected in LASER are indicated with dotted grey lines. Grey LTT line is for mosses as extracted from the tree in Shaw
and colleagues [16]. The radiation of 300 Sphagnum at 14 Myr ago is indicated with a vertical line and an arrow. Grey arrow on the left indicates the stem
age of Sphagnum at 223.8, Shaw and colleagues [16].

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both previous molecular studies and with undisputed fossil records assigned to deep nodes of eukaryotes [1,2,19]. If the rate of molecular evolution decelerated in *Arcella*, *Diffugia* and *Lesquereusia*, then using external calibrations would result in younger divergence times than expected (299 Myr). By contrast, if we calibrated these nodes with ancient dates (299 Myr fossils) and assumed a rate deceleration, then all of the inferred ages would be pushed to the past (Table S3).

4.2 Diversification of two ancient lineages: The Hyalospheniidae (Amoebozoa: Arcellinida) and *Sphagnum* mosses (Planta: Sphagnopsida)

Based on our reconstructed divergence time analyses, the diversification of arcellinids during the Phanerozoic is the most likely scenario (Figure 2). The age recovered for arcellinids *sensu stricto* (core Arcellinida: 341–409 (245–529) Myr; Table 2) is in agreement with divergence time analyses that used fossils from outside the Tubulinea (arcellinid stem age: 254–522 Myr; [19]). This is the time when land plants colonized terrestrial soils with the oldest records of spores of ca. 470 Myr [50]. Among the oldest lineages of land plants are *Sphagnum* with a fossil record of 330 Myr [14] and with slightly older divergence times from molecular phylogenetic analyses (300 Myr; [51]). Because several taxa within the Hyalospheniidae exclusively, or almost exclusively, inhabit *Sphagnum*-dominated peatlands (Figure 3), one can expect that the *Sphagnum* lineage and the Hyalospheniidae lineage (or sublineages) have experienced similar diversification dynamics through time, especially if a longstanding ecological interaction between both groups of organisms has occurred. Hubers and Kerp [14] pointed out a similar ecological role between Carboniferous (359–299 Myr ago) protosphenugales and modern *Sphagnum* in peatbogs. However while *Sphagnum* shows a diversification stasis from ca. 220 to ca. 14 Myr (with only one cladogenesis; [16]; Figure 3), the *Hyalospheniidae* seem to follow a more constant diversification pattern from ca 140 Myr until very recent times (ca. 7 Myr) when they increased the rate of diversification (Figure 3 and Figure 4).

The Cretaceous (145-65 Myr ago) was the beginning of a great change in terrestrial ecosystems [32]. The age reconstructed for the diversification of *Hyalospheniidae* matches precisely with the origin and diversification of angiosperms (ca 130 Myr ago, [53]) as well as the diversification of most mosses [52]. Therefore, the *Hyalospheniidae* certainly not only depend on the diversity of *Sphagnum* mosses but also on the diversification of different terrestrial habitats with different lineages of modern mosses and angiosperms. The relationship between *Sphagnum* and some of the *Hyalospheniidae* clades occurring only in peatlands should be interpreted as a recent specialization to the *Sphagnum*-dominated peatland ecosystem during the Miocene (5–20 Myr ago; [16]).

The radiation of the *Hyalospheniidae* detected here (7 Myr ago) occurs only after the diversification of *Sphagnum* (14 Myr ago; [16]). However, the diversification pattern found in some parts of the tree suggests that a deeper taxonomic sampling is required to improve our understanding of these ecological relationships. Long branches can indicate either long periods without diversification (because of high extinction or low speciation rates) or poor sampling. The inclusion of additional sequences from *H. papilio* (a single morphospecies) in our molecular phylogenetic analyses demonstrated its diversification in the last ca. 20 Myr. Expanding the sequence sampling (even if taxonomic diversity is still awaiting description) may lead to a pattern of similar diversification in other parts of the *Hyalospheniidae* tree. This would produce a better fit between the diversification of the *Hyalospheniidae* amoebae and *Sphagnum* during the Miocene. If other *Sphagnum*-associated morphospecies within the *Hyalospheniidae*, for instance, had a diversification pattern similar to the one described for *H. papilio*, then the hypothesis that the establishment of *Sphagnum*-dominated-peatland ecosystem influenced the diversification of clades within the *Hyalospheniidae* would be supported. Although currently unclear, it is also possible that the presence of arcellinid testate amoebae may have in turn influenced the diversification of *Sphagnum* mosses.

**Supporting Information**

Figure S1 Light micrographs illustrating six arcellinid specimens we isolated for single-cell PCR. A: *Arcella* sp (Genbank KJ544162), B: *Nebela* sp. (Genbank KJ544164), C-E: *Nebela marginata* (Genbank KJ544160, KJ544156 and KJ544157). A and C-E specimens were sampled from *Sphagnum*-dominated peatland while B was sampled from forest litter. Scale bars = 50 μm.

Table S1 List of the *Hyalospheniidae* single cells from which COI sequences were obtained.

Table S2 Sequences of the primers used in this study.

Table S3 List of publicly available COI sequences from the *Hyalospheniidae*.

Table S4 References of the fossil records not considered in this study.

