nature portfolio

# Peer Review File

# Deep origin of eukaryotes outside Heimdallarchaeia within Asgardarchaeota

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**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The precise placement of the eukaryotes within asgardarchaeota is of course of great interest
And it turns out that this question is a non-trivial phylogenetic investigation for a host of reasons, including data, and importantly methods considerations. This manuscript presents new MAGs belonging to the asgardarchaeota, reanalysis of the quality of existing njord MAGs, as well as different markers gene sets/phylogenetic method combinations. Based on these analyses the authors suggest a deeper placement of eukaryotes within asgardarchaeota. In addition geological timing via bayesian timing methods and gene content via ALE implications of this deeper placement are bioinformatically explored.

Although the new data will likely prove helpful in addressing the precise eukaryote-asgardarchaeota phylogenetic topology, it is my interpretation that on a fundamental level the question on how to derive at confidence in a specific topology is not a discussion about more data, but a discussion on on how to phylogenetically establish this. And it is in these more "computational" aspects that the current manuscript partially provides insufficient detail and partially seems to make some methodological and logical steps I am not convinced by. These points are detailed below:

1. How to deal with marker genes.
1a. Phylogenomics needs marker gene sets to make large enough supermatrices to derive . However the assignment of new genomes to marker gene sets is non trivial. In a recent study (https://doi.org/10.7554/eLife.66695) re-analysis of a proposed larger 54 marker genes concluded that many novel and promising markers showed signs of lateral evolution or hidden paralogy and they went back to only 27 markers genes. In this study marker genes seem to taken at their previous established "value", while perhaps they should be checked for their phylogenetic information as marker genes, by for example (per https://doi.org/10.7554/eLife.66695) checking how devoid of hidden paralogy or lateral transfer they seem.

1b. Related to this even when a marker set is or has previously been established for genomes/MAGs set i, it is my understanding that the markers still need to be analyzed for the new species/genome set j under study. It might be that marker genes seemed vertical for a previous data set, their behavior in a new/expanded data set might show genomes for this. This per gene phylogenetic analysis is not just to check the quality of the markers as markers but also to check whether the assignment was done correctly. Analyzing individual marker gene trees seems not to have been carried out in this study. This issue is especially important as the the argument seems to be that a majority of marker/tree combinations favors one topology over a previous suggested one, yet if is this is explained by issues in most markers sets that are absent from the minority one, I would still favor the outcome from the minority one as the currently best supported hypothesis.

2.Data vs analysis
If indeed the findings of this research are valid, they should already be possible to be obtained with the eme et al. data - without the new MAGs. Or indeed if the new MAGs make the difference (which is of course possible!) then that should be explicitly shown by showing that for the previous data, utilizing their approach they can place eukaryotes deeper, outside heimdallarchaeia. The reason this point is relevant is, as raised above and throughout this review, it might very well be a range of computational issues on which the discussion should be conducted rather than a data issue.

3. Contaminations in njord utilized as a conflated argument?

The results on the chimeric/contaminated njord MAGs are quite relevant (I understand that some people know this but I could not find a reference that states that). In any case, the manuscript seems to make the implicit argument that it seems as if the chimaeric njord are somehow responsible for the placement of eukaryotes in previous studies, especially in certain parts of the manuscript such as the abstract. However if I understand correctly what is being done, this is not the case - i.e. the placement of eukaryotes deeper in the asgardarchaeota is not dependent on njord. This potentially misleading argument/logic should be reworded or if it is actually shown should be made more explicit. In addition perhaps the nord MAG quality results should come before the trees and they should be removed from the alignments from then on. Finally insofar as I understand, and insofar as this manuscript describes, some but not all njord MAGs have issues. It is unclear to me how these presumably trustworthy MAGs behave differently from the problematic MAGs.

4. Contaminations in njord, how to deal with them in the analysis.
a. The analysis of the contamination as discussed in the main text is partly very convincing (especially panel c & d) but also insufficiently detailed w.r.t. methods and interpretation. It is completely unclear (in the main text) and somewhat unclear (from the methods) how panel a&b were generated. It seems as if a homebrew contig taxonomic assignment was used instead of (a suite) of established taxonomic assignment tools. Please use several state-of-the-art contig / read assignment tools to investigate these suspicious MAGs in detail.
b. The visualization in panel c & d is quite nice, but how where the metagenomes selected for which read depth is shown? Could it be the case that also for more reliable MAGs selective metagenomes could be found where such discontinuities can be found?
c MAG S143_49 in panel d seems to be assigned to two clusters with one being really minor and the other being the majority. This seems inconsistent with panel b where the TACK and asgard assignments are not that different in size. The other 3 MAGs in the c,d seem to fall into three clusters which is inconsistent with panel a/b. Please explain the inconsistency or perform analyses that result in a more consistent inference.

5. Eukaryotic signature proteins, how to establish their presence.
The final argument that what is now referred to as hodarchaeales are not eukaryotes closest relatives, is a re-evaluation of eukaryotic signature proteins that are now argued to not be limited to hodarchaeales. Now this is a relevant discussion, but the manuscript including the supplement does not offer sufficient detail to properly infer how this was conducted. I.e. are the domains searched using blast, hmmer, hhpred, foldseek is unclear. From the context I would infer it is either CDD search RPS BLAST or HMMER, but it is not described. A domain score threshold is mentioned (50) but it is unclear which score this is referred to (I assume bitscore). I disagree with the assertion that 50 is the default threshold for PFAM (which by the way is deprecated) as for that resource the default cut-off is the GA bitscore which differs per model. Subsequent inspection of supplementary table 9 for the assertion that DNA polE occurs in many archaea reveals a number of PFAM identifiers used, but those are not specific for polE. If I query these identifiers in uniprot there seem to be many many other proteins with these domains. If that is correct, then there seems to be an orthology / homology issue. These orthology issues are normally addressed via gene phylogenies. In summary the presence or absence of proteins in these new and existing MAGs need more methodological explanation and results to support them, including (if indeed they are orthology issues) with gene phylogenies.

6. The manuscript extensively makes use of bayesian phylogenetic inference to supplement ML approaches. I could locate in the manuscript the number iterations these were run, but not if they were converged. Given the size of the data set I expect that these approaches are potentially not converged. This should be discussed/reported on.

Referee #2

(Remarks to the Author)
The paper by Zhang et al entitled "Phylogenomic analyses with expanded taxon sampling reveal deep origin of eukaryotes outside Heimdallarchaeia" explores the evolutionary origins of eukaryotes by analyzing Asgard archaea, the closest known group of the archaeal host-ancestor of eukaryotes. By generating 223 new Asgard genomes, including 16 novel lineages, the researchers found that Njordarchaeales, previously considered part of Asgard, are chimeric genomes blending TACK and Asgard lineages, complicating previous phylogenetic interpretations. Using advanced phylogenomic methods, they propose that eukaryotes evolved before the diversification of Heimdallarchaeia, challenging previous classifications. They also date the last Asgard-eukaryote common ancestor to before the Great Oxidation Event, suggesting metabolic flexibility that might link eukaryogenesis with planetary changes, offering new insights into early cellular complexity.

This paper is a well-timed, bioinformatically rigorous contribution addressing a critical question in evolutionary biology, showcasing a substantial body of research. Moreover, I praise the authors for having provided all alignments and phylogenies in Figshare. This should be the standard for these analysis, but unfortunatly is not. I only have some methodological questions that I would like the authors to address.

1- Is the model used in the phylogenetic reconstructions the best model? If not, please justify the choice of this model instead of one that might be more appropriated for the alignment(s).

2- Although understanding the “voting” system to explain the chimerism of Njordarchaeales MAGs, it is unclear if the TACK associated contigs are derived from missassembly, gain by HGT or in alternative, TACKL having received the genes. Moreover, the local similarity (distance) method does not necessarily reflect evolutionary distance, especially if only local

similarities are considered. Thus, to avoid having to compute all of those phylogenies, using global identities, (or at least considering alignment coverage) might minimize these effects.
This might not change the conclusions of this section, but would be more accurate.

Minor:
Supplementary table 10 (Supplementary Table 10. The annotation results of the CDD (Conserved Domain Database) domain of eukaryotic signature proteins.
) not cited in main text. Also, I am not sure if the content of the table goes with the title. For instance, CoxII (subunit of complex IV) is not an ESP.

Typo in the table of contents of supplementary information

Referee #3

(Remarks to the Author)
What an exciting read, but not without problems. This is an 8 page review, for which the referee apologizes but there is a lot to say about this very exciting paper. To get to the point, this reviewer recommends acceptance after (mostly) editorial revision, no new analyses (maybe some filtering by quality), no new data, just better presentation of the massive and relevant new data at hand. The case for publication is very strong.

In this important paper, Zhang et al. report 223 new asgard metagenomic assemblies, flanked by phylogenetic analyses and metabolic reconstructions for the asgard group. Their main findings are threefold: 1) eukaryotes branch in a new place in their trees, revamping (yet again) the eukaryote host lineage sisterhood issue; 2) their metabolic reconstructions indicate that the common ancestor of asgard lineages was a methanogen, drawing tighter links to hydrogen-dependent and methanogenic lifestyles among asgard groups; 3) on the basis of better sampling, they report that some previously published asgard 'lineages' are chimeric metagenomic assembly artefacts, that is, the assembly of metagenome data led to merging two genome 'lineages' into one. This chimerism aspect (finally) brings a glaring issue in the asgard phylogeny controversies openly to the fore, as such chimaeras impact all downstream inferences, from phylogeny to metabolic reconstructions. 4) In addition, Zhang et al. offer a new narrative about archaeal evolution and eukaryote origin, this time focussing on nickel (in addition to oxygen), but as with all other asgard evolution stories, this one has fatal flaws (here regarding both Ni and O2) and was not well thought through. On balance, the paper presents a strident advance and marks a significant turning point toward greater caution in the asgard saga. As such, the manuscripts presents a very strong case for publication, but after some problems are fixed.

Issue 1: The position of eukaryotes in the trees. Readers of Nature continue to be intensely interested in which group of (yet uncultured) archaea are the sisters of eukaryotes in phylogenetic trees. This has spawned a number of papers, most recently about the "heimdallarchaeial"-ness of eukaryotes last year (ref. 3). However, no one has ever seen an heimdallarcheaon (they only exist in computers so far) and a key taxon underpinning the "heimdallarchaeial" phylogenetic result in ref. 3 (Njordarchaeales) is shown here by Zhang et al. to be non-existent in nature, an artefact of metagenome assembly procedures on the computers of the Ref. 3 team. It is imperative that this information be published as a full Nature paper, backed by the 233 new genomes, although we can be sure that the Ref. 3 team will contest this result by Zhang et al. The purpose of science is to move forward on the basis of new data as opposed to cementing old analyses into the literature (that happened with the rRNA tree of life…). It is clear that there will be a lively debate about methods and data upon publication of Zhang et al., but it is essential for that debate to occur i) *in public* and ii) *after publication* of Zhang et al., not during the peer review process. Zhang et al. have performed careful analyses, at least as careful (more so in some respects) as their competitors, they should not be forced to perform additional phylogenetic analyses that every anonymous referee would like to see. Their result negates the "heimdallarchaeial" result with 223 new genomes and a chimerism revelation – this is a good direction for the field to be moving. I recommend no new concatenated phylogenetic analyses before publication, it will be a never-ending story. The peer review process for ref. 3 was mild, with no contests on data quality. Zhang et al. should be shown the same treatment.

Figures 1 and 3: we need to see, with clearly marked and explained symbols, where the only two cultured/enriched/imaged asgards (refs 5 and 6) are located in these trees and a clear statement in the text that the remaining lineages are only known from computer assembly procedures of environmental sequence data.

Issue 2. The metabolic reconstructions. It is exciting to read that the asgard ancestor was probably methanogenic, but this information comes from one method (ALE) and it appears that ALE has failed, or the genomes have a large unrecognized bacterial component, or both. Zhang et al are to be commended for their openness of their reconstruction, it is more complete than that provided by ref. 3, but still there are are problems (hidden in ref. 3, reported in Zhang). The following points concern the reconstruction.

1. The reconstruction cannot be correct (by inference, those of ref. 3 either, because their data has a chimerism issue and the quality is not fundamentally better, whereby the quality in ref. 3 is impacted by the chimeras). In Figure 4 and Supplementary Figure 15 we see a tetrahydrofoate-(H4F-) dependent Wood-Ljungdahl pathway (the bacterial version) and a tetrahydromethanopterin-(H4MPT-) dependent Wood-Ljungdahl pathway (the archaeal version) in all four lineages shown. This is one of those "never been seen before in cultured cells" physiological phenomena, as no cultured cells have ever been shown (to this reader's knowledge) to encode both the pathways. It is another "only in metagenomes" physiology, and that sets off alarms. A given metagenome assembly can be 1.4 Mb in size or 2.9 Mb in size depending on parameters

(https://doi.org/10.1038/s41564-019-0417-6). Now the genome sizes (some of them 12 Mb) are just listed as a point estimate. The two WL pathways in the same cell cannot be correct. The bacterial is surely a contamination or binning artefact. Regarding the bacterial WL pathway and likely bacterial contamination, it comes to mind: How do bacteria and archaeal lipid synthesis genes square off here (frequencies, not ALE), and how many bacterial-specific ribosomal proteins do they find at which frequency in this data? If they are claiming that ref. 3 is an assembly artefact, the present assemblies need to be extremely clean.

