nature portfolio

Peer Review File

A timetree of Fungi dated with fossils and horizontal gene transfers

Corresponding Author: Dr Eduard Ocana-Pallares

Version 0:

Decision Letter:

9th April 2025

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Edu,

Your manuscript entitled "A timetree of Fungi dated with fossils and horizontal gene transfers" has now been seen by 3 reviewers, whose comments are attached. The reviewers have raised a number of concerns which will need to be addressed before we can offer publication in Nature Ecology & Evolution. We will therefore need to see your responses to the criticisms raised and to some editorial concerns, along with a revised manuscript, before we can reach a final decision regarding publication.

In particular, we would like to see explanations for the use of specific enzyme families in implementing maximum age constraints and for your strategy of taxon sampling when developing the phylogeny.

We invite you to revise your manuscript taking into account all reviewer and editor comments. When submitting the revision, please highlight all changes in the manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a “Response to reviewers” document detailing, point-by-point, how you addressed each reviewer comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the reviewers along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/natecolevol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

* Extended Data Figures - please ensure that any supplementary figures and tables that are crucial to the manuscript’s conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within four to eight weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Ecology & Evolution or published elsewhere.

Nature Ecology & Evolution is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

[REDACTED]

Reviewer expertise:

Reviewer #1:

Reviewer #2:

Reviewer #3:

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors present a new time-calibrated phylogeny of Fungi, utilizing an updated phylogenetic analysis and molecular clock approach utilizing both fossil and ecological absolute calibrations, as well as relative age constraints from horizontal gene transfer (HGT) events between fungi and plants and within fungi. Several molecular clock models, substitution models,sets of calibrations, and dataset site-specific sampling approaches are tested and compared, and the results thereof are clearly integrated into the conclusions.

Overall, this is very carefully done and comprehensive work, clearly explained and presented. The approaches used are well-justified and explained, and supported by sufficient supplementary data and figures. The hypotheses are carefully tested through supporting analyses. For example, re-examining the previously used constraint of pectin-related genes in fungal groups.

Importantly, the HGT inferences are well-explained and documented in the SI, and make reasonable and conservative inferences of relative and absolute age constraints given the observed topologies and their supports.

The authors' major conclusion is that crown group fungi diversified 1401-896 Ma. Additional conclusions include that this provides support for the interpretation of purported fungal fossils as old as 1025 Ma to be crown group fungal representatives. The authors also show that site-specific substitution models are more impactful than branch rate models on estimated divergence times, and that 10,000 sites are sufficient for obtaining results under these models and calibrations, a much smaller dataset than their full concatenated number of sites. These are important findings that will substantially impact and improve how molecular clocks are performed in general.

I have the following minor comments that can be addressed to further improve the manuscript.

(1) line 105: "we then selected 225 markers based on optimal metrics screening". The authors should clearly explain what 'optimal metrics screening' is.

(2) line 119: the authors provide empirical justification for using the C60 model for estimating phylogenies and branch lengths. How does this model compare to other models available in IQ-Tree, in terms of relative BIC? How does the resulting topology differ from non-site specific models that are the best fitting, if those indeed have a better fit?

(3) line 120: what is meant by "congruent with the bibliography"?

(4) discussion (Line 413): The results and SI figures seem to show that selection of slow- or fast-evolving sites are not a major contributing factor in determining the observed age distributions of the chronogram. This is an important finding and should be mentioned in the results, as subsampling sites based on their relative rates is frequently done in this kind of work--this can inform and improve future phylogenetic studies.

(5) In the conclusions, the authors make some interesting observations about the relative timing of major fungal-plant

associations, and notice a "gap" during the Neoproterozoic between these early associations and the rise of land plants. This is a striking observation, and should be expounded upon. How do these recovered ages map to planetary history in general? Are there any coincident or causal connections to be inferred here, e.g., Snowball Earth?

(5)

Reviewer #2 (Remarks to the Author):

Szánthó and colleagues present a very interesting work on estimating divergence times of fungi using phylogenomic dataset and an innovative node-calibration method based on fossils and HGT events. I find this study to be relevant for the community not only because it provides a fresh updated view on the tempo of fungi evolution, but also because it applies an innovative approach to overcome the shortcomings of estimating divergence times in groups where fossil evidence is scarce. There are also interesting results regarding the effect of using complex mixture models to infer more realistic branch lengths, which then affect inferred divergence times. These are all topics of high methodological interest and thus I think this study will be of interest to the broad readership of Nat Ecol Evol.

I found the study clear and robust. The methods adhere to current best practices in the field of phylogenomics. The assumptions are clear and have been largely tested. In this sense, I have no major concerns.

However, implementing maximum age constraints based on the presence of pectins in plants and the corresponding degrading enzymes (PSEs) in fungi might need some clarification. In comparison to seething temporal constraints based on HGTs, using the presence of enzyme families seems more controversial. For example, without being a plant biochemist, a quick literature search suggests that pectins might also be present in other streptophyte and chlorophyte algae that are more distant than the LCA of Klebormidium and embryophytes:
https://onlinelibrary.wiley.com/doi/full/10.1111/ppl.14079
https://onlinelibrary.wiley.com/doi/10.1046/j.1529-8817.2001.00179.x
This would affect different calibration scenarios tested here (constraints A, A+calib & B+C).

How were differences investigated when testing the effect of the number of alignment sites on branch length estimation? What happens to the variance? In principle, phylogenomics should increase precision, regardless of accuracy (how similar branch lengths are). This would be an interesting piece of information.

Line 365. The term ‘stem Fungi’ is inappropriate since these are all extant species.

Lines 389-390 “Dikarya, the most extensively studied fungal group, diverged into Ascomycota and Basidiomycota between 940–577 Ma and 889–550 Ma, respectively”. Please, check this sentence. The divergence of Ascomycota and Basidiomycota refers to the age of the LCA, whereas here the LCAs of their respective crown groups are provided. Also “These lineages” refer to Dikarya, right?

Line 454. What do you exactly mean by pre-embryophyte lineages?

Line 464 etc. Many streptophyte algae live exclusively on land (soils, rocks, crusts, etc.) such as members of Keblsormidiophyceae or Chlorokybophyceae (e.g. see Bierenbroodspot et al. 2024, Irisarri et al. 2021) and so, it could be said that they are fully adapted to terrestrial life. Many streptophyte algae such as Zygnema also form algal mats.

Line 195. “is” should be removed

It would be good to annotate all the clade names discussed in the paper (Holozoa, Holomycota) onto the trees.

The Fig. 2 at the end of the manuscript (not that embedded into the text) seems to have been somehow corrupted.

Reviewer #3 (Remarks to the Author):

In this study, Szánthó and collaborators have generated a timetree for fungi using genomic data from 110 fungal species. With the resulting tree topology, they estimated divergence times using the few (17) known fossil-based calibration points for fungi. To this, they added a maximum constraint on the age of Embryophyta to three phylogenetically and ecologically relevant fungal nodes. In addition to calibration points, they used 17 relative constraints derived from fungi-to-fungi HGT events. They then considered additional timing information obtained from their re-evaluation of pectin-specific enzymes’ evolution in fungi, leading to three additional separate analyses estimating divergence times. The main and central conclusion of this study is that the crown age of Fungi is older than recently reported. The authors report that their study provides a refined timescale of the diversification process of fungi and a minimum age for ancient interactions between fungi and terrestrial algae.

The authors developed and implemented an ingenious way to improve the estimation of divergence times for the tree of

Fungi. Therefore, most of the manuscript is describing the technical challenges and their innovative approach.

Main concerns:

1) Because of the complexity of this multilayered set of analyses, understandingly, the manuscript is mostly centered on the technicalities of their approach, leaving little space for evolutionary or ecological results per se. For this reason, other journals such as Systematic Biology, or Molecular Biology and Evolution, seem to be a better fit for this study.

2) The accuracy of timetree inferences rely in large part on taxon sampling. The number of taxa is relatively low compared to other comparable studies. One advantage of working with fungi, is their relatively small genomes compared to other eukaryotes. Consequently, there are far more fungal genomes available than included in this study. I could not find any justification for the selection of these specific taxa. Also, the analytical methods they used does not prevent them to include more taxa. It is not clear to me why such a low sampling of fungal taxa was used. It is important to include all fungal genomes of taxa outside Dikarya. Also, are all classes of Basidiomycota and Ascomycota for which genomes are available included in this study? This needs to be clearly stated in the methods. Using more calibration points and topological constraints but with a reduced taxon sampling, compared to other studies, does not guarantee higher accuracy or a refined timescale.

3) Low taxon sampling can also lead to phylogenetic analyses that are more prone to long-branch attraction, especially when using a concatenated dataset. On lines 115-117 the authors wrote: “the CAT model has been proven useful to solve complex scenarios of long branch attraction. However, CAT is computationally costly and only available in a Bayesian framework (Phylobayes software).” In addition to a more dense taxon sampling, genes trees could have been generated with CAT within a fully Bayesian framework to avoid long branch attraction problems, and the resulting gene trees could be used to generate a species tree for fungi (see point 4 below). Later on (lines 100-202) the authors reported that “In this regard, posterior predictive simulations showed that using CAT for phylogram sampling led to a better modelling of the input alignment than not using it (Supplementary Information 5-Table 1). Based on this, we decided to usephylograms sampled under the CAT model for the definitive dating analyses.” It would be better to move to supplementary materials the description of the alternative approach that was used, but failed, when compare to the use of the well-established CAT model, and simply proceed with the implementation of the CAT model in the main text.

4) Also, phylogenomic analyses of concatenated datasets can generate strongly supported nodes of erroneous relationships, in regions of the tree with short internodes. The tree of Fungi is known to have multiple regions with very short internodes. The authors acknowledge this reality “deep phylogenetic relationships are not yet fully resolved, including: (i) whether Chytridiomycota or Blastocladiomycota is the sister group to the rest of the fungi; (ii) the position of the flagellated group Olpidiomycota, which
is critical to understanding whether terrestrial fungal groups originated from one or multiple
terrestrialization events; and (iii) the placement of the genus Basidiobolus, which has been
variably positioned near Mucoromycota or Zoopagomycota”. For this reason, phylogenomic studies now use phylogenetic species tree methods that analyze each gene separately and use summary methodologies such as ASTRAL to avoid this bias. This is particularly important for phylogenetic trees with short internodes in parts of the tree, which are difficult to resolve with confidence, but will often result in high support for wrong relationships when using concatenated datasets derived from genomic data. An accurate phylogeny is essential for generating accurate divergence time estimates. It seems that the phylogenomic tree generated by this study was using a concatenated dataset, which raises doubts about the quality and accuracy of the resulting topology.

5) I am concerned about using HGT to justify calibration points because the placement of these calibration points on the topology is likely to be dependent on the density of the taxon sampling. What is the justification for using such a thin sampling, especially with the approach used here, which seems to be less computationally intensive. The 17 constraints based on fungi-to-fungi HGT are also sensitive to taxon sampling. Dense taxon sampling seems to be needed for this.

6) Under Figure 2 it is written “To represent node age uncertainty, node age bars show the oldest and the lowest among the 95% high posterior density (HPD) credibility interval values found for each node among the four chronogram sets reconstructed.” The resulting node age uncertainties shown of Figure 2 are huge, spanning 800 million years or more in some cases, and often spanning 500 to 400 million years. These results are in disagreement with what is written on line 421-423 claiming that the fungi-to-fungi constraints decrease uncertainty around divergence time estimates. I am not sure what the authors can conclude with such a high level of uncertainty on the core figure of this study. If the goal was to show that the uncertainty resulting from this approach is too large to conclude anything biologically relevant about most node ages, especially for the deepest nodes, which are the focus of this study, this figure would support this conclusion. This level of uncertainty does not support their main conclusions that the crown age of Fungi is older than recently reported, and that this study provides a refined timescale of the diversification process of fungi.

7) In the section “Dealing with topological uncertainty” the authors wrote: “In Metazoa and Embryophyta, maximum age calibrations (maxima) can be established based on absence
data, qualified by taphonomic outgroup controls that demonstrate that in-group
representatives would be preserved if they existed; this is possible because most lineages of animals and plants have a structured and predictable fossil record. In contrast, fungal
vegetative structures fossilise very poorly and their fossil record is, for the most part,
unstructured and unpredictable.” Yet, the uncertainty around divergence time estimates for Metazoa and Embryophyta shown on figure 2 are relatively large. This sheds even more doubts about the age estimates they obtained for Fungi with

their approach.

Other concerns:

8) One of the main results of this study is that the age of the crown fungi is older than previously reported because of recent discoveries of fungal-like fossils. On lines 434-441 the authors report that: “These results estimate the last common ancestor of Fungi (LCA-Fungi) to be between 1401–896 Ma. Compared to the age ranges reported by in Chang et al. 2021 or Lutzoni et al. 2018 (~980–650 Ma and ~950–715 Ma, respectively, based on the figures shown in these studies), the upper bound of our range (1401 Ma) accommodates not only the potential fungal identity of recently reported fossils dated to 1010–890 Ma and 810–715 Ma, but also the possibility of even older—although more uncertain—fungal fossils, such as the specimens described by Hermann and Podkovyrov 2010 (1025–1015 Ma).” It is important to realize that one of the two new, older, fossil records for fungi mentioned here (810-715 Ma), is in complete agreement with the estimates from Chang et al. (2021) and Lutzoni et al. (2018) reported here. The older new fossil record (1010-890 Ma) overlaps almost completely with the estimates from Chang et al. (2021) and Lutzoni et al. (2018), but is also well within the estimate of the origin of fungi (which is older than the LCA-Fungi node). Therefore, the most reliable, new fossils of fungi, are in agreement with previous estimates of the origin and LCA of fungi, and don’t support the older age estimates reported by this study.

