Response to "Radiolarian evolution: Analytical challenges in estimating the diversity and origin of Nature's stars"

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We appreciate Daniel Lahr's concern (Lahr, 2025) on the interpretation and conclusions of our study "**Extant diversity, biogeography, and evolutionary history of Radiolaria**" (Sandin *et al.*, 2025). Given that some of these comments were already addressed in our original study following reviewers comments and that such issues are well known in the molecular diversity and evolution fields we considered them partly resolved. However, we decided to extend such a reply and discuss it in length here for the curious reader and outsiders or newcomers to the field so that they can tackle these fields with integrity and scientific sounding.

Environmental sequencing artefacts (such as PCR amplification or sequencing errors, chimeras and contaminations) are well known in the environmental DNA community. To cope with these issues, researchers have come up with many different ways to detect, interpret and discard such artefacts. DNA sequencing indeed generates many different artefacts when reading the raw reads, however these raw sequences are normally processed and curated for the sole purpose of considering only biologically meaningful sequences (e.g., Schloss *et al.*, 2009; Mahé *et al.*, 2015; Callahan *et al.*, 2016; Frøslev *et al.*, 2017; Chwalińska *et al.*, 2025). In our original study (Sandin *et al.*, 2025), we tried our best to exclude any potential artefact beyond chimeric sequences, such as sequences containing high abundance of sequencing errors or badly annotated contaminations taking a conservative approach and discarding any doubtful sequence. Briefly, we first performed a phylogenetic curation of all environmental sequences associated with Radiolaria, starting from well defined, morphologically identified and high quality sequences, then assembled the data using stringent environmental thresholds and after all these steps of phylogenetic curation, we performed a last phylogenetic curation of the final dataset. See for example the following extracts from our original study:

> "**Taxonomic curation of environmental sequences associated to Radiolaria**": "[...] *Briefly, a backbone phylogenetic tree of Radiolaria was built including only high quality sequences from morphologically identified specimens, then environmental sequences reported in (Decelle* et al., *2012; Biard* et al., *2015; Sandin* et al., *2019, 2021) were added, followed by additional sequences retrieved from public databases*. [...]".
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> During phylogenetic curation, see "**Step 4. Check for chimeras**": "[...] *Due to the difficulties in chimera detection, I have used two different algorithms implemented in* [mothur and vsearch] [...] *A third in-house method was also used in order to detect as many chimeric or problematic sequences as possible. Here I blasted against NCBI independently the first and the last 300 bp* [basepairs] *of each sequence and compared the results. If there were less than 20 exact matches among the first 100 matches, the sequence was considered as maybe chimeric. If there are no exact matches among the first 100 matches, or it matches only with itself, the sequence was detected as chimeric.* [...] (from github.com/MiguelMSandin/radiolaria/blob/master/curation_pipeline.md).
>
> "**Data assembly**": "[...] *The conservative threshold for including only OTUs with at least 100 reads was chosen to ensure the inclusion of biologically meaningful sequences and therefore minimize the chances of dealing with PCR/sequencing errors, chimeric sequences or other artefacts.* [...]".
>
> "**Phylogenetic analyses**": "[...] *Resulting phylogenetic trees led to the manual removal of up to 16 sequences (6 OTUs from PR2 and 10 OTUs from the PacBio dataset) due to their long branches and dubious positions (Table S1).* [...]".

With these filters, we might have removed rare yet legit biological diversity, but we believed to have removed as many artefacts as possible and considered only legit biological diversity. As for any other scientific study, such arbitrary thresholds were chosen based on empirical preliminary analysis. It is also important to note that the data used and generated in our study is publicly available not only for replicability but also to be used and analyzed by the scientific community in future efforts relying on the scientific method rather than opinions.