2. The underlying metabolic reconstruction information is listed as given in “Table X” (Fig. S15 legend) but there is no Table X, although supplemental Table 7 is a start in the right direction, yet lacks all of the enzymes that are required for H4F and H4MPT synthesis. It also lacks most of the crucial information that we need to know underpinning Figure 4 and Supplementary Figure 15. The authors have clearly done analyses that lead to the metabolic reconstructions, readers need to have access to that data in a fully enumerated version of Table 7 that allows readers to check the data. All of the enzymes indicated in the figure need to be backed up by assembled and annotated genes in the Table.

3. As a prime example of a problem enzyme, Supplementary Figure 15 has Nif on the right side converting N2 into ammonia. There is no N2 importer (N2 is a gas) and Nif (nitrogenase) is a cytosolic protein, the Nif-related entries in Table 7 are lacking. MCR in Fig 4 of the main text is shown as methane/alkane oxidizing, what evidence or logic supports that scheme?

4. The lower left methanogen-like enzyme assembly in Fig. S15 is a mess. Mtr (MtrA-H?) is a pumping enzyme that generates the ion gradient for methanogen ATP synthesis in the methyl transfer from H4MPT to CoM. Free CoM from the heterodisulfide reductase is the substrate for MtrA-H, which typically takes methyl groups from methyl-H4MPT. Or is this indicating MtaABC? (see Thauer et al. Nat Rev Micro 2008 doi:10.1038/nrmicro1931). There is no methyl group for that reaction.

5. Table 7 lists chlorophyll synthesis enzymes, which can’t be right, unless there are photosystem proteins and carotenoid synthesis in the data, and then there is a problem because no archaea contain chlorophyll and no chl-photosynthesizers use the WL-pathway. Table 7 and the figures need to be complete and coherent.

6. The pta, ack, and acd enzymes present in all four (Fig S15) could, in principle, enable an acetogenic metabolism, except that Rnf is missing for pumping, although the protein that is designated as complex I could be Ech, the energy conserving hydrogenase of acetogens and methanogens ( Schoelmerich and Muller PNAS 2019 https://doi.org/10.1073/pnas.1818580116), which is indistinguishable from complex I in sequence comparisons (Schoelmerich and Muller CMLS 2020 https://doi.org/10.1007/s00018-019-03329-5). The possbility that the host lineage had an acetogenic lifestyle has been discussed (Martin 2016; DOI 10.1002/bies.201600089) although it is difficult to force modern methanogens to grow by producing acetate (Schöne et al. 2022 doi: 10.1073/pnas.2113853119).

7. In several places chemolithotrophy (a lifestyle) is written where chemolithotroph (an organism) is intended. There is no “a” in putrescine (Fig. S15). In the legend of Fig. S15 and Fig. 4, both RuMP and sp-ED are marked as ribulose monophosphate pathway.

8. Figure S15 has methyl-CoM reductase in Loki, this would make it a methanogen as some suggested early on (Sousa et al. 2016: DOI: 10.1038/NMICROBIOL.2016.34), but the pumping mtrA-H issue needs to be resolved. That 2016 paper shows lack of the bacterial WL pathway in Loki and lack of H4F biosynthesis. Maybe it would be better in the present paper to show the actual distribution of the enzymes for key pathways that reconstruct to the asgard ancestor (not all pathways, just important ones), especially contentious ones like bacterial WL pathway or folate synthesis, rather than relying on ALE not to fail.

9. To my understanding, only three archaeal environmental-genome-discovered lineages have been brought to cultivation: Stetter’s Korarchaeum cryptofilum (PNAS 2008), ref. 5 and ref. 6. All 3 are fairly mundane peptide / amino acid fermenters with unusual morphology, which seems worth mentioning.

Issue 3. The chimeric Njordarchaeales. This will immediately get the undivided attention of everyone in the metagenome and eukaryote origin community. It is essential, imperative, for Nature to publish this as an equal-weight counterbalance to Ref. 3. The chances are good that a flood of new “cryptic chimerism” papers will be the next big thing to emerge in metagenomics publishing. This analysis is direly needed to alert the community and unsuspecting readers that there are issues, severe issues, with the metagenomic data currently being used to revise, re-revise, amend re-re-revise eukaryote origin every year. People have been trusting metagenomic data as if it were real. The asgard genomes do not exist in nature (except for 2 in refs 5 and 6), they are constructed by and exist in computers. It should be pointed out that some authors were showing the problems with chimeric assembly of metagenomic *ribosomal* proteins, the ones that are being used to construct the trees. Garg et al. showed that the phylogenetic behaviour of 23 ribosomal proteins of 30 metagenomic archaeal lineages is completely anomalous in comparison to the genomes of cultured archaea (see Figure 5 of doi:10.1093/gbe/evaa238). The existence of congruent phylogenetic signals across individual ribosomal proteins (and whatever the authors use as markers) needs to be shown, not assumed, in metagenomic evolution papers. The fact that nobody looks for, or reports, conflicting vs. consistent signals in metagenomic data does not mean that they do not exist, and this paper shows dramatically (Fig 2) how a whole lineage can suddenly crumble, and dissolve into thin air, if someone looks for the chimerism problems. More people need to look for such problems, rather than focus on boostrap values or bayesian support numbers or similar. Trost et al. 2024 (DOI: 10.1093/femsle/fnae068) showed that the pangenome structure of metagenomic data only starts to look vaguely like that of natural genomes from cultured microbes for MAGs above a >90%

quality threshhold. According to Parks (ref 45) these 223 new MAGs have a 20% quality threshold (completeness 70% minus 5 x contamination 10%). That is low and one wonders how the reconstructions fare with a more robust threshold. Much of the ref. 3 data was not much better, one needs to add, and some of it was chimeric we now see.

10. All metagenome phylogeny papers (starting with this one as a good example) need to report all of the individual amino acid sequence alignments of the proteins used for concatenation, site filtering and the “big picture” phylogenetic tree. As it stands, we only get the manipulated concatenated data to inspect, leaving it up to readers to assemble, from raw data, the “same” alignments that are in the published paper if they want to inspect or reproduce the phylogenetic results. The reason is clear: The phylogenies of the individual marker genes (ribosomal and informational plus a few others) need to be available for **easy** inspection by interested parties (and the authors) to flag assembly chimerism. Transfers are not an issue for good marker proteins but the only way to demonstrate that is to do the individual protein trees and make the individual sequences and alignments (fasta) available.

Related to chimerism. Line 110. The MAG assemblies are reported as >70% complete (and less that 10% contamination), meaning that 30% of the genes that one might expect in a MAG — based on earlier sequences — are missing. This becomes particularly pressing as it regards methanogenesis, because there is an unaddressed snowballing error in the entire asgard pipeline: If one or a few early-published argard bin assemblies (‘genomes’) were missing MtrA-H (the pumping protein of methanogens) and methyl-CoM reductase, then completeness methods will not count either as missing (incomplete) because the reference assemblies for assessing completeness lack them. For a group, a gene present in 70% of its MAGs has optimal representation as present. ALE probably does not know how to handle that kind of data, because the “present vs. absent” state is uncertain in the input data. That puts quite a limitation on the ancestral reconstruction. As a remedy, one could try to counter that with reconstructions for a higher quality subset (many of us would like to know what the quality scores for the old, previously published MAGs in this data are, probably not better than 20%). Or (and in addition) downplay the reconstructions to their essentials, based on a fully enumerated version of Table S7.

Issue 4. The nickel story. The authors lean on one Nature paper (ref 27) proposing that Ni became limiting in the oceans at some point in Earth history, this caused methanogen famine, and in the narrative of this paper, caused the methanogenic ancestors of asgards to lose methyl-CoM reductase (mcr), which is however present in Loki according to Fig. S15. The problem is not Loki. The problem is that modern methanogens do not have a problem with low Ni, they produce about 1 gigatonne of methane per year (Thauer et al. NRMicro 2008), even though modern oceans only contain nanomolar amounts of Ni. There is a great story about the discovery of methanogen Ni requirement by Peter Schönheit – the methanogens dissolved nickel-containing steel to get their metal, nickel is present in vast amounts in the crust. A gigatonne of methane is 0.1% of the atmosphere, or roughly 1.4 million cubic kilometers (per year, most of it consumed by microbes). Nickel limitation? How. Worse for the Ni narrative of Zhang and team, they show five highly expressed Ni-containing enzymes in their reconstruction: CODH, acetyl-CoA synthase, and three different Ni-Fe hydrogenases. The Ni narrative does not withstand even casual inspection because they need Ni for these enzymes anyway. The oxygen part of the narrative collides with the fact that ancestral eukaryotes arose and diversified for over a billion years in extremely low oxygen environments (reviewed by Mills et al. 2022 in Nat Ecol Evol. https://doi.org/10.1038/s41559-022-01733-y). Their ancestral state is facultatively anaerobic, not aerobic. The evidence that Zhang et al. lean on for ancient or pre-GOE O2 has been shown to reflect post-depositional oxidation [Slotznick et al. Sci Adv 8, eabj7190.]. The “aerobic” state that Zhang infer for the asgard ancestor is based on only one enzyme in Loki-Heim (a terminal oxidase), which is likely contaminant (like the bacteria WL pathway) or transferred and completely incompatible with at least 10 proteins that they show in their Fig. S15 reconstruction (identified in one minute by eye): Three Ni-Fe hydrogenases, Nif, CODH (2), ACS (2), por, kor. Those are extremely O2 sensitive enzymes (easily oxidized FeS centers, doi:10.1002/1873-3468.14906, 2024) and diagnostic for anaerobic growth. The corrin synthesis list will have additional other examples, other cofactors will too. With 10% contamination, roughly 10% of the enzymes they have in their reconstruction scheme on average will be contaminants. Finally on the reconstruction, they show that all four cells in their Figure 4 are growing on glucose. Glucose is blood sugar, the main substrate for mitochondria and human parasites. In soil and marine environments, glucose is scarce. Asgard archaea cultivated so far grow on tryptone.

11. This reader suggests removing the ‘self-destructing’ nickel and oxygen narratives altogether, focus on the data and the chimerism problems, improve the reconstruction as much as possible.

In summary, this is a very important and exciting paper of immense interest to readers of Nature and all biologists especially given the editorial flag on ref. 3 on the Nature website. The points above are explained at length (my apologies) but are easily dealt with editing in a minor revision (and making the individual alignments available, which are inaccessible in other asgard papers):

Issue 1. Focus on the trees, and please make the phylogenetic data easy to access (individual unmanipulated alignments for the markers so that everyone can finally check for massive incongruence among marker genes and use their favorite simple or overparameterized method of analysis and their favourite data recoding procedure and their favourite site exclusion method). This referee is adamant about the need to present the individual analyses because this referee invented both the method of concatenating dozens of proteins for deep phylogenetic analysis and examining the compatibility of the individual trees (Martin et al. 1998 Gene transfer to the nucleus and the evolution of chloroplasts. Nature) and the method of systematically deleting highly variable sites in such large alignments (Int. J. Syst. Evol. Microbiol. (2000), 50, 1655–1663).

Issue 2. Improve the metabolic reconstructions so that they better match observations from real genomes, perhaps using a set of more stringent quality filters. The two WL pathways are unlikely to be real, their relative frequencies might resolve the issue, the H4F dependent bacterial pathway is the problem. The authors apparently see this too as only the archaeal version is shown in Figure 4b. Where is Rnf? Is complex I Ech or is it a non-pumping version, as in ndh in E.coli as opposed to nuo?

Issue 3. The Njordarchaeales chimerism issue. Focus on this crucial observation and how it impacts previous and future analyses, how it impacts metagenomic data processing pipelines, suggest ways to detect similar problems in future analyses (individual marker gene analyses and data availability), maybe present the problems surrounding the handling of these chimeras in the public databases.

Issue 4. The nickel narrative (Mcr) succumbs under the weight of the data showing Ni-dependent enzymes (CODH, ACS, three Ni hydrogenases) in these lineages. The oxygen narrative has a similar problem with FeS centers and 10 oxygen sensitive enzymes. Facultative anaerobes can have both kinds of enzymes in their genomes (O2 requiring and O2 sensitive), but differentially expressed (Martin and Müller, the hydrogen hypothesis, Nature 1998).

Other small matters:

In Figure 4, what is NR in the membrane, nitrate reductase? And where are the amino acid transporters listed (which goes back to the issue of Table S7 being incomplete)? From the figure it might seem that Hdr-Mvh complexes with Mcr? This is not reported for cultured methanogens. In addition, the common gene abbreviation is Mvh/Hdr not MH.

Some additional comments:

Line 49: probably facultative chemolithotroph and aerobic heterotroph. As explained above, this inference is problematic. The correct spelling would be “probably facultative chemolithotrophic and aerobic heterotrophic”, the more likely inference would be “probably facultative chemolithoautotrophic and facultative anaerobic” (the figures show an autotroph with a WL pathway to pyruvate synthesis).