9) Lines 160-166: Should this be a minimum age instead of a maximum age because embryophytes originated much earlier than the crown of that clade. This seems problematic because the authors seem to be assuming that these fungi originated after the origine of embryophyte. What about their potential interactions with unicellular green algae?

**********END**********

Version 1:

Decision Letter:

17th July 2025

Dear Eduard,

Thank you for submitting your revised manuscript "A timetree of Fungi dated with fossils and horizontal gene transfers" (NATECOLEVOL-24123663A). It has now been seen again by the original reviewers and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Ecology & Evolution, pending minor revisions to satisfy the reviewers' final requests and to comply with our editorial and formatting guidelines.

You will see from the reviewers' comments that while Reviewer #3 has suggested alternative techniques for phylogenomic inference and topology tests, one of the other reviewers has provided an additional report based on which we are satisfied that the current level of analysis is appropriate. You are of course welcome to justify your analytical choices by providing additional clarification in the manuscript text.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

If you have not done so already, please ensure that you also email us a completed copy of the Reporting summary :

Reporting summary: https://www.nature.com/documents/nr-reporting-summary.pdf

Next steps: Once we receive the manuscript file in an editable format, we will perform detailed checks on your paper and send you a checklist listing our editorial and formatting requirements in about a week. Please do not upload the final materials to the submission system or make any additional revisions until you receive this checklist from us.

Thank you again for your interest in Nature Ecology & Evolution. Please do not hesitate to contact me if you have any questions.

[REDACTED]

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed all of the comments made in my previous review, and have updated the revised manuscript accordingly.

Reviewer #2 (Remarks to the Author):

I found the new manuscript version improved and the conclusions to be more robust after the authors incorporated the feedback from all three reviewers. Most of my previous concerns were successfully addressed (see below) and thus I can only recommend the publication of this manuscript.

The authors claim to have modified the expression “pre-embryophyte lineages” but it continues to be present in the text (lines 37, 454, 482, 621) even though I do not think this is correct.

Also, I found the following minor errors that might be corrected.
Line 186-7. “the core set of timing informative described above”
Line 195 “it is has”

Reviewer #3 (Remarks to the Author):

The authors wrote extensive responses to my initial review of this manuscript and did new analyses. I am satisfied by their answer to the sampling issue for fungi that I brought up (points 2 and 3a of the rebuttal). The inclusion of a timetree with 662 taxa (Suppl. Fig. 1) would enable readers to see which available genomes were excluded from the core 153-taxon sampling for this study, and their phylogenetic placements.

For point 3b of the rebuttal (“On lines 115-117 the authors wrote”), the authors understood correctly the phylogenetic approach that I proposed and included the resulting timetree as Extended Data Fig. 9. Multiple studies have demonstrated that phylogenetic inferences based on concatenated dataset involving large number of genes can lead to strongly supported but incorrect phylogenetic relationships, particularly when dealing with very short internodes (including anomaly zone), incomplete lineage sorting (ILS), or horizontal gene transfer (HGT) (e.g., Kubatko and Degnan 2007, Mendes and Hahn 2018, Cloutier et al. 2019, Chafin et al. 2021, Morales-Briones et al. 2021). This study, and other previous phylogenomic studies have shown that the backbone of the fungal tree of life includes several short internodes. The species tree now included as Extended Data Fig. 9, show this as well, and shows local posterior probabilities (e.g., 0.73, 0.74) and consecutive short internodes that are potentially indicative of the anomaly zone (e.g., relationships between Zoopagomycota, Olpidiomycota, Mucoromycota, and Dikarya). In their rebuttal, the authors wrote that they consider the species tree shown in Extended Data Fig. 9 as a nice complement to the analyses based on the concatenated dataset. As far as I know (see reference at the end of this review), this is the best way to infer species trees using genome-wide data. What is the justification for not having this tree replacing the tree based on a concatenated dataset shown in Fig. 2?

In the same point 3b of the rebuttal, the authors wrote “For what it mainly concerns this study (the resolution of the main fungal groups), ASTRAL results recover the same uncertainty concerning the position of Olpidium bornovanus in the phylogeny.” The ASTRAL tree, shown in Extended Data Fig. 9, is quite different from the concatenated tree. As you can see, there are very short internodes (in coalescent units), to the point that some are polytomies that cannot be resolved (e.g., early divergences in Chytridiomycota). The shorter these internodes are, the more conflict there is among gene trees. The uncertainty is not only about the position of Olpidium. The early divergence of Chytridiomycota, the relationships between Chytridiomycota, Blastocladiomycota, and the rest of the tree, as well as the relationship between Zoopagomycota, Olpidium, and the rest of the tree (i.e., Mucoromycota with Dikarya) is also highly uncertain (short internode with conflicts among gene trees). Also, Basidiobolus is sister to Mucoromycota instead of grouping with Zoopagomycota as shown on the tree obtained from the concatenated dataset. Uncertainty about the placement of Basidiobolus was reported in previous phylogenomic analyses of the fungi (Chang et al. 2021, reference 9 in this study). To write on line 128-129 of the revised manuscript that “A complementary species tree reconstruction analysis using ASTRAL38 also recovered uncertainty concerning the position of O. bornovanus (Extended Data Fig. 9).” is an underreporting of the uncertainty associated with this dataset and discordant species tree topologies.

At the end of point 3b of the rebuttal, the authors report that “This altogether adds further support to the need of clarifying Olpidium position, something which we already addressed in our manuscript by performing AU-test analyses on gene tree-species tree reconciliation results, as it is described in the second paragraph of the ‘Dealing with topological uncertainties’ section (lines 131-137).” The AU-Test can lead to overconfidence on the wrong topology. This test was introduced by Shimodaira in 2002. The same author (Shimodaira and Terada 2019) published an article reporting that Selective Inference (SI) improves the previously proposed approximately unbiased (AU) test. However, the authors should also see this paper by Markoswski and Susko 2023. Altogether, the AU-test is not addressing or clarifying the position of Olpidium. It is important to realize that the quartet approach part of ASTRAL is exploring competing topological resolutions and it clearly shows with a local posterior probability of 0.74 (Extended Data Fig. 9) that the placement of Olpidium cannot be resolved with this dataset. It is part of a polytomy.

Point 4. The importance of modeling compositional heterogeneity across sites is well established. However, I don’t see how this supports the use of genome-wide concatenated dataset that have been shown to be inconsistent (see the papers I cited above). The large sample size obtained with concatenation is advantageous for CAT-based inferences and works well for small number of genes. The problem arises when large number of genes are used (i.e., genome-wide studies). For example,

this could be a solution: the site state frequencies could be inferred from the concatenated dataset and applied to gene tree analyses. Again, this is not a justification supporting concatenation to infer species trees using large numbers of genes, but rather to infer site state frequencies. Also, the gene tree analyses allowed the usage of CAT. Therefore, there does not seem to be a scientifically based justification to use concatenation to infer the species tree for this study, especially when there is good evidence for rapid radiations along the backbone of the fungal tree. It is important to realize that concatenation with site-heterogenous models versus quartet-based species tree methods were developed to address different challenges. One of the reasons that concatenation coupled with CAT-like models is widely used for deep phylogenetic studies was to address issues like long-branch attraction as stated in this manuscript (lines 117-119 “Among the available implemented models to handle this, the CAT model has been proven useful to solve complex scenarios of long branch attraction.)” The assumption was that this was the main challenge for solving deep phylogenetic relationships (for example, see Williams et al. 2020). However, if short internodes and rapid radiations are the main challenge, as it seems to be the case for the backbone of the fungal tree of life, then concatenation is known to be problematic, especially if the divergences are deep (Pardo De la Hoz et al. 2024).

The argument that previous studies have used a concatenated dataset approach for inferring deep species tree, including Fungi, to justify the use of a concatenation approach is not a solid scientific argument, especially when we know the circumstances under which such method fail. If this type of argument was used, science would not advance. The first example provided by the author (Aphelida) was published in 2018. This is when the realization that concatenation of genome-wide data can be problematic was starting to gain traction. Chang et al. 2021, which is very similar to the study currently considered for publication, did use a concatenated dataset but they also used extensively ASTRAL. They found extensive discrepancies between the two topologies, which is expected because the internodes are so short along the backbone of the fungal tree. This is what they reported: “the ASTRAL polytomy tests on the super-tree datasets recovered a polytomy for the branching order among Olpidium, Zoopagomycota, Mucoromycota, and Dikarya. These findings are consistent with the branches involved in these ASTRAL polytomies being one-third to one-half the lengths of the flanking backbone branches (Fig. 2). These short branches would be best captured by more quickly evolving genes/sites, however, the ancient age of these evolutionary events further compounds this problem and creates a situation that the genes/sites with the most appropriate substitution rate are also more susceptible to signal decay over time. While these nodes will be difficult to resolve using sequence-based methods, changes in genome structure and content among different lineages may provide information necessary to infer the order of divergence.” It seems that this conclusion applies directly to this study by Szánthó et al. This polytomy that Chang et al. 2021 is referring to covers a large and critical portion of the backbone of the fungal tree. The conclusion is that these internodes cannot be resolve with confidence. Galindo et al. 2021 address the problem of long-branch attraction, but not the problem of overconfidence on wrong relationships associated with genome-wide phylogenetic analyses using concatenated datasets, especially when short internodes are involved. Perhaps, they were not aware of this problem. It seems that there is a consensus in the scientific community that the backbone of the fungal tree includes short internodes that are challenging to resolve, if possible, at all. They might well be polytomies resulting from rapid radiations part of the anomaly zone.

Based on what I wrote above, I disagree with the concluding paragraph of the rebuttal for point 4 “To clarify the position of Olpidiomycota, which is the major…” I have demonstrated why using the most well-supported result based on a concatenated dataset is not reliable for the short internodes along the backbone of the fungal tree. This agrees with Chang et al. 2021 and the papers I cited above.

Line 607-608:” this first round of inference showed an overall reasonably congruent topology with recent publications in the bibliography” and Line 612-613: “In order to recover a more congruent topology, we aimed to use a more complex model such as the CAT+GTR+G4”. This gives the impression that the criterion used to evaluate the quality of, and confidence in, their species tree is congruence with previous studies. There are several issues with this approach. This is a circular argument, giving the impression that the tree topology is known, when it is not.

I have looked more closely to Suppl. Information 3. Here are some minor suggestions:

- On the seventh line of the first paragraph you wrote “signatures” twice in a row.
- Some of the figures on the subsequent pages are somewhat messy because the support values are sometimes on top of the labels at the tips of branches, making it difficult to read these values. Also, it would be good to indicate what are these values.
- Is there a figure where we can see the support values (boostrap values and/or posterior probabilities) for the tree shown in Fig. 2?

For point 6 of the rebuttal, the authors wrote “while conservative and necessarily broad for those nodes for which there is age uncertainty, do not only provide solid estimates, but are in fact informative to take important conclusions such that fungi-streptophyte interactions predated the age of embryophytes.” This was already demonstrated by Lutzoni et al. 2018 with a much better dataset for plants (see their Figure 1). Yet, this is not mentioned in this study. This is not a new result. The HPD for the Embryophyta+Streptophyte algae spans more than 700 million years in Fig. 2 of this study compared to slightly more than 400 million years in Lutzoni et al. (2018). The later study was able to take full advantage of the fossil record for plants, which is not the case for this study by Szánthó et al., which used only 10 species to represent the entire plant kingdom. Why using so few species representing plants (with an outstanding fossil record compared to Fungi) if one of the main conclusions of this study is, as written in the abstract, “providing a minimum age for ancient interactions involving fungi and the algal ancestors of embryophytes in terrestrial ecosystems (1253-797 Ma)”? With a better dataset for plants, Lutzoni et al. (2018) wrote “our conservative estimates of the minimum ages for the origins of extant terrestrial chlorophytes, estimated at 725.62 Ma (784.32–702.57; divergence of Trentepohlia and Tydemania) and 726.56 Ma (881.04–561.51; crown age of

terrestrial Trebouxiophyceae). Interestingly, losses of the fungal flagellum are associated with the origin of terrestrial green algae." This result, based on a better dataset for plants, conflicts with one of the main conclusions of the study by Szánthó et al. Indirectly, this also sheds doubts on the divergence time estimates obtained for fungi by Szánthó et al. Yet this is not discussed by Szánthó et al.

Kubatko, L. S., Degnan, J. H., 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. Systematic Biology 56:17-24

Mendes, F. K., Hahn, M. W. 2018. Why concatenation fails near the anomaly zone. Systematic Biology 67:158-169.

Cloutier, A., Sacton, T. B., Grayson, P., Clamp, M., Baker, A. J., Edwards, S. V. 2019. Whole-genome analyses resolve the phylogeny of flightless birds (Palaeognathae) in the presence of an empirical anomaly zone. Systematic Biology 68:937-955.

