

Dear Editor,

I apparently completely overlooked the comments from reviewer 3.

## 1 Round 2: Anonymous reviewer 3

- COMMENT: The author presents the article entitled “The eukaryotic last common ancestor was bifunctional for hopanoid and sterol production”. In this paper, he tries to disentangle the complicated evolutionary history of hopanoids and sterols. To do that, he constructs a new phylogeny merging existing datasets and proposing several hypotheses to explain it. In my opinion, this work is very interesting and the conclusion derived of it could help clarify the presence/absence of eukaryotes in conflictive deposits. However, he might consider the following points: In relation with the evolutionary history proposed, although the author carries out a detailed walk through the ideas, a schematic picture, instead of bullet points would be more didactic and improve the impact of the paper.
- REPLY: I hope the two additional figures help to clarify things.
- COMMENT: The main conclusion of the paper is that LECA would have had both SHC and OSC for hopanoid and sterol production. However, at the end of page 6 and beginning of page 7, the author tries to explain “Why was one of the two enzymes lost in all eukaryote lineages” arguing competitive inhibition already described. So, one can wonder whether the same effect could have affected in the same way to the LECA, then compromising bifunctionality in the LECA. Some explanations about this are required.
- REPLY: Competitive inhibition was observed in Tetrahymena, so there is some precedent. Methylococcus has both enzymes and apparently produces both sterols and hopanoids simultaneously. It would be necessary to test many more combinations of enzymes and substrates to get a sense of what extent inhibition may play a role. That is just one possibility. Otherwise, one compound might be measurably better for some other purpose, such as fluidity, or could be involved in other signaling with secondary metabolic/ hormonal pathways. I tried to reword the relevant sentence in the text.
- COMMENT: Could be interesting to date the trees of SHC and OSC? Maybe the HGT events, an acquisition of novelties at end, could have been successful in some organism because of the unstable conditions in that moment. Dating the tree could be useful to relate this event with increase/decreased O<sub>2</sub> atmospheric proportions already described in the literature.
- REPLY: I agree that this is a good consideration, but that would easily be a paper in itself.
- COMMENT: In the abstract the author mentions that the tree includes “key species” but is not mentioned anywhere why the new species included are important for the new inferred tree. This should be better explained in the methods.
- REPLY: I removed the word ”key”.
- COMMENT: At the end of the first paragraph, in” These molecules (often referred to as biomarkers) may persist for millions of years, and are detectable in some ancient rocks, giving a window into what organisms were present at the time the rock was formed.”, could be necessary citation.
- REPLY: This topic is covered by the citation immediately before.
- COMMENT: At the end of the first page the author comments that “Despite the different activity, the two enzymes are nonetheless similar at the sequence level. For instance, the bacterium Methylococcus

capsulatus is one of the few known organisms that possesses both enzymes, which show 28% identity to each other.” Maybe this shouldn’t be surprising because those conserved studies lie in domains probably needed to perform similar functions in both enzymes.

- REPLY: The intended context was to highlight that the two enzymes within one species are about as distant as those from human to bacteria. I do not know what any given reader considers to be similar or dissimilar proteins.
- COMMENT: The two first bullet points in the introduction need a full stop at the end.
- REPLY: Fixed.
- COMMENT: If the author decides to maintain the bullet points instead of the proposed picture for the evolutionary history, all sentences needs a full stop at the end.
- REPLY: Fixed.
- COMMENT: When LECA is mentioned for the first time, it should be spelled out as last eukaryote common ancestor (LECA).
- REPLY: Fixed.
- COMMENT: The author mentions that “This would therefore validate the interpretation of these fossils, arguing that they are bona fide eukaryotes, but were living in low-oxygen environments where only hopanoid synthesis was possible.” This does? Not “validate” the putative eukaryotic fossil, but increase the support for that hypothesis. The word “validate” should probably be changed for “support” or something similar.
- REPLY: Agreed, fixed.
- COMMENT: In the methods section, change -ginsi option for G-INS-I.
- REPLY: This is to precisely state what is typed in the command line.
- COMMENT: It is mentioned that the original matrix alignment was trimmed. It should be specified how this was performed.
- REPLY: I don’t think I mention the trimming at all, or any comparisons either, but in any case this was done by removing sites with 95% or more of gaps. Most of these looked like they came from misassemblies in the MMETSP data, since I doubt the protein can tolerate any large insertions.