Line 79: Heimdallarchaeota has been identified
Heimdallarchaeota have been proposed

Line 146. dataset consisting of 57 proteins of archaeal origin that excluded ribosomal proteins, ….. [just a comment by this reviewer, this is a clear example of ad hoc data handling to obtain a desired result or avoid an undesired one. No proteins evolve more vertically than the conserved ribosomal proteins].

Figure 3. The higher order archaeal (“asgardal”) taxa need to be indicated. When we read Heimdallarchaeia or similar in the text, we need to be able to see those categories in Figure 3 and Suppl figures as well.

Line 158: and other archaea compared to the NM57 tree
and other archaea, in contrast to the NM57 topology

Line 161: “…we integrated the aforesaid marker sets…” – which ones, do the authors mean the ribosomal and NM57 sets? It would be good to specify.

Line 164. See Methods.

Line 186: To uncover the mystery,
To identify the cause of this effect,

Line 212: The inclusion of such chimeras in tree reconstruction could significantly impact the evolutionary position of eukaryotes in archaea.
The inclusion of such chimeras in tree reconstruction can be expected to impact the evolutionary position of eukaryotes within archaea.

Line 274: The result indicated that the relationship between eukaryotes and Asgard archaea in the previous study was likely artifactual
The result indicated that the relationship between eukaryotes and Asgard archaea reported (ref. 3) was likely artifactual

Line 279: Totally, our phylogenetic analyses indicate
Taken together, our phylogenetic analyses indicate

Line 288 ff. See Issue 2 and Issue 4 above.

Line 321: This is hydrogen dependent autotrophy (see Martin and Müller 1998)

Line 326. the LAsCA was a chemolithotrophy. We
the LAsCA was a chemolithoautotroph. We

Line 328: lack of genes encoding methyl-coenzyme M reductase and most of tetrahydromethanopterin (H4MPT) S-methyltransferase subunits, thus the LAsCA may originate from a methanogen

In that passage, the tetrahydromethanopterin (H4MPT) S-methyltransferase to which they refer is MtrA-H, is that correct? MtrA-H is explained in (Shima S et al. (2020) Structural basis of hydrogenotrophic methanogenesis. Annu Rev Microbiol. 74:713–733).

Let us keep in mind that the genomes are not complete, some genes will be missing simply because of that.

Line 330: thus the LAsCA may originate from a methanogen.
thus the LAsCA may have been a methanogen.

Line 334: decreased eruption of nickel-rich ultramafic rocks (27).
Reference 27 fails because methanogens thrive today (1 Gigatonne per year CH4) with much lower Ni than proposed in 27, and the Ni model fails here because of the 5 other Ni dependent enzymes, CODH, ACS, three hydrogenases (Issue 4 above). Simplest solution is to delete the Ni model. This paper does not need it, it only opens up broad flanks for easy attack that the paper does not need.

Line 338: they did not abandon Ni dependent enzymes….

Line 339: This reviewer is not familiar with a report of a Hdr-Fix complex. Please, provide a definition and reference. While Hdr is an enzyme of methanogenesis, Fix is not, it is an electron carrier often found in syntrophs.

Line 341: Do we now have two asgard archaea as the host for mitochondrial symbiosis?

Line 345, The LAsCA genome encoded various NiFe/FeFe hydrogenases (Fig. 4a, Supplementary Table 7, Supplementary Fig. 15), suggesting that it was a hydrogen-dependent autotroph.

Now the authors are saying H2 dependent autotrophy. In other passages it is H2 production. This is going to get readers lost fast.

Line 350: The evidence for pre-GOE whiffs has been debunked: The oxidation of the sediments in question occurred post-deposition [Slotznick SP, Johnson JE, Rasmussen B, Raub TD, Webb SM, Zi JW, Kirschvink JL, Fischer WW. 2022 Reexamination of 2.5-Ga “whiff” of oxygen interval points to anoxic ocean before GOE. Sci. Adv. 8, eabj7190].

Line 351: This is naked speculation.

Line 358: Contrary to the result by Eme et al., our inference revealed that the Heimdallarchaeia and Hodarchaeales ancestors also possessed a complete WLP pathway (Fig. 4b), which is consistent with their geological ages because H2 was still intermittently available at that time.

This passage is not logical. First, the data here show two WL pathways, not one. Second, H2 is produced today in massive amounts by serpentinization (cubic kilometers of H2 per year). It always has been since there was water on Earth. [Tamblyn, R and Hermann, J 2023. Geological evidence for high H2 production from komatiites in the Archaean. Nature Geoscience 16:1194-1199. Sleep, N. H., Meibom, A., Fridriksson, T., Coleman, R. G., and Bird, D. K. (2004). H2-rich fluids from serpentinization: Geochemical and biotic implications. PNAS 101, 12818–12823]. It is also produced by fermentations. What H2 famine?

Line 369: besides heterotrophically

Line 371: This is exactly what the hydrogen hypothesis said (Martin and Müller, 1998). Autotrophy and methanogenesis were lost, the glycolytic pathway became established in the cytosol via gene transfer from endosymbiont to host. The first eukaryote was a facultative anaerobe.

Figure S15. Mvh/hdr is indicated twice. Again, is mtr at lower left MtrA-H? Methylamine degradation is shown, do we see pyrrolysine synthesis in the genomes?

Line 617, Figure S14 and one row in Table S4: an S98 dataset is mentioned, but not described anywhere

Some typos:
Fig. 1 legend, line 874: “Only Asgard archaeal lineages are shown…”
Fig. 3c x-axis label: “Removal”
Line 306: “improving>improve”
Line 317: “based on comparative genomics…”

In summary, this is an excellent analysis of massive new data that speak to the essence of the issue and present a major advance on a crucial evolutionary question (the heritage of our first microbial ancestors). The comments are long because the paper is interesting. The recommended revisions are short because it should go forward without delay.

W. Martin

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have performed extensive re-analysis on marker gene selection, alternative data sets, chimerisms/taxonomic assignment, and ESP definitions. In addition they have recontextualized their findings. All this work is massively appreciated.

I have three short comments, one semantic and two optional remarks / discussion points.

1. The authors perform removal of fast evolving sites to evaluate the robustness of phylogenetic signal. Thereby -for example- showing that for the NM57 (?) inference of eukaryotes within heimdallarchaeia disappears and instead eukaryotes sister to heimdallarchaeia is favored for more slowly evolving sites. This is a very insightful analysis, but the main text and rebuttal seem to refer to this as removing (highly compositionally) heterogeneous sites. Compositional heterogeneity is its "own" class of potential issues in phylogenetics (with its own set of tools see e.g. https://doi.org/10.1016/j.ympev.2013.09.011 https://doi.org/10.1016/j.isci.2022.105594) and distinct (albeit in practice likely somewhat overlapping) from homoplasies that result from fast evolving sites. I would suggest to refer to these analyses as fast site removal or something similar rather than removal of heterogeneous sites.

2. In the rebuttal/revision, the authors very graciously reanalysed the eme et al. 2023 data and markers in different contexts to try and arrive at an explanation for the discrepancy in the precise placement of eukaryotes. I am still somewhat perplexed that using more or less exactly the same marker set on more or less the exact same species using more or less the same phylogenetic methods and models, a different result is obtained. Upon closer reading the literature, the only thing that I can still think of that would explain the discrepancies between various inferences, is that the phylogenetic affinities inferred in previous studies were obtained through fast site removal combined with recoding and then phylobayes with CAT model variants. I am just mentioning this because I would like to get to the bottom of why different results are obtained so take this as FYI.

3. In the rebuttal the authors indicate correctly that ribosomal proteins are much less prone to horizontal gene transfer and other non-vertical processes than enzymes. And that this is an argument for one marker set over another. I would caution to go overboard in this line of thinking for two reasons. First the marker selection and inspection of individual trees that the authors perform should supersede (or could contradict) such general statements. Second, it is precisely the ribosomal proteins that give this problematically long branch at the base of eukaryotes that is so difficult for phylogenetic reconstructions to accommodate. And attempts at finding other markers that are vertical (within the part of the ToL here concerned with) but do not show this extremely long branch should be explored and at least somewhat appreciated rather than so easily dismissed.

Referee #2

(Remarks to the Author)
The revised version of the manuscript is very much improved and the authors addressed all of mine and the other reviewers comments. In the manuscript, besides generating 223 nearly complete metagenome-assembled genomes (MAGs) of Asgard archaea (16 previously unknown lineages) the authors show the chimeric nature of Njordarchaeales assemblies. By excluding problematic datasets and performing careful phylogenomic recosntructions, the authors concluded the ancestry of eukaryotes prior to the diversification of Heimdallarchaeia lineages sampled so far. By ancestral state reconstruction and molecular dating, the authors proposed that the last common ancestor of Asgard archaea and eukaryotes likely existed before the Great Oxidation Event (~2.3 billion years ago) and was an anaerobic, hydrogen-dependent acetogen. The major improvement of the current version is the improved metabolic reconstructions and I think, the authors do not need to perform any additional analyses to support their conclusions.

Minor:
Line 128 -130 – at least Ca. Lossiferum has a closed genome, so does it make sense to talk about completeness? Not all gene markers are present in all lineages…

Referee #3

(Remarks to the Author)

The manuscript by Zhang et al. “Phylogenomic analyses with expanded taxon sampling…” has been extensively revised and is significantly improved with respect to all criticisms of referees in the first round. The amount of new data for asgard related lineages remains an undeniable strength of the paper. Everyone will want to be working with that data moving forward. The phylogenetic analyses have been flanked by new experiments that undescore the problems of earlier reports, problem which were identified by the authors in the first version. The case for the assembly-artefact nature of the Njordarchaeales has been strengthened. The nickel dependence narrative has been removed, increasing the focus on the phylogenetic findings. The impact of these data on the new placement of the eukaryote host lineage within archaeal diversification adds significantly to developments in the field, as do the metabolic reconstructions, which now clearly point to a chemolithoautotrophic ancestry of the host lineage at eukaryote origin. These are findings about which all biologists need to know, and they shed vast amounts of new light on the humble symbiotic beginnings of our own eukaryotic lineage.

Some minor corrections remain, listed in order of of reading, most are editorial. The line numbers correspond to the numbering in the manuscript version with changes tracked.

Line 42. Delete “could”. The analyses show that this is what occurs.

Line 113: “which is significantly distinct” —> which significantly differ

Line 143: “it is vital to unveil…” —> it is essential to identify the factors underlying the conflicting…

Line 158: “not necessarily related to” —> not caused by

Line 159: “the number” —> the number and nature

Line 165. Delete “aforesaid”. The context is clear.

Line 192: “outputted” —> generated

Line 198: “owing to” —> stemming from

Line 200: “archaea.” —> archaea, specifically into the Nordarchaeales.

Line 210: delete “may”. This is what the results indicate.

Line 211: “datasets, The NM57 dataset may harbour” —> datasets, the NM57 dataset harbours

Line 213: “are selected” —> were selected

Line 221: help to resolve the placement of eukaryotes among archaea. To this end, we added

Line 224: ML trees …. within the TACK superphylum

Line 229: mitigate the effect

Line 307: delete “significantly”. The 2.26 value overlaps with the GOE.

Line 309: documented —> reported

Line 320: a gradual increase in ancestral gene content was observed

Line 330: it was inferred

Line 344: “abundant CO2 and H2 in the Archean ocean41 would” …
Please add a reference because H2 in the atmosphere [41] is not important, it diffuses to space; in the ocean, H2 produced by serpentinization fuels deep sea microbial communities locally it its sites of synthesis. Adjust to: —> …abundant CO2 in the Archean ocean[41] and marine H2 from serpentinization [41a] would…
New reference, perhaps: [41a] Tamblyn, R and Hermann, J. 2023. Geological evidence for high H2 production from komatiites in the Archaean. Nature Geoscience 16: 1194–1199.

Line 370: Something is wrong here, ref 44 is not about Heimdahlarchaeia at all or inference of archaeal acetogens, it is about Ech. The only archaea that actually grow as acetogens are generated in the laboratory through extensive genetic manipulation [Schöne et al. (2022). Deconstructing Methanosarcina acetivorans into an acetogenic archaeon. PNAS 119, e2113853119.] Maybe ref 46 was meant here. Ref 44 would fit well on line 501.

Line 511: Ref 46 in this context should be replaced with the original reference: Martin W and Müller M. 1998. The hydrogen hypothesis for the first eukaryote. Nature 392: 37–41.

Line 561: “may represent chimeric assemblies” —> likely represent chimeric assemblies

Line 567: “eukaryote common ancestor was an anaerobic H2-dependent acetogen” Perhaps it is adviseable to be more general —> “the host lineage at eukaryotic origin was an anaerobic, H2-dependent chemolithoautotroph” (this would include the possibility of acetogens)

Line 570: “will help to refining scenarios of eukaryogenesis” —> will help to refine evolutionary reconstructions [new reference] of eukaryogenesis. The new reference might be: Giger et. al. 2024. Inducing novel endosymbioses by implanting bacteria in fungi. Nature 635: 415–422. This would point to the new area of research where people are generating mitochondrion-like symbiotic relationships using technology.