Chafin, T. K., Douglas, M. R., Bangs, M. R., Martin, B. T., Mussmann, S. M., Douglas, M. E. 2021. Taxonomic uncertainty and the anomaly zone: Phylogenomics disentangle a rapid radiation to resolve contentious species (Gila robusta Complex) in the Colorado River. Genome Biology and Evolution 13:1-19.

Morales-Briones, D. F., Kadereit, G., Tefarikis, D. T., Moore, M. J., Smith, S. A., Brockington, S. F., Timoneda, A., Yim, W. C., Cushman, J. C., Yang, Y. 2021. Disentangling sources of gene tree discordance in phylogenomic data sets: Testing ancient hybridizations in Amaranthaceae s.l. Systematic Biology 70:219-235.

Shimodaira, H., and Terada, Y. 2019. Selective Inference for testing trees and edges in phylogenetics. Frontiers in Ecology and Evolution 7:174.

Markowski, E., and Susko, E. 2023. Performance of topology tests under extreme selection bias. Mol. Biol. Evol. 41:MSAD280.

Williams, T. A., Cox, C. J., Foster, P. G., Szöllosi, G. J., Embley, T. M. 2020. Phylogenomics provides robust support for a two-domains tree of life. Nature Ecology and Evolution 4:138-147.

Pardo De la Hoz, C. J., Magain, N., Piatkowski, B., Cornet, L., Dal Forno, M., Carbone, I., Miadlikowska, J., and Lutzoni, F. 2023. Ancient rapid radiation explains most conflicts among gene trees and well-supported phylogenomic trees of nostocalean cyanobacteria. Systematic Biology 72:694-712.

Additional comments from another reviewer: I have read the comments of reviewer 3 regarding the use of concatenated/coalescent analyses and the authors' responses. From my point of view, the authors have sufficiently addressed these concerns.

Reviewer #3 is correct when they point out some limitations of concatenations. But summary coalescent approaches (ASTRAL) cannot be considered the solution to these problems because they also suffer from some limitations. Namely, the reduced phylogenetic signal in single genes that are used to obtain gene trees that are considered fixed (thus correct) and used to infer the coalescent tree from them. There are many studies showing limitations of summary coalescent approaches as well. In general, concatenation approaches should be preferred when incomplete lineage sorting is low and often work well with ancient relationships because they allow using complex heterogeneous models (as done in this manuscript originally). On the other hand, coalescent approaches are preferred when incomplete lineage sorting is high and single loci are informative enough, which often does not apply to very deep divergences such as early eukaryotes. When we have high incomplete lineage sorting, deep divergences, and heterogeneous evolution, neither of them performs perfectly, and it is difficult to argue strongly in favor of one or the other method. I think this case might fall between deep divergences that require heterogeneous models (concatenation) and some presence of incomplete lineage sorting, my scenarios 1 and 3. I do not think we are talking about a high ILS scenario where ASTRAL should be favored, because we can see that the concatenated and coalescent trees are overall congruent.

The authors have performed an additional ASTRAL analysis and have specifically addressed the phylogenetic differences among the two topologies, including the position of Olpidiomycota etc., using topology tests. I consider that these analyses answer the concerns of reviewer #3 in this respect.

Note from the authors: New text in the main document and supplementary files is highlighted in yellow.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors present a new time-calibrated phylogeny of Fungi, utilizing an updated phylogenetic analysis and molecular clock approach utilizing both fossil and ecological absolute calibrations, as well as relative age constraints from horizontal gene transfer (HGT) events between fungi and plants and within fungi. Several molecular clock models, substitution models,sets of calibrations, and dataset site-specific sampling approaches are tested and compared, and the results thereof are clearly integrated into the conclusions.

Overall, this is very carefully done and comprehensive work, clearly explained and presented. The approaches used are well-justified and explained, and supported by sufficient supplementary data and figures. The hypotheses are carefully tested through supporting analyses. For example, re-examining the previously used constraint of pectin-related genes in fungal groups.

Importantly, the HGT inferences are well-explained and documented in the SI, and make reasonable and conservative inferences of relative and absolute age constraints given the observed topologies and their supports.

The authors' major conclusion is that crown group fungi diversified 1401-896 Ma. Additional conclusions include that this provides support for the interpretation of purported fungal fossils as old as 1025 Ma to be crown group fungal representatives. The authors also show that site-specific substitution models are more impactful than branch rate models on estimated divergence times, and that 10,000 sites are sufficient for obtaining results under these models and calibrations, a much smaller dataset than their full concatenated number of sites. These are important findings that will substantially impact and improve how molecular clocks are performed in general.

I have the following minor comments that can be addressed to further improve the manuscript.

(1) line 105: "we then selected 225 markers based on optimal metrics screening". The authors should clearly explain what 'optimal metrics screening' is.

We thank the reviewer for their comments. Details on the final selection of the 225 markers are available in the ‘Filtering the marker set’ (line 585) section in the Methods. In the revised version of the manuscript we explicitly reference this section of the Methods in the main text (line 108).

(2) line 119: the authors provide empirical justification for using the C60 model for estimating phylogenies and branch lengths. How does this model compare to other models available in

IQ-Tree, in terms of relative BIC? How does the resulting topology differ from non-site specific models that are the best fitting, if those indeed have a better fit?

Based on this suggestion, we have incorporated in the corresponding Methods section "*Species tree inference and selecting the most supported topology*" a mention to BIC analyses results (lines 602-606):

Using C60 provided a markedly better model fit (C60+LG+G4+F, Bayesian information criterion—BIC—score: 23,863,331) than site-homogenous alternatives (LG+G4+F, BIC score: 24,389,728), underscoring the importance of using site heterogeneous models for supermatrix-based species tree inferences.

(3) line 120: what is meant by "congruent with the bibliography"?

Based on reviewer #3's suggestion, we moved the section where this sentence belongs to to the Methods 'Species tree inference and selecting the most supported topology' (line 592). To clarify, what we meant is that the retrieved species topology by C60 "showed an overall reasonably congruent topology with recent publications in the bibliography" (lines 602-603).

(4) discussion (Line 413): The results and SI figures seem to show that selection of slow- or fast-evolving sites are not a major contributing factor in determining the observed age distributions of the chronogram. This is an important finding and should be mentioned in the results, as subsampling sites based on their relative rates is frequently done in this kind of work-- this can inform and improve future phylogenetic studies.

We agree this finding is important to be highlighted in the Results from the main text. To highlight this result in the revised version of the manuscript we write: "*progressively subsampling sites from the slowest or from the fastest-evolving markers had less impact on the retrieved ages than changing the clock model or not using CAT (Supplementary Information 5-Figs. 1)."* (lines 210-212).

(5) In the conclusions, the authors make some interesting observations about the relative timing of major fungal-plant associations, and notice a "gap" during the Neoproterozoic between these early associations and the rise of land plants. This is a striking observation, and should be expounded upon. How do these recovered ages map to planetary history in general? Are there any coincident or causal connections to be inferred here, e.g., Snowball Earth?

We have extended the Discussion, including an additional paragraph on the mapping of our timescale to planetary history and hypotheses on the relationship between Snowball Earth conditions and the potential emergence of complex macroscopic eukaryotes after the so-called 'Boring Billion' (lines 494-506).

(5)

Reviewer #2 (Remarks to the Author):

Szánthó and colleagues present a very interesting work on estimating divergence times of fungi using phylogenomic dataset and an innovative node-calibration method based on fossils and HGT events. I find this study to be relevant for the community not only because it provides a fresh updated view on the tempo of fungi evolution, but also because it applies an innovative approach to overcome the shortcomings of estimating divergence times in groups where fossil evidence is scarce. There are also interesting results regarding the effect of using complex mixture models to infer more realistic branch lengths, which then affect inferred divergence times. These are all topics of high methodological interest and thus I think this study will be of interest to the broad readership of Nat Ecol Evol.

I found the study clear and robust. The methods adhere to current best practices in the field of phylogenomics. The assumptions are clear and have been largely tested. In this sense, I have no major concerns.

However, implementing maximum age constraints based on the presence of pectins in plants and the corresponding degrading enzymes (PSEs) in fungi might need some clarification. In comparison to seething temporal constraints based on HGTs, using the presence of enzyme families seems more controversial. For example, without being a plant biochemist, a quick literature search suggests that pectins might also be present in other streptophyte and chlorophyte algae that are more distant than the LCA of Klebormidium and embryophytes:
https://onlinelibrary.wiley.com/doi/full/10.1111/ppl.14079
https://onlinelibrary.wiley.com/doi/10.1046/j.1529-8817.2001.00179.x

This would affect different calibration scenarios tested here (constraints A, A+calib & B+C).

We would like to thank the reviewer for their comments, particularly for this insightful point. We agree that the pectin-related relative constraint requires a more detailed explanation. In the revised version of the manuscript, we have re-formulated this constraint based on updated bibliographical information based in part on publications cited by the referee. In particular, the first paper [REF1 below], as well as a more recent publication from the same authors [REF2 below], provide fresh and very valuable information on the evolutionary history of pectins. These two recent references were not covered by the references that we consulted, including a recent review from 2024 (https://doi.org/10.1093/plphys/kiad384).

REF1) 'Same but different — pseudo-pectin in the charophytic alga *Chlorokybus atmophyticus*' (https://onlinelibrary.wiley.com/doi/full/10.1111/ppl.14079)

REF2) 'Pectin-like heteroxylans in the early-diverging charophyte *Klebsormidium fluitans*' (https://academic.oup.com/aob/article/134/7/1191/7745916)

Briefly, these recent results suggest that Embryophyta-like pectin, that is, pectin which shows the polysaccharide profile characteristic of Embryophyta pectins, based on calcium-bridged α-(1→4)-GalA residues and which can be digested by the endo-polygacaturonases found in extant fungi, probably originated in the streptophyte lineage leading to Embryophyta after its divergence from *Klebsormidium*.

Based on enzymatic digestion assays and polysaccharide characterization of the pectins from *Ulva*, *Chlorokybus* and *Klebsormidium* [REF1,REF2], the pectin-specific enzymes

GH28 and CE8, which all ancestral gene content reconstruction methods tested inferred to have been present in LCA-Mucoromycota+Dikarya (Fungi), should be expected to only be able to process polysaccharide compositions from classic pectins. This implies that the node age of LCA-Mucoromycota+Dikarya should probably be younger than the age of the earliest streptophyte which presented classic pectin. In this regard, classic pectin probably originated in the branch stemming from the *Klebsormidium*+Embryophyta node, this implying that this streptophyte ancestor, from the fact of having predated the origin of classic pectin, should probably be older than LCA-Mucoromycota+Dikarya. We thus have re-formulated the constraint based on this logic, and we have replaced the term 'PCW Streptophyta' by *'Klebsormidium*+Embryophyta', since as the reviewer argues, pectin, in its *sensu lato* definition, is older than the *Klebsormidium*+Embryophyta node. We provide a summary of this in lines 276-289, which is extended in Supplementary Information 4, lines 315-362).

Finally, we would like to emphasize that this pectin-related relative constraint, following a conservative strategy, was only included in two of the four sets of timing information used ('PSE constraint A', 'PSE constraint A+calib'; we explored incorporating this constraint also to the 'PSE constraints B+C' condition, but this didn't produce any remarkable change on the dates retrieved by 'PSE constraints B+C'). Also, it is important to remark that the HPDs from 'PSE constraint A' and 'PSE constraint A+calib' almost completely overlap with the HPDs from ‘Default’ and from the ‘PSE constraints B+C' conditions, and when they go beyond them, the maximum extension for a fungal node is of 27 Ma, which is a very short amount of time at the timescales involved. Thus, our combined HPDs displayed in Fig. 2 would not be significantly altered if we were not to include the ages retrieved from the 'PSE constraint A', 'PSE constraint A+calib'.

How were differences investigated when testing the effect of the number of alignment sites on branch length estimation? What happens to the variance? In principle, phylogenomics should increase precision, regardless of accuracy (how similar branch lengths are). This would be an interesting piece of information.

The effect of number of alignment sites on branch length estimation was evaluated by means of distinct analyses and graphical representations: (i) agglomerative clustering of the distinct sets of mean node ages for Supplementary Information 5-Fig. 1; (ii) difference in mean node age between each condition and the full CAT+ condition for Supplementary Information 5-Fig. 2; and (iii) scatter plots comparing comparing mean ages node by node for Supplementary Information 5-Figs. 5-6. Regarding what happens to variance, Supplementary Information 5-Fig. 3 measures how coefficient of variation (CV) changes in response to different methodological configurations. CAT+ conditions produce chronograms with lower node age variance, particularly when the number of alignment sites is larger. The fact that CV decreases in CAT+ conditions when more alignment sites are available could be because the CAT model infers the number and identity of frequency categories across sites, a process that relies on having sufficient data to accurately estimate these site-specific profiles. Having fewer sites could increase variance in the posterior across MCMC runs, leading to higher CV values. In contrast, CAT- models assume a homogeneous substitution process across all sites, so removing or downsampling sites does not substantially alter the model’s structure or parameter space. In any case, the CV values are systematically lower in the CAT+ condition than in the CAT- condition, particularly when 10,000 or more alignment sites are used (Supplementary Information 5-Fig. 3).

We have incorporated an extended comment based on this discussion in Supplementary Information 5 (lines 120-129), and incorporate a mention of this node age variance analysis in ‘Accelerated chronogram sampling based on sophisticated methods’ (lines 201-202).