An additional point to take into consideration for any given study is the bigger context of that study, and the literature that builds upon. Radiolaria has shown an outstanding environmental diversity ever since the first molecular environmental libraries for eukaryotic plankton (e.g., Moon-van der Staay *et al.*, 2001). Despite tedious work of characterizing morphologically described species of Radiolaria with their corresponding sequences (a process known as barcoding; Amaral Zettler *et al.*, 1997, 1999; Decelle *et al.*, 2012; Biard *et al.*, 2015; Sandin *et al.*, 2019; Nakamura *et al.*, 2020; Sandin *et al.*, 2021; Llopis Monferrer *et al.*, 2024), different clades still remain unexpectedly elusive. These clades are the so-called RADs (Rad-A, Rad-B, Rad-C, Rad-X and Rad-Y, as phylogenetically described in Sandin *et al.*, 2025), and do not fall within any previously morphologically described species, with the only exception of *Sticholonche* (within Rad-B). The RADs clades have been sequenced repeatedly and independently, from various research groups, using different sequencing techniques such as sanger (e.g., (Massana *et al.*, 2006; Not *et al.*, 2007; Edgcomb *et al.*, 2011; Lie *et al.*, 2014), short-read (e.g., de Vargas *et al.*, 2015; Pernice *et al.*, 2016) and long-read (e.g., Jamy *et al.*, 2022) sequencing; while they could still be artificial, the chances diminish drastically with every independent study and novel approach reporting them. In addition, repeated studies have shown a marked biogeography (Brown *et al.*, 2009; Giner *et al.*, 2020) and even hypothesised potential interactions with other lineages (Anderson *et al.*, 2024), which, as discussed in our study, support a natural and biological behaviour rather than an artifact produced in the lab.

A second central point in many molecular evolution studies is the interpretation of the fossil record, the calibration of the molecular clock and integration of the results. This is an approach that gathers two different disciplines and it is best performed within an interdisciplinary collaboration combining molecular biologists (Miguel M. Sandin and Fabrice Not) and paleobiologists (Johan Renaudie and Noritoshi Suzuki) for an accurate interpretation of both the state-of-the-art of the different disciplines and the results. Sporadic fossil occurrences are sometimes challenged both taxonomically and stratigraphically (e.g., *Rafatazmia*: Bengtson *et al.*, 2017, argued in Gibson *et al.*, 2018; Carlisle *et al.*, 2021) among others) and may dramatically affect the molecular clock calibration. However Radiolaria fossil record uniquely shines for its robustness. Cambrian formations with unambiguous, well-preserved and diverse Radiolaria assemblages are numerous and widespread (Won and Below, 1999; Maletz, 2011; Danelian and Monnet, 2021 from Canada or Sheng, Kachovich and Aitchison, 2020 from Australia) and even within Cambrian Series 2 (Pouille *et al.*, 2011, from Russia), besides references already cited in our study. See for example (Aitchison *et al.*, 2017) and references therein for a detailed review of Paleozoic (Cambrian to Permian) Radiolaria assemblages. However, and given the scope of our study, we were not able to properly credit all available references on Radiolaria fossil appearance, and thus we selected only some of the latest studies. Given that nodes can only be calibrated with a single and specific distribution (Parham *et al.*, 2012), it is of paramount importance to thoroughly select the right calibration for the right node (and adjusting the distribution shape to the fossil certainty), as those chosen and discussed in our original study. When it comes to the dating of Radiolaria, independent analysis also suggests a Neoproterozoic origin of Radiolaria and points to an even earlier possible Spumellaria fossil (ca. 540-530 Ma; Zhang *et al.*, 2021) as we have originally discussed.

Other analyses that can be done to properly assess the calibration of the molecular clock is to compare the prior and the posterior probabilities (Brown and Smith, 2018). In our study, such prior-posterior analysis found that the signal comes from the data rather than the calibration itself (prior-posterior analysis, see **Figure S4** of the original study). However, we did find a greater difference when the distribution of the prior changes, and thus Radiolaria could have appeared either at 758 Ma (Highest Posterior Density -HPD-: 943-601 Ma), as conservatively reported in our study (using a normal prior) and in agreement with Zhang *et al.*, (2021), or 855 Ma (HPD: 1081-635 Ma) with a more literal interpretation of the fossil record (using a log-normal prior). Other additional analysis can also complement molecular clock dating results. For example, in our original study we have inferred clade origin based only on fossil data, confirming our calibrations and pointing to a possible earlier appearance of Spumellaria than that reported in the fossil record (see BBB analysis, **Figure 3** in Sandin *et al.*, 2025), and suggested in Zhang *et al.*, (2021). Our conclusions were therefore rather conservative.