This referee is satisfied that they have highlighted quality as a parameter of importance.

The phylogenetic methods are sufficiently documented, the alignments are available on Figshare. Phylogeny buffs will think of a thousand new analyses that could be done with this data, and those analyses can be done by anyone who wants to — once these data are published. The authors are to be commended for hard work and straightforward phylogenetic reporting.

Overall the authors have undertaken earnest and effective efforts to improve the paper in the few areas needing attention. They present a very significant contribution to the topic of eukaryogenesis. These new genomes significantly sharpen the focus on which lineages are the sisters of eukaryotes and which genes were likely present in those genomes. The data fit well with current views about Earth history and move the field forward in many ways.

W. Martin

**Responses to reviewer's comments**

**Referee #1 (Remarks to the Author):**

The precise placement of the eukaryotes within asgardarchaeota is of course of great interest

And it turns out that this question is a non-trivial phylogenetic investigation for a host of reasons, including data, and importantly methods considerations. This manuscript presents new MAGs belonging to the asgardarchaeota, reanalysis of the quality of existing njord MAGs, as well as different markers gene sets/phylogenetic method combinations. Based on these analyses the authors suggest a deeper placement of eukaryotes within asgardarchaeota. In addition geological timing via bayesian timing methods and gene content via ALE implications of this deeper placement are bioinformatically explored.

Although the new data will likely prove helpful in addressing the precise eukaryote-asgardarchaeota phylogenetic topology, it is my interpretation that on a fundamental level the question on how to derive at confidence in a specific topology is not a discussion about more data, but a discussion on on how to phylogenetically establish this. And it is in these more "computational" aspects that the current manuscript partially provides insufficient detail and partially seems to make some methodological and logical steps I am not convinced by. These points are detailed below:

1. How to deal with marker genes.

1a. Phylogenomics needs marker gene sets to make large enough supermatrices to derive . However the assignment of new genomes to marker gene sets is non trivial. In a recent study (https://doi.org/10.7554/eLife.66695) re-analysis of a proposed larger 54 marker genes concluded that many novel and promising markers showed signs of lateral evolution or hidden paralogy and they went back to only 27 markers genes. In this study marker genes seem to taken at their previous established "value", while perhaps they should be checked for their phylogenetic information as marker genes, by for example (per https://doi.org/10.7554/eLife.66695) checking how devoid of hidden paralogy or lateral transfer they seem.

**Response:** For the S67, S97, and S150 marker sets we proposed, single marker gene

trees have been inferred in original manuscript and instances of hidden paralogous sequences were manually removed from these trees (see lines 558-560 in original manuscript). In the revision, a more detailed description about the removal of paralogs is provided in the Method section (See lines 732-737), and single-gene trees with paralogous sequences removed have been uploaded to Figshare **(**https://figshare.com/s/6e523322b0b647b91dda**).**

In addition, according to the method by Moody et al. (eLife 2022, 66695), we examined instances of lateral gene transfers between Archaea and bacteria for the three set of markers by integrating 93 bacterial genomes into original archaeal dataset (579 archaeal genomes) based on inferred sing-gene trees. These bacterial genomes are selected as representatives of 23 bacterial phyla. We observed that for 43 of the 150 markers, no or only one homologue was identified in bacteria. Among single-gene trees of the remaining markers, 51 trees failed to recover archaea and bacteria as reciprocally monophyletic domains. When excluding these markers that have potential horizontal gene transfers between archaea and bacteria, we obtained 47, 60, and 99 markers (S47, S60, and S99) from the S67, S97 and S150 marker sets, respectively. These single-gene trees recovering archaea and bacteria as reciprocally monophyletic groups have been uploaded into Figshare **(**https://figshare.com/s/6e523322b0b647b91dda**).** Using these vertically evolving markers in archaea (S47, S60, and S99), we inferred phylogenies of Asgard archaea and eukaryotes (411 Asgard archaea and 14 eukaryotes) under sophisticated evolutionary models. All of maximum likelihood phylogenomic analyses placed eukaryotes as a sister group to Heimdallarchaeia, which is consistent with the results obtained by using S67 and S97 datasets (See Supplementary results and discussion). These results suggest that inter-domain gene transfers for partial sequences of a small number of markers did not affect the position of eukaryotes in Asgard archaea. The detailed results are provided in Supplemental Materials.

1b. Related to this even when a marker set is or has previously been established for genomes/MAGs set i, it is my understanding that the markers still need to be analyzed for the new species/genome set j under study. It might be that marker genes seemed

vertical for a previous data set, their behavior in a new/expanded data set might show genomes for this. This per gene phylogenetic analysis is not just to check the quality of the markers as markers but also to check whether the assignment was done correctly. Analyzing individual marker gene trees seems not to have been carried out in this study. This issue is especially important as the argument seems to be that a majority of marker/tree combinations favors one topology over a previous suggested one, yet if is this is explained by issues in most markers sets that are absent from the minority one, I would still favor the outcome from the minority one as the currently best supported hypothesis.

**Response:** As mentioned above, individual marker gene trees for the expanded archaeal dataset have been inferred in original manuscript and paralogs have been removed from these trees. Furthermore, the previously used marker sets were refined to exclude these markers with inter-domain gene transfers. Based on these reduced marker sets, phylogenomic analyses also supported our previous conclusion.

2.Data vs analysis

If indeed the findings of this research are valid, they should already be possible to be obtained with the eme et al. data - without the new MAGs. Or indeed if the new MAGs make the difference (which is of course possible!) then that should be explicitly shown by showing that for the previous data, utilizing their approach they can place eukaryotes deeper, outside heimdallarchaeia. The reason this point is relevant is, as raised above and throughout this review, it might very well be a range of computational issues on which the discussion should be conducted rather than a data issue.

**Response:** This is a good suggestion. Based on the three sets of newly selected markers (S47, S60, and S99), we constructed three supermatrices: smS47, smS60, and smS99, using the data of Eme et al. (175 Asgard archaea + 14 eukaryotes). ML phylogenomic analyses of the three supermatrices under LG+C60+F+G+PMSF model supported Heimdallarchaeia-sister topology (See Supplementary Figure 15). Furthermore, we investigated the influence of compositional heterogeneity on the placement of eukaryotes by progressively reducing compositional heterogeneity of these alignments

and inferring maximum-likelihood phylogenies. For the smS47 dataset, the support for Heimdallarchaeia-sister was unstable during the removal of heterogeneity and significant support was only detected when 20% of the fastest-evolving sites were excluded, while no support for Hodarchaeales-sister was observed. For the smS60 and smS99 datasets, the support for Heimdallarchaeia-sister remained robust until 50% and 80% of sites were excluded, respectively (Supplementary Fig. 16b and c). The results suggest that larger datasets retain more phylogenetic information for resolving the placement of eukaryotes.

Similarly, the site-exclusion method was also applied to the supermatrices (exS47, exS60 and exS99) constructed using our expanded Asgard archaeal genomes and the concatenations of S47, S60 and S99 markers. We observed that in the exS47 and exS60 supermatrices, the loss of phylogenetic signals supporting the Heimdallarchaeia-sister relationship occurs at a significantly slower rate following the removal of the fastest-evolving sites (Supplementary Fig. 17), compared to the corresponding supermatrices constructed using Eme et al.'s data. More importantly, as shown in Fig. 3c, when the NM57 marker set selected by Eme et al. was used to construct a supermatrix for our expanded Asgard archaeal sampling, phylogenomic analyses of the supermatrix strongly support Heimdallarchaeia-sister topology after the exclusion of heterogeneous sites. These results indicate that our expanded Asgard archaeal sampling helps to disentangle true phylogenetic signals from noise and potential artefacts.

The NM57 marker set consists of 57 functional proteins involved in diverse informational, metabolic and cellular processes, with ribosomal proteins completely excluded. As shown previously (Moody et al., 2022, eLife), non-ribosomal markers have a greater number of horizontal gene transfers and cases of mixed paralogs than ribosomal proteins. Thus, the supermatrix constructed using these non-ribosomal proteins is likely to contain a higher level of compositional heterogeneity. In contrast, the widespread inclusion of ribosomal proteins in our marker sets complements this flaw as ribosomal proteins have been shown to be exceptionally conserved, with rare occurrences of gene duplication, transfer, and loss (Ramulu et al., 2014, Mol

Phylogenet Evol).

Taken together, our conclusions can be attributed to various factors including careful marker selection, removal of outgroups and Njordarchaeales, expanded genomic sampling of Asgard archaea, use of complex site-heterogeneous evolution models, and so on.

3. Contaminations in njord utilized as a conflated argument?

The results on the chimeric/contaminated njord MAGs are quite relevant (I understand that some people know this but I could not find a reference that states that). In any case, the manuscript seems to make the implicit argument that it seems as if the chimaeric njord are somehow responsible for the placement of eukaryotes in previous studies, especially in certain parts of the manuscript such as the abstract. However if I understand correctly what is being done, this is not the case - i.e. the placement of eukaryotes deeper in the asgardarchaeota is not dependent on njord. This potentially misleading argument/logic should be reworded or if it is actually shown should be made more explicit. In addition perhaps the nord MAG quality results should come before the trees and they should be removed from the alignments from then on. Finally insofar as I understand, and insofar as this manuscript describes, some but not all njord MAGs have issues. It is unclear to me how these presumably trustworthy MAGs behave differently from the problematic MAGs.

**Response:** Thank you for your suggestion. In fact, we have already clearly stated in original article that these Njordarchaeales genomes can influence the true evolutionary position of eukaryotes in archaea. The detailed information is presented as follows: 1) In lines 135-140, given that Njordarchaeales often branched with eukaryotes in previous phylogenomic analyses, it is vital to unveil the puzzle underlying the conflicting topologies among different trees for position of Njordarchaeales within archaea. The statement is put forward based on a previous study, laying the groundwork for evaluating the position of Njordarchaeales in archaea. Eme et al., (2023, Nature) observed that in the tree inferred using 56 concatenated ribosomal proteins, Njordarchaeales branched as a sister clade to Korarchaeota while eukaryotes branched at the base of the clade formed by

Korarchaeota and Njordarchaeales. 2) In lines 211-212, given that Njordarchaeales mainly comprise TACK and Asgard archaeal sequences, we conclude that the inclusion of such chimeras in tree reconstruction could significantly impact the evolutionary position of eukaryotes in archaea. 3) In lines 216-222, we performed a ML phylogenomic analysis of S67 markers for a phylogenetically diverse set of archaeal genomes, and observed that eukaryotes were placed within TACK superphylum as sister clade to Njordarchaeale (Suppl. Fig. 8a). It is inferred that this may be caused by the high compositional similarity between the Asgard archaeal sequences in the Njordarchaeales genomes and eukaryotic sequences. Based on all the analyses above, we have excluded the Njordarchaeales genomes from the alignments from then on (see lines 222-278). When the S67 dataset excluding Njordarchaeales genomes was used to infer ML tree, eukaryotes were recovered as a sister clade to Heimdallarchaeia within Asgard archaea. 4) Finally, in the revision, we clearly addressed in the Abstract that "the Njordarchaeales could artefactually draw eukaryotes into TACK superphylum as its sister clade in phylogenomic trees" (see Abstract).

Based on the taxonomic profiles of Njordarchaeales contigs and their unstable evolutionary positions in phylogenetic trees, we concluded that all 10 Njordarchaeales MAGs likely have issues (PS: a total of 15 MAGs were found in public databases and 10 were obtained after removing redundant MAGs). However, we analyzed the sequence composition and coverage patterns of only four MAGs across metagenomes because these were the only MAGs whose contigs could be fully mapped by reads from multiple metagenomes.

4. Contaminations in njord, how to deal with them in the analysis.

a. The analysis of the contamination as discussed in the main text is partly very convincing (especially panel c & d) but also insufficiently detailed w.r.t. methods and interpretation. It is completely unclear (in the main text) and somewhat unclear (from the methods) how panel a&b were generated. It seems as if a homebrew contig taxonomic assignment was used instead of (a suite) of established taxonomic assignment tools. Please use several state-of-the-art contig / read assignment tools to

investigate these suspicious MAGs in detail.

**Response:** In the revision, two state-of-the art contig assignment tools (CAT and MMseqs2) were used to determine the taxonomic identity of Njordarchaeales contigs (von Meijenfeldt et al., 2019, 20:217, Genome Biology; Mirdita et al., 2021, 37:3029-3031, Bioinformatics). The results were comparable to those obtained using a voting scheme in original manuscript. In fact, the voting scheme we used followed the procedure proposed by Al-Shayeb et al. (2021, Nature, https://doi.org/10.1038/s41586-022-05256-1). In addition, a detailed description of the contamination analyses in njord is provided in the Methods section (see 765-778).

b. The visualization in panel c & d is quite nice, but how where the metagenomes selected for which read depth is shown? Could it be the case that also for more reliable MAGs selective metagenomes could be found where such discontinuities can be found?