Line 365. The term ‘stem Fungi’ is inappropriate since these are all extant species.

We have replaced the term “stem” by “the lineage leading to” Fungi.

Lines 389-390 “Dikarya, the most extensively studied fungal group, diverged into Ascomycota and Basidiomycota between 940–577 Ma and 889–550 Ma, respectively”. Please, check this sentence. The divergence of Ascomycota and Basidiomycota refers to the age of the LCA, whereas here the LCAs of their respective crown groups are provided. Also “These lineages” refer to Dikarya, right?

Yes, we are referring to the ages of the LCAs of both groups. We have updated the text (lines 405-406):

“*From Dikarya, the most extensively studied fungal group, the Ascomycota and Basidiomycota clades originated between 940–577 Ma and 889–550 Ma, respectively*”.

Line 454. What do you exactly mean by pre-embryophyte lineages?

By “pre-embryophyte lineages”, we used this term to refer to algae lineages that predated Embryophyta. We have replaced this potentially ambiguous term along the manuscript by “the algae ancestors of modern land plants/Embryophytes” or “streptophyte algae”.

Line 464 etc. Many streptophyte algae live exclusively on land (soils, rocks, crusts, etc.) such as members of Keblsormidiophyceae or Chlorokybophyceae (e.g. see Bierenbroodspot et al. 2024, Irisarri et al. 2021) and so, it could be said that they are fully adapted to terrestrial life. Many streptophyte algae such as Zygnema also form algal mats.

We agree with the referee that streptophyte habitat is heterogeneous and is distributed across a wide range from fresh-water to organisms which may be considered as fully terrestrial. We have improved the text (lines 509-510) “*Given this, and given also that streptophyte algae share some adaptations found in embryophytes to terrestrial life, …*”

Line 195. “is” should be removed

Fixed.

It would be good to annotate all the clade names discussed in the paper (Holozoa, Holomycota) onto the trees.

Thanks for pointing this out. In the revised version of the manuscript we have updated Fig. 2 accordingly.

The Fig. 2 at the end of the manuscript (not that embedded into the text) seems to have been somehow corrupted.

We have made available the full resolution main figures available in the figshare link just in case this happens again ('ALL_MAIN_FIGS.pdf' https://figshare.com/s/c08efaba6bfdcd242d06).

Reviewer #3 (Remarks to the Author):

In this study, Szánthó and collaborators have generated a timetree for fungi using genomic data from 110 fungal species. With the resulting tree topology, they estimated divergence times using the few (17) known fossil-based calibration points for fungi. To this, they added a maximum constraint on the age of Embryophyta to three phylogenetically and ecologically relevant fungal nodes. In addition to calibration points, they used 17 relative constraints derived from fungi-to-fungi HGT events. They then considered additional timing information obtained from their re-evaluation of pectin-specific enzymes' evolution in fungi, leading to three additional separate analyses estimating divergence times. The main and central conclusion of this study is that the crown age of Fungi is older than recently reported. The authors report that their study provides a refined timescale of the diversification process of fungi and a minimum age for ancient interactions between fungi and terrestrial algae.

The authors developed and implemented an ingenious way to improve the estimation of divergence times for the tree of Fungi. Therefore, most of the manuscript is describing the technical challenges and their innovative approach.

Main concerns:

1) Because of the complexity of this multilayered set of analyses, understandingly, the manuscript is mostly centered on the technicalities of their approach, leaving little space for evolutionary or ecological results per se. For this reason, other journals such as Systematic Biology, or Molecular Biology and Evolution, seem to be a better fit for this study.

We thank the reviewer for their comments. Our choice of publishing venue was determined by the breadth of relevance of the results—whether they are relevant only to specialists of fungal taxonomy/phylogeneticists, or to broader interdisciplinary audiences across evolutionary and ecological research. The topology and timescale of Fungi has long been of general interest, with previous studies published in leading interdisciplinary journals (Floudas et al. 2012 Science 336: 1715-1719; Li et al. 2021 Current Biology 31: 1653-1665; Shen et al. 2020 Science Advances 6: eabd0079; James et al. 2020 Ann Rev Microbiol 74: 291-313). Beyond our methodological contributions, the resulting timetree and the results stemming from it are of broad relevance, including our timescale for fungal diversification and the age estimates for fungi-pre-Embryophyta interactions, as also highlighted by the other two referees. In the revised version of the manuscript we have extended the Discussion and the Abstract to remark on these. In addition, our study offers a general framework for inferring evolutionary timescales in clades with sparse or uneven fossil records. The manuscript is carefully structured to present a clear and accessible narrative, with technical details not central to the main discussion appropriately placed in the Supplementary Information. Reviewers 1 and 2 support the view that our study is of general scientific interest and well suited for the audience that Nature Ecology & Evolution attracts.

2) The accuracy of timetree inferences rely in large part on taxon sampling. The number of taxa is relatively low compared to other comparable studies. One advantage of working with fungi, is their relatively small genomes compared to other eukaryotes. Consequently, there are far more fungal genomes available than included in this study. I could not find any justification for the selection of these specific taxa. Also, the analytical methods they used does not prevent them to include more taxa. It is not clear to me why such a low sampling of fungal taxa was used. It is important to include all fungal genomes of taxa outside Dikarya. Also, are all classes of Basidiomycota and Ascomycota for which genomes are available included in this study? This needs to be clearly stated in the methods. Using more calibration points and topological constraints but with a reduced taxon sampling, compared to other studies, does not guarantee higher accuracy or a refined timescale.

We would argue that in this case, the resolution of timetree inference is first and foremost determined by an adequate balance between (i) taxon sampling, (ii) methodological complexity, (iii) amount of phylogenetic signal/markers, and (iv) availability of timing information. As we discuss below, if we had incorporated a taxon sampling even larger than the 153 species sampled, this would not only have limited the amount of phylogenetic markers that we could have used, but most importantly, would have limited the possibility of using site-heterogeneous models for the inference of the topology and the branch lengths. To illustrate this, our analysis, based on a taxon sampling of 153 species and using site-heterogeneous models, already (i) took 6 days and 5.5 hours on 48 CPU threads of Intel Silver 4116 @ 2.1 GHz CPUs to sample a guide tree for CAT-pmsf, (ii) 37 days on 240 CPU threads of Intel Silver 4116 CPU @ 2.1 GHz to infer the amino acid exchangeabilities and site-state frequencies with the CAT-pmsf pipeline, and (iii) 22 days on 192 threads of Intel Silver 4116 CPUs to sample phylograms as a required input for Mcmcdate (information on these running times have been added in the Methods section). We provide substantial detail over the manuscript on why the usage of site-heterogeneous models was important for our study (see particularly 'Dealing with topological uncertainties' and 'Accelerated chronogram sampling based on sophisticated methods' sections).

Taking this into consideration, we designed our 153-taxon sampling aiming to cover a broad diversity of fungi while minimizing taxonomic redundancy. It includes 43 outgroup species and 110 fungi representing the main clades within the major fungal groups. Within Fungi, 67 are from Dikarya and 43 from outside Dikarya. Dikarya is slightly overrepresented to take full advantage of fossil calibrations within this group. See ‘Taxon sampling’ section from the Methods for further details. Recently dated timetrees of Fungi that we mention in our discussion used chronograms of relatively similar sizes than ours, but they either used a much reduced phylogenetic marker set, or used site homogeneous evolutionary models for the inference of the topology and the branch lengths. Extending our taxon sampling would have mainly added taxonomic redundancy at the expense of renouncing to site-heterogeneous models for the inference and datation of the topology.

Notwithstanding this, for those readers that may be missing some specific taxon of interest in our Fig. 2 main timetree, we now also provide an extra timetree of Fungi in Supplementary Fig. 1.

This new timetree includes 662 taxa and was built based on the dataset provided by Strassert et al https://doi.org/10.1016/j.cub.2022.06.057. We however remark that this very

large tree had to be inferred and dated based on site-homogeneous models which, as argued above and at least for our 153-taxon sampling for which site heterogeneous models could be used and compared for site-homogeneous models, showed a worse model fit and a worse modelling of the input alignment than site heterogeneous models.

3) Low taxon sampling can also lead to phylogenetic analyses that are more prone to long-branch attraction, especially when using a concatenated dataset. …

We believe a similar rationale applies here as above, increased taxon sampling in this case would be adding crown diversity to already sampled groups, in particular Dikarya, and as result would not 'cut' long branches. The best available approach is to use complex substitution models that take into account across site compositional heterogeneity. As argued above, our study is designed to make an optimal compromise between model complexity and dataset size.

On lines 115-117 the authors wrote: "the CAT model has been proven useful to solve complex scenarios of long branch attraction. However, CAT is computationally costly and only available in a Bayesian framework (Phylobayes software)." In addition to a more dense taxon sampling, gene trees could have been generated with CAT within a fully Bayesian framework to avoid long branch attraction problems, and the resulting gene trees could be used to generate a species tree for fungi (see point 4 below). …

If we understood it correctly, the reviewer suggests an alternative approach to infer the species tree topology than the one we followed. Instead of concatenating the phylogenetic markers, the reviewer suggests reconstructing a gene tree for each phylogenetic marker, using the CAT model, and then, according to point 4 below, building a species tree based on the reconstructed gene trees using the tool ASTRAL. We thank the reviewer for the suggested analysis. We consider it a nice complement to the analyses done, and hence we have incorporated it in Extended Data Fig. 9. We incorporate a reference to this new analysis in the first paragraph of the 'Dealing with topological uncertainties' section (lines 128-129). For what it mainly concerns this study (the resolution of the main fungal groups), ASTRAL results recover the same uncertainty concerning the position of *Olpidium bornovanus* in the phylogeny. This altogether adds further support to the need of clarifying *Olpidium* position, something which we already addressed in our manuscript by performing AU-test analyses on gene tree-species tree reconciliation results, as it is described in the second paragraph of the 'Dealing with topological uncertainties' section (lines 131-137).

… Later on (lines 100-202) the authors reported that "In this regard, posterior predictive simulations showed that using CAT for phylogram sampling led to a better modelling of the input alignment than not using it (Supplementary Information 5-Table 1). Based on this, we decided to usephylograms sampled under the CAT model for the definitive dating analyses." It would be better to move to supplementary materials the description of the alternative approach that was used, but failed, when compare to the use of the well-established CAT model, and simply proceed with the implementation of the CAT model in the main text.

We thank the reviewer for this suggestion. For the results on species tree inference, we have simplified (lines 120-121) our mention to the tested approaches that preceded the CAT-pmsf

approach to Methods, leaving details for the 'Species tree inference and selecting the most supported topology' section from Methods, lines 598-626). Regarding the results on dating the topology ('Accelerated chronogram sampling based on sophisticated methods' section, lines 185-212), the current paragraph only mentions some important results from the comparison of the CAT vs noCAT dating analyses, leaving details for Supplementary Information 5.

4) Also, phylogenomic analyses of concatenated datasets can generate strongly supported nodes of erroneous relationships, in regions of the tree with short internodes. The tree of Fungi is known to have multiple regions with very short internodes. The authors acknowledge this reality "deep phylogenetic relationships are not yet fully resolved, including: (i) whether Chytridiomycota or Blastocladiomycota is the sister group to the rest of the fungi; (ii) the position of the flagellated group Olpidiomycota, which is critical to understanding whether terrestrial fungal groups originated from one or multiple terrestrialization events; and (iii) the placement of the genus Basidiobolus, which has been variably positioned near Mucoromycota or Zoopagomycota". For this reason, phylogenomic studies now use phylogenetic species tree methods that analyze each gene separately and use summary methodologies such as ASTRAL to avoid this bias. This is particularly important for phylogenetic trees with short internodes in parts of the tree, which are difficult to resolve with confidence, but will often result in high support for wrong relationships when using concatenated datasets derived from genomic data. An accurate phylogeny is essential for generating accurate divergence time estimates. It seems that the phylogenomic tree generated by this study was using a concatenated dataset, which raises doubts about the quality and accuracy of the resulting topology.

The reviewer raises concerns on the concatenate approach for the reconstruction of species topologies. We believe the concatenate approach is well suited for employing sophisticated methods for modeling compositional heterogeneity across sites, a factor that has been shown to be crucial for deep species reconstructions (e.g., https://doi.org/10.1093/sysbio/syad013). The concatenate approach is particularly appropriate for CAT-based inferences, since then site state frequencies can be inferred from sufficiently large sample sizes. Also, independently of the usage of CAT, the concatenate approach is widely used for deep species tree reconstructions including Fungi. e.g., Torruella 2018 (https://doi.org/10.1038/s42003-018-0235-z), an important work which established the phylogenetic position of Aphelida to Fungi; Chang 2021 (https://www.nature.com/articles/s41598-021-82607-4), an important phylogenomic study on *Olpidium*; Galindo 2021 (https://www.nature.com/articles/s41467-021-25308-w), an important paper to establish the position of Sanchytriomycota; or Strassert 2022 (https://doi.org/10.1016/j.cub.2022.06.057), or Li 2021 (https://doi.org/10.1016/j.cub.2021.01.074), etc. These works display a concatenate-based phylogeny in the main figures to illustrate the inferred topologies. Notwithstanding our preference for the concatenate approach, we agree that incorporating results from alternative approaches e.g., ASTRAL is a nice complement to our main analyses; this same criteria was followed by some of the cited works. In the revised version of our manuscript we incorporated the suggested analysis by 'Reviewer #3' on providing a species topology inferred with ASTRAL (Extended Data Fig. 9). Remarkably, our results from ASTRAL and our results from the concatenate approach are largely congruent; they display the same main certainties and uncertainties regarding the internal resolution of the main fungal nodes.