Table S5 Divergence times from 5 protein + SSU datasets combined and from 5 protein and SSU independently under different calibration schemes. Divergence times for major clades are indicated in Myr (million years). Capital letters represent fossil calibrations (upper and lower bounds) for the corresponding node (Figure 2): A) root <770 Myr, B) *Centropyxis* >220<770 Myr, C) *Hyalospheniidae* >100<770 Myr, D) *Arcella* >100<105 Myr, unless otherwise specified; * In the 5 proteins dataset, *Hyalospheniidae* did not include the basal split between *Pudangiella* and the rest of *Hyalospheniidae*. E) corresponds to the node *Diffugia-Lesquereusia.*

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**Author Contributions**

Conceived and designed the experiments: OFP TJH. Performed the experiments: OFP TJH. Analyzed the data: OFP TJH. Contributed reagents/materials/analysis tools: BSL. Wrote the paper: OFP TJH BSL. Conception of the work: OFP. Acquisition of data (lab work): TJH. Acquisition of data (databases): OFP.
References

1. Birney C, Pavlovska J (2006) A molecular time-scale for eukaryote evolution recalibrated with the continuous microfossil record. Proceedings of the Royal Society B 273: 1087–1097. doi:10.1098/rspb.2006.3553
2. Parfrey LW, Lahr DJG, Knoll AH, Katz LA (2013) Estimating the timing of early eukaryotic diversification with multicellular molecular clocks. Proceedings of the National Academy of Sciences of the United States of America 108: 13624–13629. doi:10.1073/pnas.1110633108. PubMed: 21109099
3. Adler SM, Simpson AGB, Lane CE, Luketa J, Bass D, et al. (2012) The revised classification of eukaryotes. Journal of Eukaryotic Microbiology 59: 429–493.
4. Lahr DJG, Grant JR, Katz LA (2013) Multigene phylogenetic reconstruction of the Tubulinea (Amoeba) corroborates four of the six major lineages, while additionally revealing that shell composition does not predict phylogeny in the Arcellinida. Protist 164: 323–339.
5. Smirnov AV, Chao E, Nasonova ES, Caver-Smith T (2011) A revised classification of naked ciliate amoebae (Amoeba: Lobosa). Protist 162: 545–570.
6. Pavlovska J, Audic S, Adler S, Bass D, Belbahri L, et al. (2012) CBOL, protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS Biology 10(11): e1001419. doi:10.1371/journal.pbio.1001419
7. Golmavsky V (2007) Testate Amoebas and Monothalamous Foraminifera (Protozoa) from the Bulgarian Black Sea Coast. In: Fei V, Popov A, editors. Biogeography and Ecology of Bulgaria. pp. 555–570.
8. Meisterfeld R (2002) Order Arcellinida Kent, 1880. In: Lee JJ, LeeLGE, Bradbury P, editors. The illustrated guide to the protozoa. Second edition ed. Lawrence, Kansas, USA: Society of protozoologists. pp. 827–860.
9. Bouckaert R, Zhang J, Xiu X, Drummond AJ (2009) Bayesian phylogenetic comparative study of testate amoebae inhabiting Sphaugnum-dominated peatlands in the Great Lakes and Rocky Mountain regions of North America. Diversity and distributions 11: 577–590.
10. Deflandre G (1936) Étude monographique sur le genre Nolidae Leidy. Annales de Protologie 5: 201–286.
11. Mitchell EAD, Gilbert D, Amblard C, Grosvernier P, et al. (2003) Paleobiogeography of the Tertiary Ancistrina/Ambrosia (Protozoa): the paedomorphic phase of the biodiversity crisis. Palaeontologia Electronica 5: 115.
12. Heger TJ, Mitchell EAD, Leander BS (2013) Holarctic phylogeography of the Tabulinae (Amoeba) corroborates four of the six major lineages, while additionally revealing that shell composition does not predict phylogeny in the Arcellinida. Protist 164: 323–339.
13. Smirnov AV, Chao E, Nasonova ES, Caver-Smith T (2011) A revised classification of naked ciliate amoebae (Amoeba: Lobosa). Protist 162: 545–570.
14. Mitchell EAD, Gilbert D, Amblard C, Grosvernier P, et al. (2003) Paleobiogeography of the Tertiary Ancistrina/Ambrosia (Protozoa): the paedomorphic phase of the biodiversity crisis. Palaeontologia Electronica 5: 115.
15. Porter S (2011) The rise of predators. Geology 39: 607–608.
16. Shaw AJ, Devos N, Cox CJ, Boles SB, Shaw B, et al. (2010) Peatmoss Structure of microbial communities in Sphagnum from the Rocky Mountain regions of North America. Diversity and Distributions 11: 577–590.
17. Lara E, Heger TJ, Ekelund F, Lamentowicz M, Mitchell EAD (2008) Ribosomal DNA analysis of Sphagnum species corroborates geographical and genetic diversity explained more by environment than dispersal limitation. Systematic Biology 58: 390–399.
18. Chim 48: 145–147.
19. Porter S (2011) The rise of predators. Geology 39: 607–608.
20. Schütte-Kowallik K, Tielens A, Lagrange H, Léonard S, Vergnaud C, et al. (2009) Divergence of early eukaryotes: A single-celled eukaryote as a test case. Trends in Ecology & Evolution 24: 390–396.
21. Seifert K, Chen P, Guo Y, Wang T, Pan X, et al. (2010) Early fossil records of land plants: a review of new molecular evidence. Palaeontology 56: 841–859.
22. Schütte-Kowallik K, Tielens A, Lagrange H, Léonard S, Vergnaud C, et al. (2009) Divergence of early eukaryotes: A single-celled eukaryote as a test case. Trends in Ecology & Evolution 24: 390–396.
23. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.