**Response:** In NCBI database, it is shown that Njordarchaeales MAGs were recovered from metagenomes generated from deep-sea hydrothermal vent sediments in Gulf of California and Pacific Ocean. We downloaded 17 metagenomes in these projects (PRJNA546572, PRJNA495050, PRJNA417281, PRJNA362212, PRJNA469236), all of which originated from similar habitats. These MAGs, for which the contigs were fully matched by reads, were used to analyze the sequence composition and coverage of their contigs.

From the selected metagenomes, we chose an additional four MAGs (2 Archaea, 2 Bacteria) that could be fully matched by reads (Fig. r1), all of which exhibited stable evolutionary positions in the phylogenetic trees. We analyzed their sequence composition and coverage patterns across the metagenomes. Unlike the Njordarchaeales MAGs, the contigs of these MAGs were grouped into two distinct clusters: Cluster 1 and Cluster 2. The contigs in the larger cluster (Cluster 2) exhibited similar coverage values within the same metagenome, which were either significantly higher or lower than those of the contigs in the smaller cluster. The smaller cluster (Cluster1) contained 5-9 contigs that added up to a total length of 16-55 kbp, accounting for 1.0-2.4% of the full genome length. The mean coverage of contigs in

these smaller clusters differed significantly from that of contigs in the larger clusters, suggesting that the smaller clusters likely represent contamination. We used the CAT tool to classify the contigs of the four MAGs and observed that the contigs in the larger cluster (Cluster 2) could be assigned to the same taxonomic level as determined by the GTDB tool. However, the contigs in the smaller cluster (Cluster 1) are either of unknown classification or assigned to different taxonomic units, often within different phyla. These results further indicate that the contigs in the smaller clusters are contaminants or mobile genetic elements (Valentin-Alvarado et al., 2024, Genome Research, 34:1-15). Despite the presence of these contaminants, they do not impact the evolutionary position of the MAGs, as no markers were found in the contaminated contigs, and the contamination level falls within the range reported by CheckM.

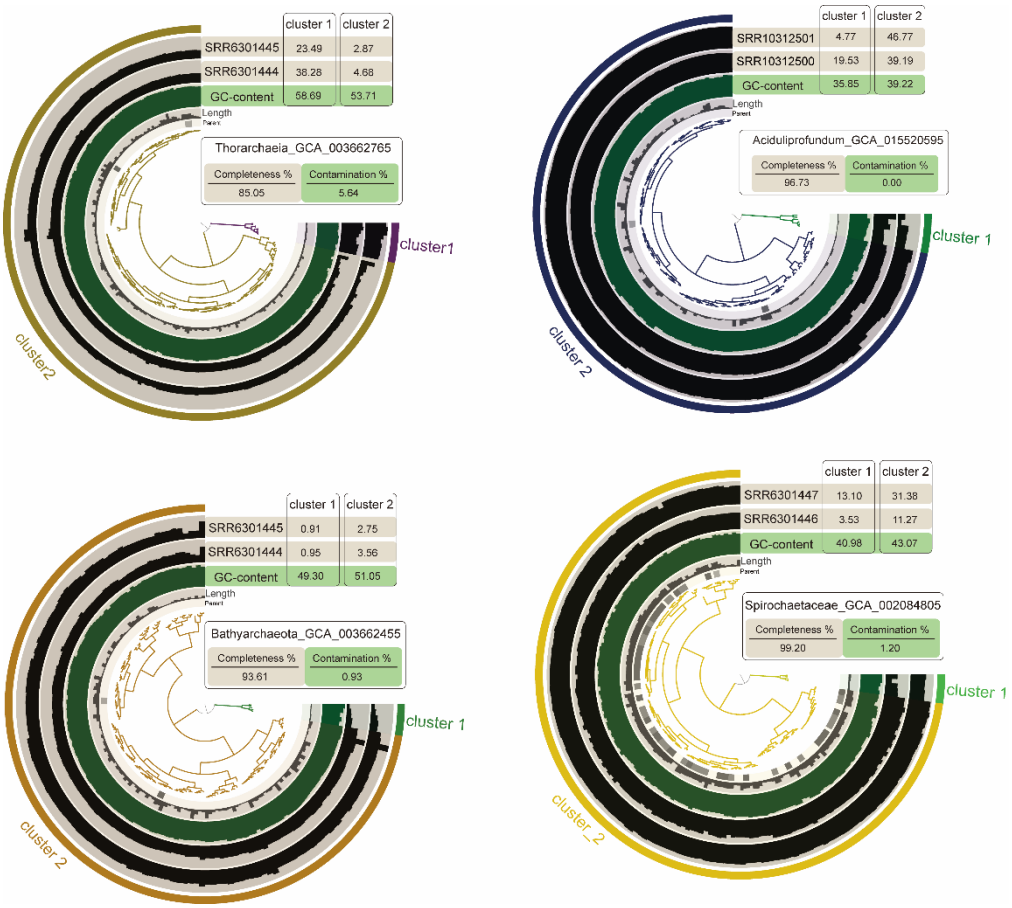


**Fig. r1.** The hierarchical clustering of contigs in an additional four non-

Njordarchaeales MAGs, reconstructed from the same metagenomes in which Njordarchaeales MAGs were recovered, based on their sequence composition and differential mean coverage across different metagenomes.

c MAG S143_49 in panel d seems to be assigned to two clusters with one being really minor and the other being the majority. This seems inconsistent with panel b where the TACK and asgard assignments are not that different in size. The other 3 MAGs in the c,d seem to fall into three clusters which is inconsistent with panel a/b. Please explain the inconsistency or perform analyses that result in a more consistent inference.

**Response:** This inconsistence arises from the different methods used. In panel c and d, the contigs are clustered based on coverage patterns, while in panel a and b, they are classified based on protein homologies to a reference database. For a novel lineage, if a contig contains protein sequences that are too divergent from those in the reference database, it may not be assigned to a specific classification. This explains why 20-73% of the contigs in each MAG were not assigned in Figure 2. Even for the contigs that have already been classified, we cannot guarantee that the classification of all the contigs is entirely accurate, as this only indicates that the sequences in these contigs are more closely related to sequences in the reference database. It may reflect a general trend, but not a definitive classification. This trend in sequence classification aligns with the unstable evolutionary positions of the lineage in the species tree. In a word, our findings should be viewed in a broader context. The results from the species tree, contig classification, and contig coverage patterns across different metagenomes should be put together to assess the quality of Njordarchaeales MAGs.

5. Eukaryotic signature proteins, how to establish their presence.

The final argument that what is now referred to as hodarchaeales are not eukaryotes closest relatives, is a re-evaluation of eukaryotic signature proteins that are now argued to not be limited to hodarchaeales. Now this is a relevant discussion, but the manuscript including the supplement does not offer sufficient detail to properly infer how this was conducted. I.e. are the domains searched using blast, hmmer, hhpred,

foldseek is unclear. From the context I would infer it is either CDD search RPS BLAST or HMMER, but it is not described. A domain score threshold is mentioned (50) but it is unclear which score this is referred to (I assume bitscore). I disagree with the assertion that 50 is the default threshold for PFAM (which by the way is deprecated) as for that resource the default cut-off is the GA bitscore which differs per model. Subsequent inspection of supplementary table 9 for the assertion that DNA polE occurs in many archaea reveals a number of PFAM identifiers used, but those are not specific for polE. If I query these identifiers in uniprot there seem to be many many other proteins with these domains. If that is correct, then there seems to be an orthology / homology issue. These orthology issues are normally addressed via gene phylogenies. In summary the presence or absence of proteins in these new and existing MAGs need more methodological explanation and results to support them, including (if indeed they are orthology issues) with gene phylogenies.

**Response:** Thank you for your valuable suggestions. In the revision, we used the methods you recommended to identify the eukaryotic signature proteins, including the use of the GA bitscore for Pfam, gene phylogeny inference and multiple sequence alignment. The results still support our previous conclusion that these eukaryotic signature proteins are not be limited to hodarchaeales. The detailed methods and results are provided in Supplemental Materials (Supplemental Methods and Results).

The following illustrates this with the example of identifying DNA polymerase Pol ε. In eukaryotes, Pol ε contains four subunits: Pol2, Dpb2, Dpb3, Dpb4. The Pol2 subunit harbors the catalytic DNA polymerase and 3'-5' exonuclease in the N-terminal half. We noticed that the Pol ε identified by Eme et al. (2023, Nature) is the Pol2 subunit. The Pol2 in eukaryotes contains three domains: PF03104, PF00136, and PF10108. We annotated these genomes in our database using HMMER with a GA bitscore cutoff for the three domains. To identify homologs in eukaryotes, the coexistence of all three domains was required, and the resulting sequences were manually verified against the UniProt database. For Asgard archaea, homologs with any one of the three domains were considered. All identified sequences were used to infer a phylogeny. We observed that the Pol2 sequences of eukaryotes formed a

distinct cluster with those of Jordarchaeia, Wenzhongarchaeales, Hodarchaeales (designated as Cluster 1) (See Supplementary Fig. 19). In addition, two other clusters (Cluster 2 and 3), closely related to Cluster 1, contain sequences from multiple Asgard lineages (excluding Hodarchaeales). These sequences in the cluster 2 and 3 also harbor the PF03104, PF00136, and PF10108 domains. It is inferred that the Asgard archaeal proteins in Cluster 1, 2, and 3 may all be orthologs of the Pol2 subunit of eukaryotic Pol ε.

6. The manuscript extensively makes use of bayesian phylogenetic inference to supplement ML approaches. I could locate in the manuscript the number iterations these were run, but not if they were converged. Given the size of the data set I expect that these approaches are potentially not converged. This should be discussed/reported on.

**Response:** In all Bayesian inferences, two independent Markov chains were run until a sufficient effective sample size was achieved (effsize > 300). However, as you said, the two chains did not converge, likely duo to the large size of the supermatrices and computational limitations. This has been reported in the Method section (see lines 567-572).

**Referee #2 (Remarks to the Author):**

The paper by Zhang et al entitled "Phylogenomic analyses with expanded taxon sampling reveal deep origin of eukaryotes outside Heimdallarchaeia" explores the evolutionary origins of eukaryotes by analyzing Asgard archaea, the closest known group of the archaeal host-ancestor of eukaryotes. By generating 223 new Asgard genomes, including 16 novel lineages, the researchers found that Njordarchaeales, previously considered part of Asgard, are chimeric genomes blending TACK and Asgard lineages, complicating previous phylogenetic interpretations. Using advanced phylogenomic methods, they propose that eukaryotes evolved before the diversification of Heimdallarchaeia, challenging previous classifications. They also date the last Asgard-eukaryote common ancestor to before the Great Oxidation Event, suggesting metabolic flexibility that might link eukaryogenesis with planetary

changes, offering new insights into early cellular complexity.

This paper is a well-timed, bioinformatically rigorous contribution addressing a critical question in evolutionary biology, showcasing a substantial body of research. Moreover, I praise the authors for having provided all alignments and phylogenies in Figshare. This should be the standard for these analysis, but unfortunatly is not. I only have some methodological questions that I would like the authors to address.

1- Is the model used in the phylogenetic reconstructions the best model? If not, please justify the choice of this model instead of one that might be more appropriated for the alignment(s).

**Response:** In maximum-likelihood phylogenies, we did not use the best model IQ-TREE recommended. Instead, we applied site-heterogeneous evolution model: LG + C60 + F + G, to infer ML trees. The sophisticated mixture model we used has been reported to be able to capture sequence heterogeneity in the substitution process across different sites of a protein alignment (Quang et al., 2008, Bioinformatics, 24: 2317-2323; Martijn et al., 2018, Nature 557:101-105; Eme et al., 2023, Nature, https://doi.org/10.1038/s41586-023-06186-2), which will reduce the risk of tree reconstruction artifacts. An explanation for use of the model was added in the revision (see line 743-744). For Bayesian inferences, we selected the CAT + GTR model because it is significantly more robust against long-branch attraction artifacts compared to all other models (see the Phylobayes-MPI manual by Nicolas Lartillot et al.)

2- Although understanding the “voting” system to explain the chimerism of Njordarchaeales MAGs, it is unclear if the TACK associated contigs are derived from missassembly, gain by HGT or in alternative, TACKL having received the genes. Moreover, the local similarity (distance) method does not necessarily reflect evolutionary distance, especially if only local similarities are considered. Thus, to avoid having to compute all of those phylogenies, using global identities, (or at least considering alignment coverage) might minimize these effects.

This might not change the conclusions of this section, but would be more accurate.

**Response:** According to your suggestion, we considered alignment coverage (50% of alignment length) in addition to the local similarity (cutoff: e-value < 1e-5) for identifying best-hit matches in a reference database. The resulting taxonomic profiles of contigs of Njordarchaeales MAGs were similar to the initial results. In addition, as per other reviewer's suggestion, in the revision, two state-of-the art contig assignment tools (CAT and MMseqs2) were used to determine the taxonomic identity of Njordarchaeales contigs (von Meijenfeldt et al., 2019, 20:217, Genome Biology; Mirdita et al., 2021, 37:3029-3031, Bioinformatics). The results were comparable to those obtained using a voting scheme in original manuscript (see Fig. 2ab and Supplementary Figure 5).