ASTRAL recovered Chytridiomycota with full support as the sister-group to the rest of Fungi, consistently with the CAT-pmsf topology, but, in contrast to CAT-pmsf, failed to recover *Basidiobolus* (Zoopagomycota) as the sister-group to Zoopagomycota. The position of Olpidiomycota was found to be uncertain by both methods (see 'Dealing with topological uncertainties' section).

To clarify the position of Olpidiomycota, which is the major uncertainty recovered at the level of splits between the main fungal groups (both by the concatenated and by ASTRAL), we performed advanced topology tests to finally chose the topology that minimizes the gene tree-species tree reconciliation likelihood for a total of 38,837 gene family trees (lines 131-137). Altogether, we chose to date the topology that gathered more support from the whole set of approaches explored. We consider the dated topology the most credible based on extant data and the available methodology.

5) I am concerned about using HGT to justify calibration points because the placement of these calibration points on the topology is likely to be dependent on the density of the taxon sampling. What is the justification for using such a thin sampling, especially with the approach used here, which seems to be less computationally intensive. The 17 constraints based on fungi-to-fungi HGT are also sensitive to taxon sampling. Dense taxon sampling seems to be needed for this.

In this study, we have used the embryophyta-to-fungi HGT inferred to set one calibration point, and the inferred fungi-to-fungi HGTs to set 17 relative age constraints. Being aware of the risks and challenges of inferring HGTs, we have been particularly conservative in our analyses, and we provide detailed justification on all the calibrations and relative constraints used in the Supplementaries, as acknowledged by other reviewers.

Regarding the taxon sampling: in a previous answer (see point 2), we have provided a detailed justification of why a taxon sampling size of our range was optimal for this study, considering all the factors exposed. For the HGT analyses, as explained in Supplementary information 2, the original taxon sampling (Supplementary Table 1) was extended (Supplementary Table 3), representing a total of 171 eukaryotic species, and also incorporated non-eukaryotic sequences in the phylogenies to minimize the risk of confounding HGT from prokaryotes with intra-eukaryotic HGT. Altogether, the HGT dataset included 8,861,117 sequences. In fact, we had to remove redundancy by for example excluding species-specific paralogs, this in order to obtain reasonable alignment sizes so that we could run phylogenetic inferences by using standard maximum-likelihood phylogenetic methods instead of relying on fast tree reconstruction methods which can be quick, but come at the expense of losing accuracy in the phylogenetic reconstruction. In any case, the possibility that hypothetically missing taxa could create some bias in HGT inferences will always be a factor to be taken into consideration unless we could incorporate genomic data from all fungal species. Because of that, below we discuss distinct scenarios regarding how this potential issue may have impacted (1) the HGT-derived calibration and (2) the HGT-derived relative constraints inferred. We conclude that taxon sampling issues are unlikely to have led to the inclusion of wrong information for the dating analysis, and even if this were the case, the hypothetically wrong information incorporated wouldn't have been problematic for the main results of this work, as we explain in point (3) below.

(1) On the potential impact of missing taxon sampling for the HGT-derived calibration point. To put this calibration point into context, the branching of the Ascomycota clade within embryophytes is robust and implies that at least the ancestor of this clade descends from an Ascomycota branch that acquired the HGT from embryophytes. Having a larger taxon sampling may lead to three possible scenarios. Scenario 1: the added sampling does not branch within or near the clade. This would add no information regarding this HGT story. Scenario 2: the added sampling branches within the clade. This also doesn't add any information regarding the HGT story. Scenario 3: the added taxon is a sister branch of the HGT clade. This would imply that it would also be possible to transfer the max age calibration of embryophytes to a parent node of the currently calibrated node, but setting the max age calibration of embryophytes to the currently calibrated node would still be correct. Conclusion: an extension of the taxon sampling can't be expected to lead to results that would illegitimate the fact of having set the max age calibration of embryophytes to the currently calibrated Ascomycota node.

(2) The same logic applies to the HGT-derived relative constraints inferred. First of all, for the inference of fungi-to-fungi HGTs, in order to minimize the risk of missinferences due to taxon sampling / deep paralogy issues, we were already conservative and specifically focused on candidate HGT topologies that involved phylogenetic associations only between distantly related fungal groups. Second, we have only considered HGT clades with very high bootstrap support, and for which the phylogenetic relationships between the taxa belonging to distinct major fungal groups is also clearly supported by gene-tree independent sequence similarity analyses (DIAMOND all-against-all alignments between sequences). Based on this, we considered the inferred HGT clades from which we set the relative constraints to be robust. (The methodology used, as well as the justification of each relative constraint, are described with detail in the corresponding supplementary). Having clarified this, if we envision, as we did above for the HGT-derived max age calibration, how the fact of having used a larger taxon sampling may have impacted the inference of the HGT-derived relative constraints, we here develop the following hypothetical scenarios. Scenario 1: the added sampling does not branch within or near the donor HGT clade or the receptor HGT clade. Obviously, this would not alter the relative constraint. Scenario 2: the added sampling branches within either the donor HGT clade, the receptor HGT clade, or within both. This also wouldn't make any alteration to the relative constraint. Scenario 3.1: the added taxa extends the receptor HGT clade so that now this clade, which is used to infer the youngest among the pair of nodes involved in the older-younger relative constraint relationship, represents an older ancestor. This may in fact turn the constraint into a more informative constraint, but would not invalidate the current version of the constraint, as the currently constrained node would be a descendant of the newly constrained node, i.e., also younger than the oldest node of the constraint. Scenario 3.2: the added taxa extends the donor HGT clade so that now this clade represents an older ancestor. We were particularly careful when setting up the constraints precisely to minimize this potential issue. To prevent it, in those cases in which the inferred donor was quite distant in the species tree to the species represented in the immediate sister-clade of the HGT clade, then the donor was considered to be an older node than the node which was originally inferred to be the donor, as we detail case-by-case in the corresponding Supplementary. Finally, it is important to remark that for the Bayesian molecular clock analysis, the HGT-relative constraints were also set as relaxed soft bounds priors (see Methods), so that the sampled ages can "break" from the constraint if the molecular sequence data is strongly in disagreement with the constraint.

(3) Despite the above rationale for why we believe our HGT constraints are robust to sampling, to explore the effect of possible errors in relative age constraints, we re-ran Mcmcdate using the 'Default' set of timing information, but excluding each time one of the HGT-derived relative constraints used (see Extended Data Fig. 10). As our new results show, no individual relative constraint has a systematic influence on the global ages obtained in our study nor does any single constraint have any relevant impact on the deep nodes for which the evolutionary and ecological implications are discussed in the manuscript. This indicates that our main results and conclusion are robust to misspecification of the HGT-derived relative age constraints.

6) Under Figure 2 it is written "To represent node age uncertainty, node age bars show the oldest and the lowest among the 95% high posterior density (HPD) credibility interval values found for each node among the four chronogram sets reconstructed." The resulting node age uncertainties shown of Figure 2 are huge, spanning 800 million years or more in some cases, and often spanning 500 to 400 million years. These results are in disagreement with what is written on line 421-423 claiming that the fungi-to-fungi constraints decrease uncertainty around divergence time estimates. I am not sure what the authors can conclude with such a high level of uncertainty on the core figure of this study. ...

We thank the reviewer for this comment, as it made us realize that we could have put more effort in offering the readers a better visualization of node age uncertainty in our dataset, as well as additional files to allow exploiting more detailed node age information beyond the conservative HPD bars shown in Fig. 2. To solve this, we have incorporated, on the one hand, Supplementary Figure 3, which offers a clear view on how node age uncertainty is distributed in our study. This figure shows how the nodes for which there is greater age uncertainty are outside Fungi. Among the nodes outside Fungi, the only nodes that are of higher interest for our study are those in Streptophyta, and these have lower uncertainty thanks to the calibrations available for LCA-Embryophyta. Within Fungi, the nodes with most age uncertainty are specifically within Chytridiomycota and some clades of Mucoromycota. We don't discuss the ages of these nodes on purpose. Finally, the vast majority of the fungal tree is poorly impacted by node age uncertainty, also those nodes found in the most internal parts which are relevant for our study. For example, we retrieved 286 Myr of HPD width (95%CIhigh - 95%CIlow) for LCA-Fungi in the 'Default' condition, see Supplementary Figure 3A. To put this value into context, Chang et al. 2021 or Lutzoni et al. 2018 retrieved HPD widths of a similar range for this node of ~335 and ~235 Ma, respectively.

Regarding Fig. 2, it is important to remark that the HPDs presented combine results from four separate dating analyses ('Default', 'PSE-constraint A', 'PSE-constraint A+calib', and 'PSE-constraints B+C'). This necessarily leads to broader uncertainty
ranges (e.g., from 286 Ma in the 'Default' condition to 504 Ma of HPD width in 'Aggregated' for LCA-Fungi, see panel Supplementary Figure 3B). To achieve narrower age estimates, we could have (i) assigned fossil calibrations as strict maximum age calibrations—implying absence of evidence equates evidence of absence—which is problematic due to the sparse fossil record; (ii) used contentious maximum age calibrations from earlier studies, such as the previously employed pectin-related calibrations (Chang et al. 2015, 2021), which our re-analysis found to be unreliable as they were implemented in these studies (see main text); (iii) applied hard-bound priors only instead of using soft-bound priors to reflect

uncertainty inherent in fossil interpretations; or (iv) presented results from only one dating analysis instead of combining all four. However, the inclusion of multiple analyses, particularly the PSE-related conditions, allowed us to integrate valuable evolutionary timing information from Streptophyta into the fungal age estimates. The relative constraints implemented in the PSE-related conditions rely on plausible evolutionary scenarios which, despite being built on reasonable interpretations on the evolutionary history of PSE in Streptophyta and Fungi, inevitably carry some uncertainty. Therefore, we took a conservative approach by aggregating the broader uncertainty across analyses in Fig. 2. For readers interested in narrower estimates conditional on more equivocal assumptions or results from specific scenarios, we provide separate chronograms in Extended Data Figures 3 and 6–8 (now referenced in the Fig. 2 caption). In sum, we have sought to achieve accuracy through the exploration of methodological uncertainties.

The node age estimates provided in Fig. 2, while conservative and necessarily broad for those nodes for which there is age uncertainty, do not only provide solid estimates, but are in fact informative to take important conclusions such that fungi-streptophyte interactions predated the age of embryophytes. We however agree that readers would benefit from the possibility of obtaining fine-grained information on the distribution of sampled node ages for any specific nodes of interest. On the one hand, we have incorporated node age cumulative probability distribution plots for all nodes in the phylogeny as Supplementary Fig. 2. On the other hand, Supplementary Data now incorporates two additional files, which we have computed based on the distribution of timetrees retrieved from each chronogram analysis. A first file reports the fraction of sampled timetrees showing each node being older than any of the ages shown in our timescale, and a second file shows, for each pair of nodes, the fraction of timetrees in which node A is older than node B. These files have been made available for each separate datation analyses as well as for the aggregated set of chronogram data. We believe these new files will allow readers to navigate our results and obtain specific, while still rigorous, datation information.
-Example 1: The Ascomycota clade (node 142) is older than the Basidiomycota clade (node 59) in 81.3% of sampled timetrees (file Data/Chronograms/Consensus_Figure2/nodeA_olderThanNodeB_fractionChronograms.tsv)
-Example 2: 50% of timetrees support that the Ascomycota clade (node 142) existed 764 My ago (file Data/Chronograms/Consensus_Figure2/node_olderOriginThanCertainAge.tsv).

We have incorporated a mention to the availability of these new files and data in the caption of Fig. 2.

… This level of uncertainty does not support their main conclusions that the crown age of Fungi is older than recently reported, and that this study provides a refined timescale of the diversification process of fungi."

“Our summary timescale, integrating analytic uncertainties, suggests older ages for crown-Fungi (1401–896 Ma) than recently reported”. This is what we claim in the abstract. Quantitatively, 91.78% of the aggregated sampled chronogram data from the four dating conditions support LCA-Fungi being older than 980 Ma, the oldest age among the 95% HPD CI upper bounds reported by the previously published timetrees of fungi mentioned in the discussion. Thus, despite the marginal overlap between our conservative age range for

LCA-Fungi and those provided by previous studies, our chronograms provide age estimates for LCA-Fungi that are systematically older than those from previous studies.

Regarding our timescale of Fungi, we believe there are two possible options that readers may follow. They can reference the age bars from Fig. 2, which are necessarily broader than standard age bars since they account for the different sources of uncertainty, or they can consult perhaps more specific information from the node age probability distributions (summarized in Supplementary Fig. 2, but fully available in Supplementary Data). We believe the claim that our study provides a refined timescale of Fungi is supported by 1) The species tree topology and the dating process has been done using models that provide a better fit than the models used by previous studies. 2) We have incorporated many more calibrations than previous studies, as well as multiple relative constraint information derived from HGT events. 3) Our timescale covers all plausible evolutionary scenarios that relate Fungi and Streptophyta in the evolutionary history of pectin/pectinases (Lutzoni et al. 2018 didn't incorporate information on pectin/pectinases, and Chang et al. 2015 and 2021 incorporated—as we discuss in the manuscript—a problematic version of the pectin-related calibration which we have reformulated in this study). 4) As an improvement to previous studies and as argued in previous response points, beyond the conservative age ranges provided in Fig. 2, we are providing multiple data files that allow a fine-grained exploration of the retrieved node age information, as well as a detailed discussion along the manuscript of the challenges and limitations of dating an old eukaryotic group with a poor fossil record such is the case of Fungi.