When it comes to the tree topology, as we have discussed in our original study, the relationships between Foraminifera and Radiolaria are still debatable (the so-called "Retaria hypothesis"). See for example:

Discussion: "[...] *The support of both Polycystinea and Spasmaria still needs further validation, as do their phylogenetic relationships with Foraminifera (i.e., either sister groups (Krabberød et al., 2017) or Radiolaria paraphyletic (Irwin et al., 2019; Sierra et al., 2022) are hitherto low supported hypothesis). Testing such hypotheses with rDNA markers has been inconclusive (Moreira et al., 2007; Pawlowski and Burki, 2009; Krabberød et al., 2011) and it would require more effort in collecting additional molecular data (e.g., protein-coding genes) and the use of different modeling strategies* [...]"

At present, Radiolaria genomes are not available, and transcriptomic data is still poor and patchy. However, it is still possible to obtain consistent patterns such as that between Acantharia and Spumellaria, both consistent groups that always appear monophyletic (**Figure 1**). Therefore, considering the direct evidence that Spumellaria fossils are at least ~515 Ma, and the phylogenetic distance between Spumellaria and Acantharia, their last common ancestor is certainly precambrian and well established in the Neoproterozoic.

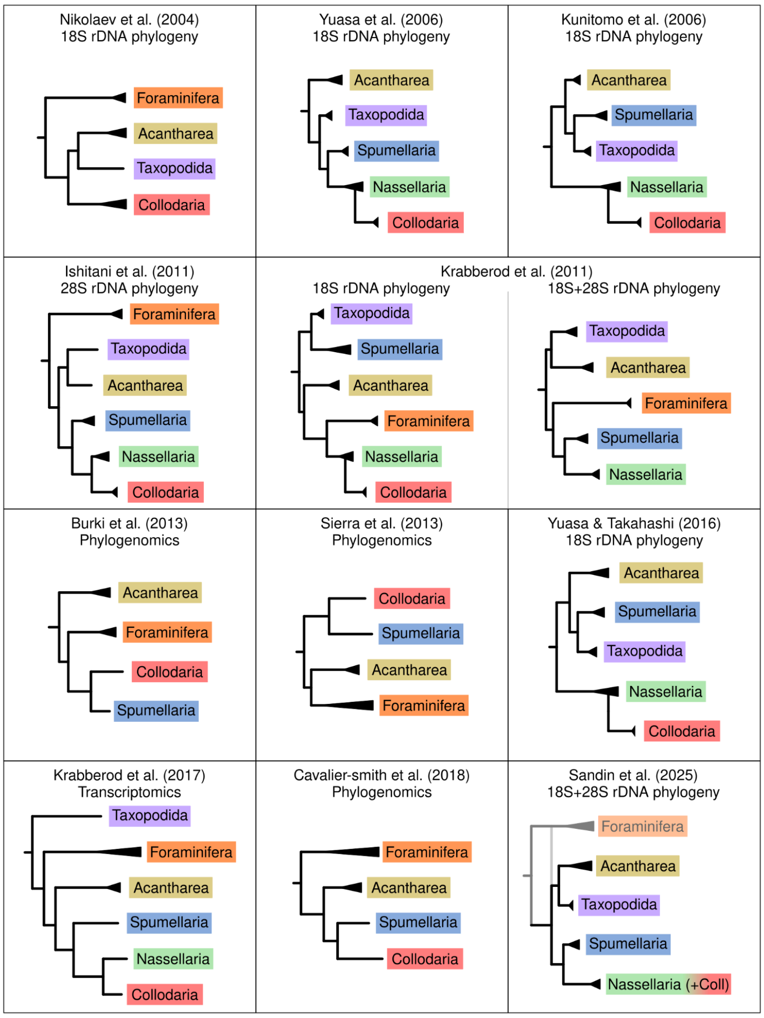

Figure 1. Schematic representation of phylogenetic trees of Radiolaria/Retaria inferred among different studies. Below the citation it is mentioned the main marker/approach used to obtain the phylogenetic tree. Modified from (Sandin, 2019).

In summary, the findings of our study (Sandin *et al.*, 2025) are not coming as unfounded and provocative but rather as conclusive and conservative. We have acknowledged and taken into consideration the most common analytical challenges known for decades in the field, addressed them and interpreted them accordingly. We agree morphological characterization is of paramount importance to understand the nature of environmental lineages, as well as fossil evidence for literal interpretation of evolutionary history. However, in the original study, not only did we check thoroughly for potential spurious results due to method artefacts but we also tested and confirmed our results using several independent lines of evidence. While the criticisms brought on by Lahr (2025) all come from a place of reasonable doubt of outstanding scientific results and novel methodology, all of them were addressed in the original study and, in our opinion, none of them stand up to scrutiny.

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