Based on the current results, we cannot determine whether these TACK associated contigs are the result of missassembly, acquired by HGT, or if TACK archaea have received these genes. Nevertheless, if we assume that Njordarchaeales is an Argard archaeon, it would be highly unusual for as much as 24-60% of the contigs in its MAGs to originate from TACK archaea. More importantly, the chimeric nature of Njordarchaeales have affected its evolutionary history in archaea, causing it to shift between the TACK superphylum and Asgardarchaeota when different marker sets are used.

Minor:

Supplementary table 10 (Supplementary Table 10. The annotation results of the CDD (Conserved Domain Database) domain of eukaryotic signature proteins.

) not cited in main text. Also, I am not sure if the content of the table goes with the title. For instance, CoxII (subunit of complex IV) is not an ESP.

**Response:** The Supplementary table 10 was cited in the section "distribution of eukaryotic signature proteins (ESPs)" in Supplemental Materials. The table described domains from Pfam database found in ESPs, but it did not serve as annotations of the ESPs themselves. We observed that homologs of some ESPs in eukaryotes are multidomain proteins, whereas these domains exist independently in proteins of Asgard archaea. A given domain can appear in a variety of different proteins. It is inferred that homologs of these ESPs in eukaryotes may have been created by

recombining archaeal domains in various arrangements. Finally, given that the results in this Table 10 are not closely related to the theme of the article, we have deleted the Table in the revision.

Typo in the table of contents of supplementary information

**Response:** Corrected.

**Referee #3 (Remarks to the Author):**

What an exciting read, but not without problems. This is an 8 page review, for which the referee apologizes but there is a lot to say about this very exciting paper. To get to the point, this reviewer recommends acceptance after (mostly) editorial revision, no new analyses (maybe some filtering by quality), no new data, just better presentation of the massive and relevant new data at hand. The case for publication is very strong.

In this important paper, Zhang et al. report 223 new asgard metagenomic assemblies, flanked by phylogenetic analyses and metabolic reconstructions for the asgard group. Their main findings are threefold: 1) eukaryotes branch in a new place in their trees, revamping (yet again) the eukaryote host lineage sisterhood issue; 2) their metabolic reconstructions indicate that the common ancestor of asgard lineages was a methanogen, drawing tighter links to hydrogen-dependent and methanogenic lifestyles among asgard groups; 3) on the basis of better sampling, they report that some previously published asgard 'lineages' are chimeric metagenomic assembly artefacts, that is, the assembly of metagenome data led to merging two genome 'lineages' into one. This chimerism aspect (finally) brings a glaring issue in the asgard phylogeny controversies openly to the fore, as such chimaeras impact all downstream inferences, from phylogeny to metabolic reconstructions. 4) In addition, Zhang et al. offer a new narrative about archaeal evolution and eukaryote origin, this time focussing on nickel (in addition to oxygen), but as with all other asgard evolution stories, this one has fatal flaws (here regarding both Ni and O2) and was not well

thought through. On balance, the paper presents a strident advance and marks a significant turning point toward greater caution in the asgard saga. As such, the manuscripts presents a very strong case for publication, but after some problems are fixed.

Issue 1: The position of eukaryotes in the trees. Readers of Nature continue to be intensely interested in which group of (yet uncultured) archaea are the sisters of eukaryotes in phylogenetic trees. This has spawned a number of papers, most recently about the “heimdallarchaeial”-ness of eukaryotes last year (ref. 3). However, no one has ever seen an heimdallarcheaon (they only exist in computers so far) and a key taxon underpinning the “heimdallarchaeial” phylogenetic result in ref. 3 (Njordarchaeales) is shown here by Zhang et al. to be non-existent in nature, an artefact of metagenome assembly procedures on the computers of the Ref. 3 team. It is imperative that this information be published as a full Nature paper, backed by the 233 new genomes, although we can be sure that the Ref. 3 team will contest this result by Zhang et al. The purpose of science is to move forward on the basis of new data as opposed to cementing old analyses into the literature (that happened with the rRNA tree of life…). It is clear that there will be a lively debate about methods and data upon publication of Zhang et al., but it is essential for that debate to occur i) *in public* and ii) *after publication* of Zhang et al., not during the peer review process. Zhang et al. have performed careful analyses, at least as careful (more so in some respects) as their competitors, they should not be forced to perform additional phylogenetic analyses that every anonymous referee would like to see. Their result negates the “heimdallarchaeial” result with 223 new genomes and a chimerism revelation – this is a good direction for the field to be moving. I recommend no new concatenated phylogenetic analyses before publication, it will be a never-ending story. The peer review process for ref. 3 was mild, with no contests on data quality. Zhang et al. should be shown the same treatment.

Figures 1 and 3: we need to see, with clearly marked and explained symbols, where the only two cultured/enriched/imaged asgards (refs 5 and 6) are located in these trees

and a clear statement in the text that the remaining lineages are only known from computer assembly procedures of environmental sequence data.

**Response:** Corrected, please see lines 1146, 1176-1177 and Fig. 1 and 3.

Issue 2. The metabolic reconstructions. It is exciting to read that the asgard ancestor was probably methanogenic, but this information comes from one method (ALE) and it appears that ALE has failed, or the genomes have a large unrecognized bacterial component, or both. Zhang et al are to be commended for their openness of their reconstruction, it is more complete than that provided by ref. 3, but still there are are problems (hidden in ref. 3, reported in Zhang). The following points concern the reconstruction.

1. The reconstruction cannot be correct (by inference, those of ref. 3 either, because their data has a chimerism issue and the quality is not fundamentally better, whereby the quality in ref. 3 is impacted by the chimeras). In Figure 4 and Supplementary Figure 15 we see a tetrahydrofoate-(H4F-) dependent Wood-Ljungdahl pathway (the bacterial version) and a tetrahydromethanopterin-(H4MPT-) dependent Wood-Ljungdahl pathway (the archaeal version) in all four lineages shown. This is one of those “never been seen before in cultured cells” physiological phenomena, as no cultured cells have ever been shown (to this reader’s knowledge) to encode both the pathways. It is another “only in metagenomes” physiology, and that sets off alarms. A given metagenome assembly can be 1.4 Mb in size or 2.9 Mb in size depending on parameters (https://doi.org/10.1038/s41564-019-0417-6). Now the genome sizes (some of them 12 Mb) are just listed as a point estimate. The two WL pathways in the same cell cannot be correct. The bacterial is surely a contamination or binning artefact. Regarding the bacterial WL pathway and likely bacterial contamination, it comes to mind: How do bacteria and archaeal lipid synthesis genes square off here (frequencies, not ALE), and how many bacterial-specific ribosomal proteins do they find at which frequency in this data? If they are claiming that ref. 3 is an assembly artefact, the present assemblies need to be extremely clean.

**Response:** Thank you for your suggestions. For the genomes in ref. 3, we only

thought that the Njordarchaeale genomes have issues because they show an unstable evolutionary position in phylogenetic trees when different marker sets are used. In contrast, the remaining genomes are reliable. Lines of existing studies have shown that the MAGs from environmental samples closely resemble the genomes of the corresponding organisms that were later enriched or isolated (Imachi et al., 2020, Nature, https://doi.org/10.1038/s41586-019-1916-6; Rodrigues-Oliveira et al., 2022, Nature, https://doi.org/10.1038/s41586-022-05550-y; Krukenberg et al., 2024, https://doi.org/10.1038/s41586-024-07829-8; Wu et al., 2024, Nature, https://doi.org/10.1038/s41586-024-07728-y).

We carefully annotated our archaeal genomes by comparison with arCOGs, Pfam, TIGRFAMs and nr databases, and manually retrieved and verified genes involved in archaeal WLP or bacterial WLP. As expected and as shown in a previous study (Adam et al., 2019, Nature Microb, 4:2155-2163), the majority of analyzed archaeal genomes contained a complete or nearly complete archaeal WLP (using $H_4$MPT as cofactor carriers) (Fig. r2). Surprisingly, we identified homologues of four key enzymes for bacterial WLP (FdhA, Fhs, FolD and MetF) in a substantial proportion of these archaeal genomes (Fig. r2), including two cultured Asgard archaea, five cultured Euryarchaea, and six cultured TACK archaea. Specifically, 21.1% of genomes contained FdhA genes, 32% contained Fhs genes, 72.3% contained FolD genes, and 68% contained MetF genes. The presence of these bacterial WLP genes in genomes of isolated pure cultures strongly indicates that they are not artifacts of contamination from binning errors. In the case of the cultured Asgard archaeon-*Candidatus Prometheoarchaeum syntrophicum* strain MK-D1, its genome harbored a complete $H_4$F methyl branch of the bacterial WLP, where a molybdopterin-dependent oxidoreductase was identified as a homolog of FdhA, but it lacked a complete archaeal WLP (only Ftr and CdhE were present). It has been suggested that the $H_4$F methyl branch in MK-D1 may be responsible for converting histidine, serine, and glycine into formate for interspecies electron transfer (Imachi et al., 2020, Nature 577:519-528). It is likely that a similar metabolic process may occur in other Asgard archaea containing the $H_4$F methyl branch. In addition, it cannot be excluded that this

$H_4F$ methyl branch may be connected to the serine cycle for C1-assimilation.

Interestingly, previous studies have found that the $H_4MPT$ branch of archaeal WLP appears as a module in bacteria or as standalone, playing roles in carbon assimilation, formaldehyde detoxification and energy conservation (Adam et al., 2019, Nature Microb, 4:2155-2163). Considering the limited distribution of the $H_4MPT$ branch in bacteria, it has been suggested that this branch may originate in Archaea and was later transferred to Bacteria. Similarly, the full $H_4F$ methyl branch of the bacterial WLP was found in only 51 out of 411 Asgard archaea, thus it is inferred that the branch in Asgard archaea might have been acquired later from bacteria. The evolutionary history of the $H_4F$ methyl branch in both Archaea and Bacteria will be the subject of further study in the future.

We examined the distribution of genes encoding bacterial-specific ribosomal proteins and genes involved in bacterial lipid synthesis in the Asgard archaeal dataset (Fig. r2). A total of 18 bacterial-specific ribosomal protein genes were identified in 18 out of 411 Asgard archaea, while 9 genes related to bacterial lipid synthesis were found in 15 genomes. No homologs of these genes were detected in the two cultured Asgard archaea. These results suggest that the contamination of these archaeal genomes is within a reasonable range and does not impact their evolutionary placement or the reconstruction of their metabolic pathways.

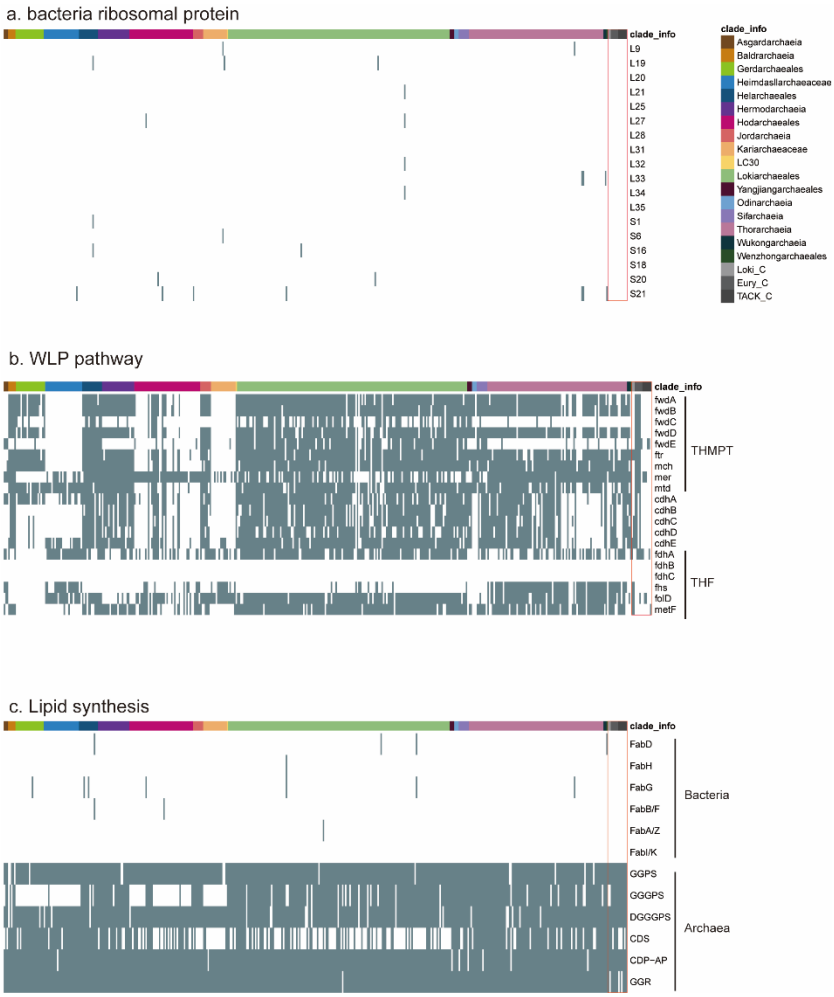


**Fig. r2.** The distribution of bacterial-specific ribosomal protein genes and genes involved in WLP and lipid synthesis in 411 Asgard archaea, five cultured Euryarchaea, and six cultured TACK archaea.