7) In the section "Dealing with topological uncertainty" the authors wrote: "In Metazoa and Embryophyta, maximum age calibrations (maxima) can be established based on absence data, qualified by taphonomic outgroup controls that demonstrate that in-group representatives would be preserved if they existed; this is possible because most lineages of animals and plants have a structured and predictable fossil record. In contrast, fungal vegetative structures fossilise very poorly and their fossil record is, for the most part, unstructured and unpredictable." Yet, the uncertainty around divergence time estimates for Metazoa and Embryophyta shown on figure 2 are relatively large. This sheds even more doubts about the age estimates they obtained for Fungi with their approach.

Here we would like to emphasize that the age bars shown for LCA-Metazoa and LCA-Embryophyta in Fig. 2, 574-693 and 431-612 Ma, respectively, are not standard 95% HPD CIs, but aggregate 95% HPD CIs from four different chronogram sampling conditions. If we focus for example in the 'Default' condition, the HPD width (95%CIhigh - 95%CIlow) for both nodes are, respectively, 118 and 102 Myr. Using regression to model the relationship between node mean age and HPD width gives expected HPD widths of 276 and 224 for LCA-Metazoa and LCA-Embryophyta, respectively. We thus retrieved much lower HPD width values than expected for both nodes (158 and 122 Myr less than expected). This was thanks to the fact that the relaxed molecular clock analyses could take advantage of the availability of max and min fossil calibrations for both nodes (we retrieved much higher HPD widths in a modified version of the 'Default' condition, in which the calibrations for both nodes are excluded: 682 Ma and 213 My of HPD width for LCA-Metazoa and for LCA-Embryophyta, respectively). In the new Supplementary Figure 3, it can also be observed how the nodes corresponding to LCA-Metazoa and to LCA-Embryophyta present substantially less age uncertainty than their neighbour nodes for which calibration information is not available.

Other concerns:

8) One of the main results of this study is that the age of the crown fungi is older than previously reported because of recent discoveries of fungal-like fossils. On lines 434-441 the authors report that: “These results estimate the last common ancestor of Fungi (LCA-Fungi) to be between 1401–896 Ma. Compared to the age ranges reported by in Chang et al. 2021 or Lutzoni et al. 2018 (~980–650 Ma and ~950–715 Ma, respectively, based on the figures shown in these studies), the upper bound of our range (1401 Ma) accommodates not only the potential fungal identity of recently reported fossils dated to 1010–890 Ma and 810–715 Ma, but also the possibility of even older—although more uncertain—fungal fossils, such as the specimens described by Hermann and Podkovyrov 2010 (1025–1015 Ma).” It is important to realize that one of the two new, older, fossil records for fungi mentioned here (810-715 Ma), is in complete agreement with the estimates from Chang et al. (2021) and Lutzoni et al. (2018) reported here. The older new fossil record (1010-890 Ma) overlaps almost completely with the estimates from Chang et al. (2021) and Lutzoni et al. (2018), but is also well within the estimate of the origin of fungi (which is older than the LCA-Fungi node). …

We thank the reviewer for this observation. Our intention was to emphasize that our broader upper bound estimate (1401 Ma) is not only compatible with the two fossil age ranges (as are the estimates from Chang et al. and Lutzoni et al.), but also accommodates the possibility of even older fungal-like fossils (e.g., Hermann and Podkovyrov 2010), thereby expanding the temporal window in which fungal diversification could be interpreted. We have revised the paragraph to clarify this point (lines 469-477):

“*The age range retrieved for crown-Fungi (1401–896 Ma, Fig. 2), as well as the age ranges reported in Chang et al. 20219 or Lutzoni et al. 201861 (~980–650 Ma and ~950–715 Ma, respectively, based on the figures shown in these studies), are compatible with the potential fungal identity of recently reported fossils dated to 1010–890 Ma63 and 810–715 Ma64. However, our timescale is also compatible with older—although more uncertain—fungal fossils, such as the specimens described by Hermann and Podkovyrov65 (1025–1015 Ma), and more generally, with the possibility that bona fide fungal fossils from the Mesoproterozoic (1.6–1.0 Ga) could be reported in future studies (LCA-Fungi is > 1 Ga in 88.5% of our chronograms, Supplementary Figure 2 and Supplementary Data)*.”

…Therefore, the most reliable, new fossils of fungi, are in agreement with previous estimates of the origin and LCA of fungi, and don’t support the older age estimates reported by this study.

Since fossils are not indicative of maximum ages for clades, particularly in groups such as Fungi for which the fossil record is scarce, the fact that our upper bound for LCA-Fungi is older than the mentioned fossils doesn’t provide support for or against our timescale.

We believe interpreting fungal fossils as minimum ages for the calibrated groups is the most conservative choice. In this regard, and taking the upper bounds of the discussed fossils into consideration, 86.9% of our chronograms retrieved older ages for LCA-Fungi than 1,010 Ma, and 84% than 1,025 Ma. These values increase to 94.5% and 92.9% when considering the parent node to LCA-Fungi, which fully covers the origin of Fungi. This altogether indicates

that only a very little fraction of our chronograms is incompatible with the potential fungal identity of the discussed fossils. We could not find the full chronogram data from Chang et al. 2021 and Lutzoni et al. 2018 in their supplementary, and hence we could not do this same analysis with their data. However, from the HPD intervals of ~980–650 Ma and ~950–715 Ma reported for LCA-Fungi in these studies, we could expect a higher fraction of chronogram samples being incompatible with the potential fungal identity of the discussed fossils than in our study. Having discussed this, in our manuscript, we prefer to be conservative and restrict our discussion on age compatibility analysis just to the 95% HPD credibility values reported by each study. In this regard, the values reported by our study are fully compatible with the age ranges of the tree fossils discussed, and hence with the possibility that they could correspond to fungi. Still, and as we acknowledge in the discussion, temporal compatibility between fossil and clade ages is not in itself evidence of affinity.

9) Lines 160-166: Should this be a minimum age instead of a maximum age because embryophytes originated much earlier than the crown of that clade. This seems problematic because the authors seem to be assuming that these fungi originated after the origine of embryophyte. What about their potential interactions with unicellular green algae?

To our knowledge, there is no evidence of a fungal association between algae species and these crown clades, with the rare exception of *Geosiphon pyriformis* (https://www.cell.com/current-biology/fulltext/S0960-9822(21)00371-7). While this Glomeromycotina species can form endosymbiosis with a cyanobacterium, the authors of this work identified that this species retains the Glomeromycotina plant cell wall degradation gene set. The authors discuss that this could reflect an intrinsic capacity for *G. pyriformis* to undergo classic mycorrhizal associations with plants under the right conditions, and that this lineage probably switched at some point from regular arbuscular mycorrhizal symbiosis to a fungal-cyanobacteria symbiosis. The identification of a conserved gene set for arbuscular mycorrhizal symbiosis across Glomeromycotina (including *G. pyriformis*) altogether supports that the last common ancestor of Glomeromycotina was most likely capable of establishing symbiotic associations with embryophytes (justification for calibration nº7B has been extended to incorporate this). Consequently, we consider the age of crown-embryophytes as a conservative maximum constraint on the age of crown-Glomeromycotina. In any case, we implemented all the maximum age calibrations as soft bound calibrations, and as our analyses measuring the impact of these maximum age calibrations from the ‘Default’ set shows (Extended Data Fig. 5B), we can discard any potential pejorative impact of these two calibrations on the global evolutionary timescale.

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed all of the comments made in my previous review, and have updated the revised manuscript accordingly.

Reviewer #2 (Remarks to the Author):

I found the new manuscript version improved and the conclusions to be more robust after the authors incorporated the feedback from all three reviewers. Most of my previous concerns were successfully addressed (see below) and thus I can only recommend the publication of this manuscript.

The authors claim to have modified the expression “pre-embryophyte lineages” but it continues to be present in the text (lines 37, 454, 482, 621) even though I do not think this is correct.

To be more accurate, we have replaced “pre-embryophytes” by “the algal ancestors of embryophytes” (lines 46-47,537,703-704 in track changes PDF version), and “pre-Embryophyta era” by “before the rise of embryophytes“ (line 534 in track changes)

Also, I found the following minor errors that might be corrected.
Line 186-7. “the core set of timing informative described above”
Line 195 “it is has”

Solved. Thanks for pointing this out.

Reviewer #3 (Remarks to the Author):

The authors wrote extensive responses to my initial review of this manuscript and did new analyses. I am satisfied by their answer to the sampling issue for fungi that I brought up (points 2 and 3a of the rebuttal). The inclusion of a timetree with 662 taxa (Suppl. Fig. 1) would enable readers to see which available genomes were excluded from the core 153-taxon sampling for this study, and their phylogenetic placements.

For point 3b of the rebuttal (“On lines 115-117 the authors wrote”), the authors understood correctly the phylogenetic approach that I proposed and included the resulting timetree as Extended Data Fig. 9. Multiple studies have demonstrated that phylogenetic inferences based on concatenated dataset involving large number of genes can lead to strongly supported but incorrect phylogenetic relationships, particularly when dealing with very short internodes (including anomaly zone), incomplete lineage sorting (ILS), or horizontal gene transfer (HGT) (e.g., Kubatko and Degnan 2007, Mendes and Hahn 2018, Cloutier et al. 2019, Chafin et al. 2021, Morales-Briones et al. 2021). This study, and other previous phylogenomic studies have shown that the backbone of the fungal tree of life includes several short internodes. The species tree now included as Extended Data Fig. 9, show this as well, and shows local posterior probabilities (e.g., 0.73, 0.74) and consecutive short internodes that are potentially indicative of the anomaly zone (e.g., relationships between Zoopagomycota, Olpidiomycota, Mucoromycota, and Dikarya). In their rebuttal, the authors wrote that they consider the species tree shown in Extended Data Fig. 9 as a nice

complement to the analyses based on the concatenated dataset. As far as I know (see reference at the end of this review), this is the best way to infer species trees using genome-wide data. What is the justification for not having this tree replacing the tree based on a concatenated dataset shown in Fig. 2?

We summarize our preference for the concatenated approach, which is aligned with what another anonymous reviewer commented (see bottom of this document). Preventing LBA is crucial for deep species phylogenies [e.g., REFS1-3 below]. Employing site-heterogeneous models (CAT) is important to reduce systematic errors leading to LBA [e.g., REFS1-2, REF4]. It is not only that the usage of these parameter-rich models, particularly CAT, is potentially problematic in short alignments (constrained sample sizes reduce the chance of estimating the right parameters), but the concatenated approach is also more robust to stochastic errors. Partitioned datasets and ASTRAL-like methods are more prone to LBA due to the small sample bias problem (gene trees built from short alignments) [REF5]. Even when models are correctly specified, there can be a tendency towards estimating a particular type of tree when sample size is small [REF6]. Based on that, we have proceeded as it is usual for works related to the reconstruction of the fungal tree [e.g., REFS 7-10], and in general for deep phylogenetic reconstructions of eukaryotes [e.g., REFS 11-14]. In particular, we have relied on the concatenated approach+site-heterogeneous models for our main analyses, but we have also incorporated results from the coalescent approach, which coincide with the concatenated approach in highlighting which are the main uncertainties on the earliest splits of the fungal tree of life (discussed below).

[REF1: Lartillot, N., Brinkmann, H. & Philippe, H. Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. BMC Evol Biol 7 (Suppl 1), S4 (2007)]
[REF2: Improved Modeling of Compositional Heterogeneity Supports Sponges as Sister to All Other Animals. Current Biology 27, 3864–3870, 2017]
[REF3: Williams, T.A., Cox, C.J., Foster, P.G. et al. Phylogenomics provides robust support for a two-domains tree of life. Nat Ecol Evol 4, 138–147 (2020)]
[REF4: Lénárd L Szánthó, Nicolas Lartillot, Gergely J Szöllősi, Dominik Schrempf, Compositionally Constrained Sites Drive Long-Branch Attraction, Systematic Biology, Volume 72, Issue 4, July 2023, Pages 767–780]
[REF5: Sebastien Roch et al. Long-Branch Attraction in Species Tree Estimation: Inconsistency of Partitioned Likelihood and Topology-Based Summary Methods, Systematic Biology, Volume 68, Issue 2, March 2019, Pages 281–297]
[REF6: Huai-Chun Wang et al. The Relative Importance of Modeling Site Pattern Heterogeneity Versus Partition-Wise Heterotachy in Phylogenomic Inference, Systematic Biology, Volume 68, Issue 6, November 2019, Pages 1003–1019]
[REF7: Torruella, G., Grau-Bové, X., Moreira, D. et al. Global transcriptome analysis of the aphelid Paraphelidium tribonemae supports the phagotrophic origin of fungi. Commun Biol 1, 231 (2018)]
[REF8: Chang, Y., Rochon, D., Sekimoto, S. et al. Genome-scale phylogenetic analyses confirm Olpidium as the closest living zoosporic fungus to the non-flagellated, terrestrial fungi. Sci Rep 11, 3217 (2021)]
[REF9: Galindo, L.J et al. Phylogenomics of a new fungal phylum reveals multiple waves of reductive evolution across Holomycota. Nat Commun 12, 4973 (2021)]
[REF10: Strasser et al.. Phylogenomic insights into the early diversification of fungi. Current Biology, Volume 32, Issue 16, 22 August 2022, Pages 3628-3635.e3]
[REF11: Karnkowska A et al. A Eukaryote without a Mitochondrial Organelle. Curr Biol. 2016 May 23;26(10):1274-84]
[REF12: Janouškovec J et al. A New Lineage of Eukaryotes Illuminates Early Mitochondrial Genome Reduction. Curr Biol. 2017 Dec 4;27(23):3717-3724.e5]
[REF13: Matthew W Brown et al. Phylogenomics Places Orphan Protistan Lineages in a Novel Eukaryotic Super-Group, Genome Biology and Evolution, Volume 10, Issue 2, February 2018, Pages 427–433]
[REF14: Williamson, et al. A robustly rooted tree of eukaryotes reveals their excavate ancestry. Nature 640, 974–981 (2025)]
[REF15: Hector Baños et al. Is Over-parameterization a Problem for Profile Mixture Models?, Systematic Biology, Volume 73, Issue 1, January 2024, Pages 53–75]

In the same point 3b of the rebuttal, the authors wrote “For what it mainly concerns this study (the resolution of the main fungal groups), ASTRAL results recover the same uncertainty concerning the position of Olpidium bornovanus in the phylogeny.” The ASTRAL tree, shown in Extended Data Fig. 9, is quite different from the concatenated tree. As you can see, there are very short internodes (in coalescent units), to the point that some are polytomies that cannot be resolved (e.g., early divergences in Chytridiomycota). The shorter these

internodes are, the more conflict there is among gene trees. The uncertainty is not only about the position of Olpidium. The early divergence of Chytridiomycota, the relationships between Chytridiomycota, Blastocladiomycota, and the rest of the tree, as well as the relationship between Zoopagomycota, Olpidium, and the rest of the tree (i.e., Mucoromycota with Dikarya) is also highly uncertain (short internode with conflicts among gene trees). Also, Basidiobolus is sister to Mucoromycota instead of grouping with Zoopagomycota as shown on the tree obtained from the concatenated dataset. Uncertainty about the placement of Basidiobolus was reported in previous phylogenomic analyses of the fungi (Chang et al. 2021, reference 9 in this study). To write on line 128-129 of the revised manuscript that "A complementary species tree reconstruction analysis using ASTRAL38 also recovered uncertainty concerning the position of O. bornovanus (Extended Data Fig. 9)." is an underreporting of the uncertainty associated with this dataset and discordant species tree topologies.

Here we would argue that the main uncertainties shown by the ASTRAL topology (Extended Data Fig. 9), which are highlighted by the reviewer in the paragraph above, are also shown in the concatenated topology (Extended Data Fig. 2). i) "*The early divergence of Chytridiomycota, the relationships between Chytridiomycota, Blastocladiomycota, and the rest of the tree*": both ASTRAL and concatenated recover uncertainty regarding the earliest splits within Chytridiomycota, and both ASTRAL and the concatenated recover Chytridiomycota as sister to the rest of Fungi. ii) "*as well as the relationship between Zoopagomycota, Olpidium, and the rest of the tree (i.e., Mucoromycota with Dikarya) is also highly uncertain (short internode with conflicts among gene trees)*": the concatenated approach also recover uncertainty concerning the Zoopago/Olpidium/Mucoro/Dikarya relationships. This is why we employed phylogenomic reconciliation to find the topology that provides a better reconciliation likelihood (Olpidium,(Zoopago,Mucoro,Dikarya) the most supported one). iii) "*Uncertainty about the placement of Basidiobolus*": this uncertainty is also recovered by the concatenated approach (see lines 132-135 track changes). Overall, a description of the challenging parts of the fungal tree and the approaches done to handle the found uncertainty are explained in the sections 'Dealing with topological uncertainties' (main text) and 'Species tree inference and selecting the most supported topology' (methods).

We have slightly modified a Results paragraph to clarify this point (lines 131-140 track changes) "*We also employed a complementary species tree reconstruction analysis using ASTRAL to capture topological uncertainty in the ToF. The CAT-PMSF tree (Extended Data Fig. 2) but not the ASTRAL tree shows B. meristosporus grouped with Zoopagomycota, as expected based on its taxonomic classification37. On the other hand, Chytridiomycota branched as a sister-group to the rest of Fungi by both approaches, consistent with most recently published ToFs which also used site heterogeneous models12,13,16,17. Finally, the placement of the flagellated fungi O. bornovanus was unresolved (<95% UFBoot bootstrap support), also in the ASTRAL topology*."

At the end of point 3b of the rebuttal, the authors report that "This altogether adds further support to the need of clarifying Olpidium position, something which we already addressed in our manuscript by performing AU-test analyses on gene tree-species tree reconciliation results, as it is described in the second paragraph of the 'Dealing with topological uncertainties' section (lines 131-137)." The AU-Test can lead to overconfidence on the wrong topology. This test was introduced by Shimodaira in 2002. The same author (Shimodaira and

Terada 2019) published an article reporting that Selective Inference (SI) improves the previously proposed approximately unbiased (AU) test. However, the authors should also see this paper by Markoswski and Susko 2023. Altogether, the AU-test is not addressing or clarifying the position of Olpidium. It is important to realize that the quartet approach part of ASTRAL is exploring competing topological resolutions and it clearly shows with a local posterior probability of 0.74 (Extended Data Fig. 9) that the placement of Olpidium cannot be resolved with this dataset. It is part of a polytomy.

According to Shimodaira and Terada 2019, "the previously proposed non-selective version of approximately unbiased p-values are still useful for testing candidate trees to claim that they are rejected if $p < \alpha$". This is what we consider to be doing in our work; we don't believe to be here under a selection bias case. This would be the case if we had employed phylogenomic reconciliation to find the topology that maximizes the likelihood among all possible topologies, and then run the phylogenomic reconciliation AU test including that topology among the tested set. Instead, what we did is to evaluate and compare under the phylogenomic reconciliation framework three topologies, none of which has been selected from the fact of being the highest likelihood one in phylogenomic reconciliation analyses (this approach has already been employed in previous studies, see e.g., https://www.science.org/doi/10.1126/science.abe0511). It's true that one of the 3 topologies comes from the best topology inferred by species tree reconstruction with IQ-TREE (i.e., by a different method than the one employed for the AU test of the 3 final topologies), but this topology is precisely one of the topologies that got rejected by the phylogenomic reconciliation AU-test approach. In any case, even if we had to ignore AU-test results, the Olpidium topology shown in Fig. 2, with Olpidium branching as sister-group to Zoopago+Mucoro+Dikarya, is the species tree topology that sums the highest likelihood among the three topologies compared. We thus consider that we have operated correctly in selecting this resolution of the Olpidium topology (Olpidium branching as sister-group to Zoopago+Mucoro+Dikarya) as the most likely topology to date the fungal tree. We have modified the text to emphasize that it is not among our claims that our manuscript has provided a definitive resolution for this challenging phylogenetic position (lines 148-150 track changes): "We thus consider the recovered monophyly of the non-flagellated terrestrial fungi as the most likely topology. This agrees with recent findings …".

Point 4. The importance of modeling compositional heterogeneity across sites is well established. However, I don't see how this supports the use of genome-wide concatenated dataset that have been shown to be inconsistent (see the papers I cited above). The large sample size obtained with concatenation is advantageous for CAT-based inferences and works well for small number of genes. The problem arises when large number of genes are used (i.e., genome-wide studies). For example, this could be a solution: the site state frequencies could be inferred from the concatenated dataset and applied to gene tree analyses. Again, this is not a justification supporting concatenation to infer species trees using large numbers of genes, but rather to infer site state frequencies. …

If we understand correctly, the reviewer suggests running CAT-Phylobayes on the concatented and using the estimated site profiles to perform single gene tree inferences. While this non-standard strategy may be potentially interesting to perform single gene tree inferences with perhaps more accurate site profile estimates, this would not prevent from the small sample bias problem (gene trees built from short alignments) [REFS 5-6 above].

… Also, the gene tree analyses allowed the usage of CAT. …

It is technically possible to run CAT or GTR in single gene tree analyses. However, there is uncertainty regarding how much we can rely on phylogenetic inferences done on short alignments with parameter-rich models such as CAT and/or GTR when there is a potentially limited availability of data (amino acid sites). This uncertainty is possibly why the following recommendation was made in https://doi.org/10.1093/bioinformatics/btn445 "empirical mixtures are especially suited to phylogenetic analyses using small alignments (e.g. single gene analyses), whereas large analyses should preferably be conducted under the more flexible nonparametric CAT model developed previously". CAT usually provides a better fit than empirical mixtures (CXX models, e.g., C60), also in our work. We thus relied on our CAT-based phylogenetic species tree inference based on the concatenated.

… Therefore, there does not seem to be a scientifically based justification to use concatenation to infer the species tree for this study, especially when there is good evidence for rapid radiations along the backbone of the fungal tree. It is important to realize that concatenation with site-heterogenous models versus quartet-based species tree methods were developed to address different challenges. One of the reasons that concatenation coupled with CAT-like models is widely used for deep phylogenetic studies was to address issues like long-branch attraction as stated in this manuscript (lines 117-119 "Among the available implemented models to handle this, the CAT model has been proven useful to solve complex scenarios of long branch attraction.)" The assumption was that this was the main challenge for solving deep phylogenetic relationships (for example, see Williams et al. 2020). However, if short internodes and rapid radiations are the main challenge, as it seems to be the case for the backbone of the fungal tree of life, then concatenation is known to be problematic, especially if the divergences are deep (Pardo De la Hoz et al. 2024).

Here we would argue that LBA is a problem in most if not every deep phylogeny, including the Tree of Fungi. In fact, the phylogenetic position of Microposporidia is a historical example of LBA that is used to emphasize the importance of employing site-heterogeneous models (e.g., REF4 and REF15 above). Long branches are not rare in Fungi (e.g., Sanchytriomycota, Olpidium, yeast groups, …). Our results show how employing the best fitting site-heterogeneous models, as shown by the topological differences associated with the fact of using CAT vs C60, has a clear impact on the results (e.g., recovering Chytridiomycota or Blastocladiomycota as the sister-clade to the rest of Fungi, or Basidiobolus grouping with Zoopagomycota, in accordance with its taxonomic classification). This reinforces the importance of employing the best fitting site-heterogeneous model (CAT) and, in this context, the concatenation-approach is not only the standard way to employ the CAT model, but it is also free from the small sample bias problem. With this we are not arguing neither that the concatented approach is perfect nor that the coalescent approach is not useful for this study. We believe that having incorporated a coalestent-based phylogeny, as suggested by Reviewer 3, has been a nice addition to our study, as it displays phylogenetic uncertainty associated with some early bipartitions of the phylogeny. Notwithstanding this, we also argue that this uncertainty (early-branching Chytridiomycota vs Blastocladiomycota, the position of Basidiobolus and Olpidiomycota) was already explicit based on the discordances between the C60 and the CAT-based concatenate-based phylogenies, which we already discussed before incorporating the coalescent analyses (see

section ‘Dealing with topological uncertainties’ in the main text, and section and ‘Species tree inference and selecting the most supported topology’ in the Methods).

The argument that previous studies have used a concatenated dataset approach for inferring deep species tree, including Fungi, to justify the use of a concatenation approach is not a solid scientific argument, especially when we know the circumstances under which such method fail. If this type of argument was used, science would not advance. …

By putting forward what the previous works have done we just aimed to show that the concatenated approach is not a rare choice but rather a standard approach employed by the community to address deep phylogenetic reconstructions including the tree of Fungi. Notwithstanding whether it is a standard method or not, a methodological justification on why we employed the concatenated approach has been provided above.

… The first example provided by the author (Aphelida) was published in 2018. This is when the realization that concatenation of genome-wide data can be problematic was starting to gain traction. Chang et al. 2021, which is very similar to the study currently considered for publication, did use a concatenated dataset but they also used extensively ASTRAL. They found extensive discrepancies between the two topologies, which is expected because the internodes are so short along the backbone of the fungal tree. This is what they reported: “the ASTRAL polytomy tests on the super-tree datasets recovered a polytomy for the branching order among Olpidium, Zoopagomycota, Mucoromycota, and Dikarya. These findings are consistent with the branches involved in these ASTRAL polytomies being one-third to one-half the lengths of the flanking backbone branches (Fig. 2). These short branches would be best captured by more quickly evolving genes/sites, however, the ancient age of these evolutionary events further compounds this problem and creates a situation that the genes/sites with the most appropriate substitution rate are also more susceptible to signal decay over time. While these nodes will be difficult to resolve using sequence-based methods, changes in genome structure and content among different lineages may provide information necessary to infer the order of divergence.”