2. The underlying metabolic reconstruction information is listed as given in “Table X” (Fig. S15 legend) but there is no Table X, although supplemental Table 7 is a start in the right direction, yet lacks all of the enzymes that are required for H4F and H4MPT synthesis. It also lacks most of the crucial information that we need to know underpinning Figure 4 and Supplementary Figure 15. The authors have clearly done analyses that lead to the metabolic reconstructions, readers need to have access to that data in a fully enumerated version of Table 7 that allows readers to check the data. All of the enzymes indicated in the figure need to be backed up by assembled and annotated genes in the Table.

**Response:** Corrected. All of the enzymes shown in Supplementary Figure 25 are provided in Supplementary Table 6-7. Table X in Fig. S15 legend has been corrected.

3. As a prime example of a problem enzyme, Supplementary Figure 15 has Nif on the right side converting N2 into ammonia. There is no N2 importer (N2 is a gas) and Nif (nitrogenase) is a cytosolic protein, the Nif-related entries in Table 7 are lacking. MCR in Fig 4 of the main text is shown as methane/alkane oxidizing, what evidence or logic supports that scheme?

**Response:** This is a mistake. Nif (nitrogenase) has been placed into cytoplasm and added into Supplementary Table 6. In Fig. 4, only Loki ancestor contained the MCR complex for methane/alkane oxidization while other ancestors do not contain the MCR complex. Lokiarchaeia included Helarchaeales and Lokiarchaeales. All members of Helarchaeales are found to contain an MCR-like enzyme that are similar to that found in butane-oxidizing archaea. A detailed description for metabolic feature of Helarchaeales can be found in a previous study (Seitz et al., 2019, Nature Commun. 10: 1822).

4. The lower left methanogen-like enzyme assembly in Fig. S15 is a mess. Mtr (MtrA-H?) is a pumping enzyme that generates the ion gradient for methanogen ATP synthesis in the methyl transfer from H4MPT to CoM. Free CoM from the heterodisulfide reductase is the substrate for MtrA-H, which typically takes methyl groups from methyl-H4MPT. Or is this indicating MtaABC? (see Thauer et al. Nat Rev Micro 2008 doi:10.1038/nrmicro1931). There is no methyl group for that reaction.

**Response:** You are correct. This is a wrong connection. No reaction occurs here. In addition, only the Mtr H subunit is present in Asgard archaea, thus it is inferred that the reaction from $CH_3$-CoM to $CH_3$-$H_4$MPT, catalyzed by the MtrA-H complex, may not necessarily occur in these archaea.

5. Table 7 lists chlorophyll synthesis enzymes, which can't be right, unless there are photosystem proteins and carotenoid synthesis in the data, and then there is a problem because no archaea contain chlorophyll and no chl-photosynthesizers use the WL-pathway. Table 7 and the figures need to be complete and coherent.

**Response:** The identification of enzymes related to chlorophyll synthesis should be ascribed to incorrect annotation using KEGG database. In ancestral metabolic reconstruction section of original manuscript, we annotated representative sequences in homologous families clustered by MMseqs2 by searching against KEGG database using kofamscam tool. However, we found that the best hits for some protein sequences obtained using the KEGG tool were inaccurate. For example, the top hit in KEGG for the representative sequence in cluster 8645 was anaerobic magnesium-protoporphyrin IX monomethyl ester cyclase, which is involved in the tetrapyrrole biosynthetic pathways leading to chlorophyll and bacteriochlorophyll. However, when searching nr database using BLASTP, the top 20 hits for this sequence were all B12-binding domain-containing radical SAM proteins. These metalloenzymes catalyze a wide range of radical-based reactions in various biosynthetic pathways, including vitamin, cofactor and metallic center biosynthesis, nucleic acid modifications, DNA repair, and protein post-translational modifications. Additionally, searches using InterPro database revealed that the protein contained two domains: B12 binding and Radical SAM. Searching for arCOG database returned a top hit as a radical SAM superfamily enzyme. Given the reliability of the BLASTP algorithm, the authority of the nr database, and the specificity of the arCOG database, we concluded that KEGG annotations were inaccurate and potentially misleading. This discrepancy may arise because the KEGG database primarily focuses on model organisms, and annotation information for non-model organisms is often limited. Therefore, in the revised manuscript, we excluded the KEGG database and instead used the arCOG, nr, and InterPro databases for genome annotation in ancestral metabolic reconstruction. Gene functions were further verified by searching against SwissProt database using BLASTP.

6. The pta, ack, and acd enzymes present in all four (Fig S15) could, in principle, enable an acetogenic metabolism, except that Rnf is missing for pumping, although the protein that is designated as complex I could be Ech, the energy conserving hydrogenase of acetogens and methanogens ( Schoelmerich and Muller PNAS 2019 https://doi.org/10.1073/pnas.1818580116), which is indistinguishable from

complex I in sequence comparisons (Schoelmerich and Muller CMLS 2020 https://doi.org/10.1007/s00018-019-03329-5). The possbility that the host lineage had an acetogenic lifestyle has been discussed (Martin 2016; DOI 10.1002/bies.201600089) although it is difficult to force modern methanogens to grow by producing acetate (Schöne et al. 2022 doi: 10.1073/pnas.2113853119).

**Response:** Thank you for your valuable suggestions. We have reviewed the references you provided. According to updated gene annotations and improved ALE inference using high-quality genomes (completeness > 80%, contamination > 5%), we found that genes encoding phosphotransacetylase (*pta*), ADP-forming acetyl-CoA synthase (*acd*), and acetyl-CoA synthase (*acs*) are widely distributed across Asgard archaea, except for acetate kinase (*ack*) (Fig. r3). Although Complex I (possibly acting as the energy conserving hydrogenase Ech) was also widely present in Asgard archaea, but a large amount of gene loss occurs in Lokiarchaeia (including Lokiarchaeles and Helarchaeales). If the *ack* gene is required for acetogenesis, it can be inferred that only the ancestor of Heimdallarchaeia might have had an acetogenic lifestyle.

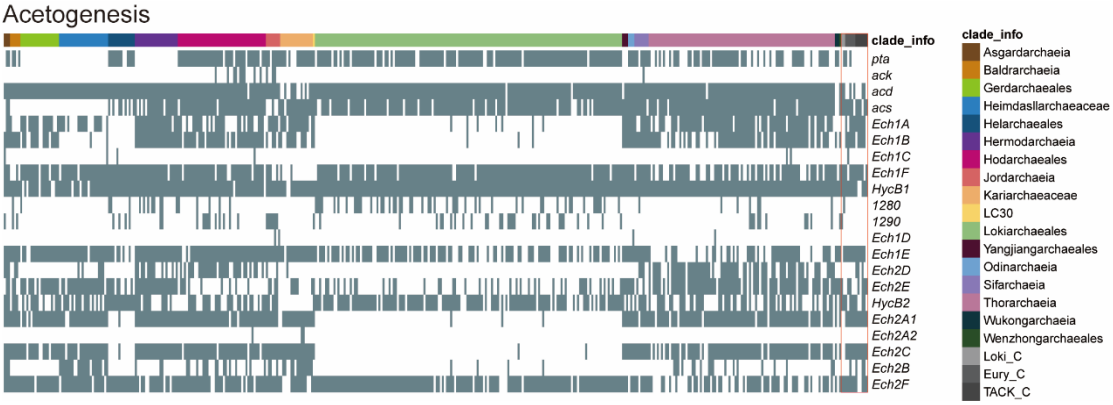


**Fig. r3.** The distribution of genes related to acetogenesis in 411 Asgard archaea

7. In several places chemolithotrophy (a lifestyle) is written where chemolithotroph (an organism) is intended. There is no “a” in putrescine (Fig. S15). In the legend of Fig. S15 and Fig. 4, both RuMP and sp-ED are marked as ribulose monophosphate pathway.

**Response**: Corrected.

8. Figure S15 has methyl-CoM reductase in Loki, this would make it a methanogen as some suggested early on (Sousa et al. 2016: DOI: 10.1038/NMICROBIOL.2016.34), but the pumping mtrA-H issue needs to be resolved. That 2016 paper shows lack of the bacterial WL pathway in Loki and lack of H4F biosynthesis. Maybe it would be better in the present paper to show the actual distribution of the enzymes for key pathways that reconstruct to the asgard ancestor (not all pathways, just important ones), especially contentious ones like bacterial WL pathway or folate synthesis, rather than relying on ALE not to fail.

**Response:** As you suggested, in the revision, we carefully annotated the genomes by a combination of multiple databases, rather than depending on the function of representative sequences in homologous families clustered by MMseqs2. Based on these more accurate gene annotations, we examined the distribution of enzymes involved in key pathways across Asgard archaea. Related results are provided in Supplementary Table 7. As mentioned earlier, although genes associated with the $H_4F$ methyl branch of the bacterial WLP (fdhA, fhs, folD, metF) are present in lots of Asgard archaeal genomes, the fhs gene was only found in a small number of genomes in Lokiarchaeles (13 out of 152). Notably, the cultured Lokiarchaeota MK-D1 and its two closely related MAGs contain a complete $H_4F$ methyl branch (Supplementary Figure 24), which is suggested to be involved in the degradation of glycine, serine, and histidine. In the study by Sousa et al. 2016, only a Lokiarchaeon genome with 90% completeness was analyzed, so many metabolic features of Lokiarchaeles and Lokiarchaeia are still incomplete. We also investigated the distribution of genes involved in folate synthesis and found that very few genomes contain the folB, folK, folC and folA genes (Fig. r4). Additionally, the cultured Lokiarchaeota MK-D1 appears to lack key genes for folate synthesis, as shown in the previous study by Imachi et al. (2020, Nature, https://doi.org/10.1038/s41586-019-1916-6). The results likely suggest that organisms harboring the $H_4F$ methyl branch may instead absorb folate from their environment.

At present, almost all members of Helarchaeales in Lokiarchaeia (13 MAGs)

encode a methyl-CoM reductase (Fig. r4), which has been suggested to play a role in short-chain alkane oxidation, rather than methanogenesis. In this case, a complete Mtr complex may be not required (only MtrH is identifiable in Helarchaeales). Using the ALE tool, it was inferred that the ancestor of Lokiarchaeia may have acquired a methyl-CoM reductase, suggesting that it may be able to oxidize short-chain alkanes.

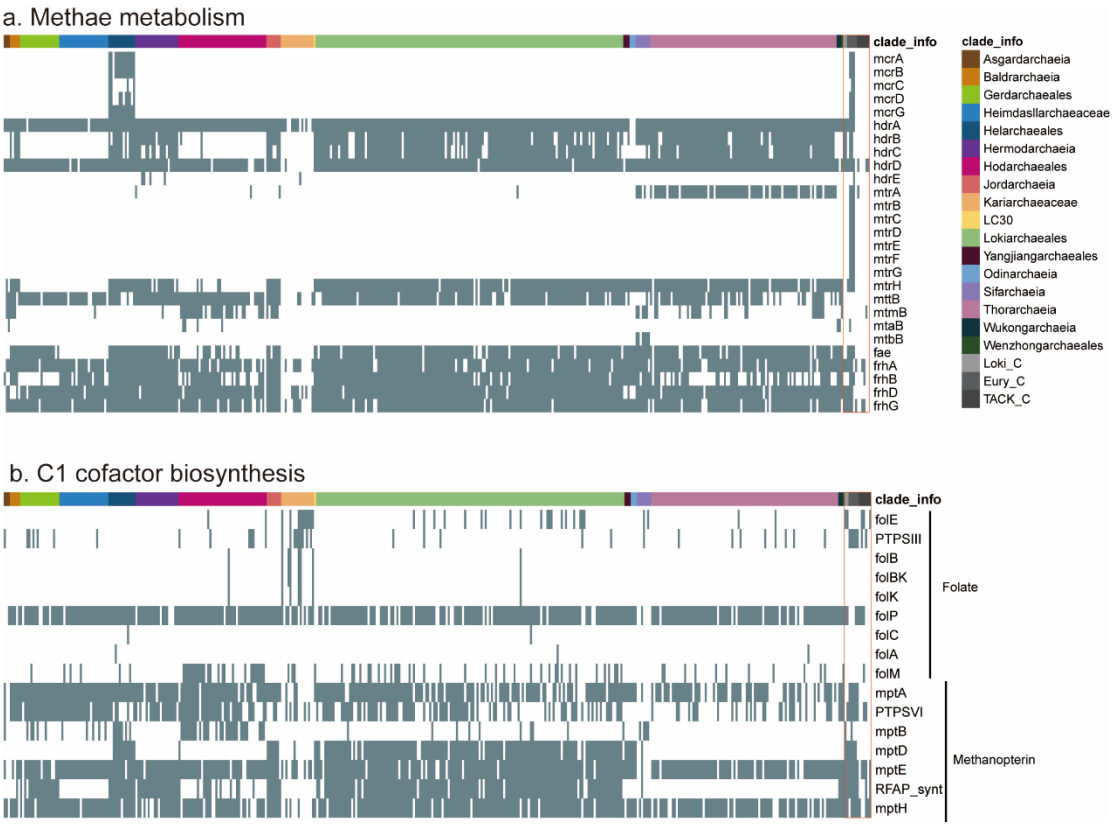


**Fig. r4.** The distribution of genes related to methane, alkane metabolism, and C1 cofactor synthesis in 411 Asgard archaea

9. To my understanding, only three archaeal environmental-genome-discovered lineages have been brought to cultivation: Stetter's Korarchaeum cryptofilum (PNAS 2008), ref. 5 and ref. 6. All 3 are fairly mundane peptide / amino acid fermenters with unusual morphology, which seems worth mentioning.