It seems that this conclusion applies directly to this study by Szánthó et al. This polytomy that Chang et al. 2021 is referring to covers a large and critical portion of the backbone of the fungal tree. The conclusion is that these internodes cannot be resolve with confidence. Galindo et al. 2021 address the problem of long-branch attraction, but not the problem of overconfidence on wrong relationships associated with genome-wide phylogenetic analyses using concatenated datasets, especially when short internodes are involved. Perhaps, they were not aware of this problem. It seems that there is a consensus in the scientific community that the backbone of the fungal tree includes short internodes that are challenging to resolve, if possible, at all. They might well be polytomies resulting from rapid radiations part of the anomaly zone.

Based on what I wrote above, I disagree with the concluding paragraph of the rebuttal for point 4 “To clarify the position of Olpidiomycota, which is the major…” I have demonstrated why using the most well-supported result based on a concatenated dataset is not reliable for the short internodes along the backbone of the fungal tree. This agrees with Chang et al. 2021 and the papers I cited above.

Above we detailed our reasons for employing the concatenated approach which, summarized here, are that a long concatenated alignment is the standard methodological choice for the usage of site-heterogeneous models, and that long alignments are not sensitive to the small sample bias problem. We however acknowledge that the concatenated approach is well complemented by the coalescent approach, and this is why we also incorporated this second approach into our analysis. Then, regarding the quoted paragraph from Chang et al. 2021, we can't do anything other than agreeing with what they reported concerning the topological uncertainty at the first splits of the fungal tree. We would however like to remark two interesting points from the quoted paragraph by the reviewer:

1) "While these nodes will be difficult to resolve using sequence-based methods, *changes in genome structure and content among different lineages may provide information necessary to infer the order of divergence*". →This is exactly what we explored, aiming to solve Olpidium's position: Since the concatenated approach (as well as the coalescent approach) both recover uncertainty regarding the position of Olpidium, we employed phylogenomic reconciliation to find the species tree that maximizes the reconciliation likelihood on a whole genome-level analysis, that is, the species tree that better explains the evolutionary history every gene family found in fungal genomes. This analysis recovered Olpidium as sister to the non-flagellated fungal groups, and this is the topology we chose for the dating analyses.
2) Chang et al. 2021 are explicit with the uncertainty concerning the phylogenetic position of Olpidium. However, despite employing the coalescence and other analyses to explore topological uncertainties, they ended up choosing one particular topology (the one from the concatenated) to provide a dated version of the tree. We believe that this is an acceptable way to proceed and is also similar to how we proceeded: We performed a large battery of phylogenetic analyses in order to find and report which are the challenging positions in the tree (sections 'Dealing with topological uncertainties' and 'Species tree inference and selecting the most supported topology'), and then we chose for the dating analyses the topology which altogether we consider to have gathered more support from our analyses.

Line 607-608:" this first round of inference showed an overall reasonably congruent topology with recent publications in the bibliography" and Line 612-613: "In order to recover a more congruent topology, we aimed to use a more complex model such as the CAT+GTR+G4". This gives the impression that the criterion used to evaluate the quality of, and confidence in, their species tree is congruence with previous studies. There are several issues with this approach. This is a circular argument, giving the impression that the tree topology is known, when it is not.

We incorporated a new analysis in which we tested model adequacy by employing a recently proposed approach and observed that CAT-pmsf provides a better fit to the input alignment than C60 (lines 643-652 track changes PDF), this reinforcing our decision to finally rely on the topology retrieved by CAT-pmsf over the topology retrieved by the C60 model.

I have looked more closely to Suppl. Information 3. Here are some minor suggestions:

- On the seventh line of the first paragraph you wrote "signatures" twice in a row.
- Some of the figures on the subsequent pages are somewhat messy because the support values are sometimes on top of the labels at the tips of branches, making it difficult to read these values. Also, it would be good to indicate what are these values.

We have implemented these suggestions in Supplementary Information 3. In the event that some visualisation can be challenging in the main PDF, each phylogeny has been included as a separate PDF file in Data/S3_SupplementaryInformation_3_data/phylogenies.zip

- Is there a figure where we can see the support values (boostrap values and/or posterior probabilities) for the tree shown in Fig. 2?

Fig. 2 topology is a modification of the CAT-pmsf topology, in which we rearranged *Olpidium*'s position according to what we found to be the most supported topology on the phylogenomic reconciliation analyses (lines 154-155 track changes). The CAT-pmsf topology, bootstrap support values included, is shown in Extended Data Fig 2.

For point 6 of the rebuttal, the authors wrote "while conservative and necessarily broad for those nodes for which there is age uncertainty, do not only provide solid estimates, but are in fact informative to take important conclusions such that fungi-streptophyte interactions predated the age of embryophytes." This was already demonstrated by Lutzoni et al. 2018 with a much better dataset for plants (see their Figure 1). Yet, this is not mentioned in this study. This is not a new result. …

We would argue that reconstructing a timescale, like Lutzoni et al. 2018 did, provides information on which lineages were possibly contemporary to each other, and based on that, it is possible to hypothesize that some of these lineages may have (although not necessarily) been involved in ecological interactions. However, temporal compatibility per se doesn't provide evidence for interactions and for when lineages started to interact. In our work we not only reconstructed a timescale, but most importantly, we integrated into that timescale our ancestral gene content reconstruction results concerning the inference of pectin enzymes in fungi (Fig. 5). It is only by integrating that information into the timescale (how old was the oldest fungal ancestor for which we inferred the presence of pectin degrading enzymes) that we can provide evidence of a minimum age estimate for interactions involving fungi and streptophytes. We have slightly modified a paragraph in the discussion to put emphasis into this, as well as to reference the results sections in which we explain our evaluation and implementation of the pectin-based calibration from its original proposition:

Lines 496-500 track changes: "*Our results also have implications concerning ancient fungi-algae interactions preceding the emergence of crown-embryophytes. Previously hypothesized based on comparative timescale information (e.g., Lutzoni et al. 2018), following [52] (see our Results sections 'Re-evaluating the pectin-related maximum age calibration' and 'Exploring a relative constraint involving streptophytes and early terrestrial fungi'), our findings on PSE evolution and aggregated chronogram data (Fig. 5) provide evidence for such interactions and suggest a minimum age for early interactions involving ancestral streptophytes and Fungi (1253–797 Ma, LCA-Mucoromycota+Dikarya), predating by hundreds of million years the emergence of modern land plants (LCA-Embryophyta, 612–431 Ma)"*

… The HPD for the Embryophyta+Streptophyte algae spans more than 700 million years in Fig. 2 of this study compared to slightly more than 400 million years in Lutzoni et al. (2018). The later study was able to take full advantage of the fossil record for plants, which is not the case for this study by Szánthó et al., which used only 10 species to represent the

entire plant kingdom. Why using so few species representing plants (with an outstanding fossil record compared to Fungi) if one of the main conclusions of this study is, as written in the abstract, “providing a minimum age for ancient interactions involving fungi and the algal ancestors of embryophytes in terrestrial ecosystems (1253-797 Ma)”? With a better dataset for plants …

Lutzoni et al. 2018 put significant effort in describing potential co-diversification events that may have involved fungi and plant lineages particularly a posteriori to the emergence of embryophytes. Since this is not among the aims of our study, incorporating further sampling from embryophytes would not have added significant information concerning the minimum age estimate for ancient interactions involving fungi and the algal ancestors of embryophytes, since this is an evolutionary event that occurred before the emergence of embryophytes. Furthermore, including an extended embryophyte sampling would have forced us to employ site-homogeneous instead of site-heterogeneous models to date the tree (the phylogram sampling process under the CAT model already took us 22 days on 192 threads with the current sampling size). We believe that renouncing to employ site-heterogeneous models would have been detrimental to our study, as one of the major strengths of our work is precisely the fact of having reconstructed a timetree of Fungi that incorporates substantial methodological improvements compared to previous works.

…, Lutzoni et al. (2018) wrote “our conservative estimates of the minimum ages for the origins of extant terrestrial chlorophytes, estimated at 725.62 Ma (784.32–702.57; divergence of Trentepohlia and Tydemania) and 726.56 Ma (881.04–561.51; crown age of terrestrial Trebouxiophyceae). Interestingly, losses of the fungal flagellum are associated with the origin of terrestrial green algae.” This result, based on a better dataset for plants, conflicts with one of the main conclusions of the study by Szánthó et al. Indirectly, this also sheds doubts on the divergence time estimates obtained for fungi by Szánthó et al. Yet this is not discussed by Szánthó et al.

In our view, it is not possible to associate a specific evolutionary event (e.g., loss of the fungal flagellum) with the origin of a particular group based solely on temporal compatibility. Having said that, the quoted paragraph above, that “losses of the fungal flagellum are associated with the origin of terrestrial green algae (terrestrial chlorophytes?)”, is not incompatible with the reported evidence in our study for early interactions involving fungi and streptophyte algae with classic-pectin. It is true that the evidence that we report for these interactions doesn’t include chlorophytes (because they don’t present classic pectin), but since absence of evidence doesn’t imply evidence of absence, our results don’t reject the possibility that early fungi also interacted with chlorophytes, and that these interactions could have driven the loss of the fungal flagellum. In this regard, the age that we recovered for the last common ancestor of the terrestrial fungal groups that lost the flagellum, LCA-Zoopagomycota+Mucoromycota+Dikarya (1287-820 Ma), is compatible (overlaps) with the above-mentioned age range for crown terrestrial Trebouxiophyceae (881-561 Ma). And regarding the divergence of Trentepohlia and Tydemania, we believe there is at least uncertainty to claim that the ancestor of these two algae was terrestrial since only Trentepohlia seems to have a terrestrial habitat. Having said this, in our work, we preferred not to discuss the potential connection between fungi-algae interactions and the loss of the fungal flagellum since we believe there is no direct evidence for this scenario (we report

evidence for early co-existence between fungi and streptophytes, but we don't find evidence to claim that to be associated with the loss of the fungal flagellum).

Kubatko, L. S., Degnan, J. H., 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. Systematic Biology 56:17-24

Mendes, F. K., Hahn, M. W. 2018. Why concatenation fails near the anomaly zone. Systematic Biology 67:158-169.

Cloutier, A., Sacton, T. B., Grayson, P., Clamp, M., Baker, A. J., Edwards, S. V. 2019. Whole-genome analyses resolve the phylogeny of flightless birds (Palaeognathae) in the presence of an empirical anomaly zone. Systematic Biology 68:937-955.

Chafin, T. K., Douglas, M. R., Bangs, M. R., Martin, B. T., Mussmann, S. M., Douglas, M. E. 2021. Taxonomic uncertainty and the anomaly zone: Phylogenomics disentangle a rapid radiation to resolve contentious species (Gila robusta Complex) in the Colorado River. Genome Biology and Evolution 13:1-19.

Morales-Briones, D. F., Kadereit, G., Tefarikis, D. T., Moore, M. J., Smith, S. A., Brockington, S. F., Timoneda, A., Yim, W. C., Cushman, J. C., Yang, Y. 2021. Disentangling sources of gene tree discordance in phylogenomic data sets: Testing ancient hybridizations in Amaranthaceae s.l. Systematic Biology 70:219-235.

Shimodaira, H., and Terada, Y. 2019. Selective Inference for testing trees and edges in phylogenetics. Frontiers in Ecology and Evolution 7:174.

Markowski, E., and Susko, E. 2023. Performance of topology tests under extreme selection bias. Mol. Biol. Evol. 41:MSAD280.

Williams, T. A., Cox, C. J., Foster, P. G., Szöllosi, G. J., Embley, T. M. 2020. Phylogenomics provides robust support for a two-domains tree of life. Nature Ecology and Evolution 4:138-147.

Pardo De la Hoz, C. J., Magain, N., Piatkowski, B., Cornet, L., Dal Forno, M., Carbone, I., Miadlikowska, J., and Lutzoni, F. 2023. Ancient rapid radiation explains most conflicts among gene trees and well-supported phylogenomic trees of nostocalean cyanobacteria. Systematic Biology 72:694-712.

Additional comments from another reviewer: I have read the comments of reviewer 3 regarding the use of concatenated/coalescent analyses and the authors' responses. From my point of view, the authors have sufficiently addressed these concerns.

Reviewer #3 is correct when they point out some limitations of concatenations. But summary coalescent approaches (ASTRAL) cannot be considered the solution to these problems because they also suffer from some limitations. Namely, the reduced phylogenetic signal in single genes that are used to obtain gene trees that are considered fixed (thus correct) and used to infer the coalescent tree from them. There are many studies showing limitations of summary coalescent approaches as well. In general, concatenation approaches should be preferred when incomplete lineage sorting is low and often work well with ancient relationships because they allow using complex heterogeneous models (as done in this manuscript originally). On the other hand, coalescent approaches are preferred when incomplete lineage sorting is high and single loci are informative enough, which often does not apply to very deep divergences such as early eukaryotes. When we have high incomplete lineage sorting, deep divergences, and heterogeneous evolution, neither of them performs perfectly, and it is difficult to argue strongly in favor of one or the other method. I think this case might fall between deep divergences that require heterogeneous models (concatenation) and some presence of incomplete lineage sorting, my scenarios 1 and 3. I do not think we are talking about a high ILS scenario where ASTRAL should be favored, because we can see that the concatenated and coalescent trees are overall congruent.

The authors have performed an additional ASTRAL analysis and have specifically addressed the phylogenetic differences among the two topologies, including the position of Olpidiomycota etc., using topology tests. I consider that these analyses answer the concerns of reviewer #3 in this respect.