**Response:** Corrected.

Issue 3. The chimeric Njordarchaeales. This will immediately get the undivided

attention of everyone in the metagenome and eukaryote origin community. It is essential, imperative, for Nature to publish this as an equal-weight counterbalance to Ref. 3. The chances are good that a flood of new “cryptic chimerism” papers will be the next big thing to emerge in metagenomics publishing. This analysis is direly needed to alert the community and unsuspecting readers that there are issues, severe issues, with the metagenomic data currently being used to revise, re-revise, amend re-re-revise eukaryote origin every year. People have been trusting metagenomic data as if it were real. The asgard genomes do not exist in nature (except for 2 in refs 5 and 6), they are constructed by and exist in computers. It should be pointed out that some authors were showing the problems with chimeric assembly of metagenomic *ribosomal* proteins, the ones that are being used to construct the trees. Garg et al. showed that the phylogenetic behaviour of 23 ribosomal proteins of 30 metagenomic archaeal lineages is completely anomalous in comparison to the genomes of cultured archaea (see Figure 5 of doi:10.1093/gbe/evaa238). The existence of congruent phylogenetic signals across individual ribosomal proteins (and whatever the authors use as markers) needs to be shown, not assumed, in metagenomic evolution papers. The fact that nobody looks for, or reports, conflicting vs. consistent signals in metagenomic data does not mean that they do not exist, and this paper shows dramatically (Fig 2) how a whole lineage can suddenly crumble, and dissolve into thin air, if someone looks for the chimerism problems. More people need to look for such problems, rather than focus on boostrap values or bayesian support numbers or similar. Trost et al. 2024 (DOI: 10.1093/femsle/fnae068) showed that the pangenome structure of metagenomic data only starts to look vaguely like that of natural genomes from cultured microbes for MAGs above a >90% quality threshhold. According to Parks (ref 45) these 223 new MAGs have a 20% quality threshold (completeness 70% minus 5 x contamination 10%). That is low and one wonders how the reconstructions fare with a more robust threshold. Much of the ref. 3 data was not much better, one needs to add, and some of it was chimeric we now see.

**Response:** Thank you for your good suggestions. For MAGs from environmental samples, a ≥ 50% quality threshold is typically used as a filtering criterion (Parks et

al., 2017, Nature Microb, 2:1533-1542). Although we used a cutoff of >70% completeness and <10% contamination, the majority of MAGs far exceeds this threshold. Based on the quality measure (completeness minus 5 x contamination), we observed that 363 out of 411 genomes (88%) have a quality of more than 50%, and 120 out of 136 new MAGs we used exceed the 50% quality threshold.

In the revision, to improve metabolic reconstruction, only genomes with >80% completeness and <5% contamination (with a mean completeness of 88.5% and mean contamination of 2.7%) were retained for ALE analyses. Using the criterion, a total of 235 Asgard archaea were included, with a mean quality score of 75% (the lowest quality value being 51%). We understand that using near-complete genomes (completeness >90%, contamination <5%) may be much better for reconstruction, but this will result in many Asgard lineages having only one genome remaining or disappearing entirely, which inevitably affects our understanding of the evolution and metabolic characteristics of Asgard archaea. From a statistical perspective, using more genomes can help reduce incorrect inferences about certain metabolic pathways, especially when these pathways are widely distributed across a large number of genomes. Finally, the majority of microbes in nature are still difficult to isolate and culture, and metagenomic assembly techniques remain one of the most important tools for understanding the microbial world on Earth.

10. All metagenome phylogeny papers (starting with this one as a good example) need to report all of the individual amino acid sequence alignments of the proteins used for concatenation, site filtering and the “big picture” phylogenetic tree. As it stands, we only get the manipulated concatenated data to inspect, leaving it up to readers to assemble, from raw data, the “same” alignments that are in the published paper if they want to inspect or reproduce the phylogenetic results. The reason is clear: The phylogenies of the individual marker genes (ribosomal and informational plus a few others) need to be available for **easy** inspection by interested parties (and the authors) to flag assembly chimerism. Transfers are not an issue for good marker proteins but the only way to demonstrate that is to do the individual protein trees and make the individual sequences and alignments (fasta) available.

**Response:** In the revision, for all markers we used, their individual amino acid sequence alignments and individual-gene trees have been uploaded to Figshare (https://figshare.com/s/6e523322b0b647b91dda).

Related to chimerism. Line 110. The MAG assemblies are reported as >70% complete (and less that 10% contamination), meaning that 30% of the genes that one might expect in a MAG — based on earlier sequences — are missing. This becomes particularly pressing as it regards methanogenesis, because there is an unaddressed snowballing error in the entire asgard pipeline: If one or a few early-published argard bin assemblies ('genomes') were missing MtrA-H (the pumping protein of methanogens) and methyl-CoM reductase, then completeness methods will not count either as missing (incomplete) because the reference assemblies for assessing completeness lack them. For a group, a gene present in 70% of its MAGs has optimal representation as present. ALE probably does not know how to handle that kind of data, because the "present vs. absent" state is uncertain in the input data. That puts quite a limitation on the ancestral reconstruction. As a remedy, one could try to counter that with reconstructions for a higher quality subset (many of us would like to know what the quality scores for the old, previously published MAGs in this data are, probably not better than 20%). Or (and in addition) downplay the reconstructions to their essentials, based on a fully enumerated version of Table S7.

**Response:** As mentioned above, although we used a cutoff of >70% completeness and <10% contamination, the majority of MAGs far exceeds this threshold, with a mean completeness of 85.3% and mean contamination of 3.6%. Given that genes related to methanogenesis, such as MtrA-H and methyl-CoM reductase, are missing in the two cultured Asgard archaea, they may be also absent in members of most of Asgard archaeal lineages.

In the revision, for ALE inference, we have used a higher quality subset of genomes, including a total of 235 Asgard archaea (71 newly generated genomes and 164 previously published genomes), with a mean quality score of 75% (the lowest quality value being 51%). In addition, to compensate for the limitations of ALE inference, the distribution of genes related key pathways in Asgard archaea was

analyzed and is presented in Supplementary Table 7.

Issue 4. The nickel story. The authors lean on one Nature paper (ref 27) proposing that Ni became limiting in the oceans at some point in Earth history, this caused methanogen famine, and in the narrative of this paper, caused the methanogenic ancestors of asgards to lose methyl-CoM reductase (mcr), which is however present in Loki according to Fig. S15. The problem is not Loki. The problem is that modern methanogens do not have a problem with low Ni, they produce about 1 gigatonne of methane per year (Thauer et al. NRMicro 2008), even though modern oceans only contain nanomolar amounts of Ni. There is a great story about the discovery of methanogen Ni requirement by Peter Schönheit – the methanogens dissolved nickel-containing steel to get their metal, nickel is present in vast amounts in the crust. A gigatonne of methane is 0.1% of the atmosphere, or roughly 1.4 million cubic kilometers (per year, most of it consumed by microbes). Nickel limitation? How. Worse for the Ni narrative of Zhang and team, they show five highly expressed Ni-containing enzymes in their reconstruction: CODH, acetyl-CoA synthase, and three different Ni-Fe hydrogenases. The Ni narrative does not withstand even casual inspection because they need Ni for these enzymes anyway. The oxygen part of the narrative collides with the fact that ancestral eukaryotes arose and diversified for over a billion years in extremely low oxygen environments (reviewed by Mills et al. 2022 in Nat Ecol Evol. https://doi.org/10.1038/s41559-022-01733-y). Their ancestral state is facultatively anaerobic, not aerobic. The evidence that Zhang et al. lean on for ancient or pre-GOE O2 has been shown to reflect post-depositional oxidation [Slotznick et al. Sci Adv 8, eabj7190.]. The “aerobic” state that Zhang infer for the asgard ancestor is based on only one enzyme in Loki-Heim (a terminal oxidase), which is likely contaminant (like the bacteria WL pathway) or transferred and completely incompatible with at least 10 proteins that they show in their Fig. S15 reconstruction (identified in one minute by eye): Three Ni-Fe hydrogenases, Nif, CODH (2), ACS (2), por, kor. Those are extremely O2 sensitive enzymes (easily oxidized FeS centers, doi:10.1002/1873-3468.14906, 2024) and diagnostic for anaerobic growth. The corrin synthesis list will have additional other examples, other

cofactors will too. With 10% contamination, roughly 10% of the enzymes they have in their reconstruction scheme on average will be contaminants. Finally on the reconstruction, they show that all four cells in their Figure 4 are growing on glucose. Glucose is blood sugar, the main substrate for mitochondria and human parasites. In soil and marine environments, glucose is scarce. Asgard archaea cultivated so far grow on tryptone.

**Response:** In the revision, based on newly selected high-quality genomes and accuracy gene annotation, ALE inference was re-analyzed. Ni and oxygen story, and Fig. S15 and Table S7 have been modified.

11. This reader suggests removing the 'self-destructing' nickel and oxygen narratives altogether, focus on the data and the chimerism problems, improve the reconstruction as much as possible.

**Response:** Corrected.

In summary, this is a very important and exciting paper of immense interest to readers of Nature and all biologists especially given the editorial flag on ref. 3 on the Nature website. The points above are explained at length (my apologies) but are easily dealt with editing in a minor revision (and making the individual alignments available, which are inaccessible in other asgard papers):

Issue 1. Focus on the trees, and please make the phylogenetic data easy to access (individual unmanipulated alignments for the markers so that everyone can finally check for massive incongruence among marker genes and use their favorite simple or overparameterized method of analysis and their favourite data recoding procedure and their favourite site exclusion method). This referee is adamant about the need to present the individual analyses because this referee invented both the method of concatenating dozens of proteins for deep phylogenetic analysis and examining the compatibility of the individual trees (Martin et al. 1998 Gene transfer to the nucleus and the evolution of chloroplasts. Nature) and the method of systematically deleting highly variable sites in such large alignments (Int. J. Syst. Evol. Microbiol. (2000), 50, 1655–1663).

**Response:** In the revision, for all markers we used, their individual unmanipulated

amino acid sequence alignments and individual-gene trees have been uploaded to Figshare (https://figshare.com/s/6e523322b0b647b91dda).

Issue 2. Improve the metabolic reconstructions so that they better match observations from real genomes, perhaps using a set of more stringent quality filters. The two WL pathways are unlikely to be real, their relative frequencies might resolve the issue, the H4F dependent bacterial pathway is the problem. The authors apparently see this too as only the archaeal version is shown in Figure 4b. Where is Rnf? Is complex I Ech or is it a non-pumping version, as in ndh in E.coli as opposed to nuo?

**Response:** In the revision, the metabolic reconstructions have been improved as follows: 1) Genomes were carefully annotated by a combination of multiple databases, including nr, arCOGs, Pfam, InterPro, and SwissProt databases, rather than depending on the function of representative sequences in homologous families clustered by MMseqs2. The strategy was necessary because we observed that other sequences within the same family, as clustered by MMseqs2, can exhibit functional differences compared to their representative sequences. 2) Based on these more accurate gene annotations, we examined the distribution of enzymes involved in key pathways across Asgard archaea. Related results are provided in Supplementary Table 7. 3) For ALE inference, we have used a higher quality subset of genomes, comprising 235 Asgard archaea in total (71 newly generated genomes and 164 previously published genomes). The mean quality score of genomes in this subset was 75%, with the lowest quality value being 51% (See Methods). Protein sequences with the same function annotation, manually verified, were clustered to infer individual-gene trees.

For the two WL pathways, as mentioned above, the $H_4F$ dependent bacterial pathway is not a contamination. It was present in the two cultured Asgard strains and is inferred to be involved in degradation of amino acids. Gene frequency analysis revealed that genes associated with the $H_4F$ methyl branch of the bacterial WLP (fdhA, fhs, folD, metF) are present in lots of Asgard archaeal genomes (see Supplementary Figure 24).

We did not identify an Rnf complex in Asgard archaea. But we found that many

subunits of Complex I have high amino acid sequence similarity with subunits of group 4-type energy-converting hydrogenase (Ech). To retrieve homologues of Ech from Asgard archaeal genomes, we built a database using protein sequences from two Ech gene clusters in *Thermoanaerobacter kivui* (Schoelmerich and Muller, 2019, PNAS) and performed BLASTP searches. Top hits for many proteins were assigned to proteins that are named as Ni,Fe-hydrogenase I large subunit/small subunit, and NADH dehydrogenase subunits L, M, H, C, and B in arCOG database. Since the Ech complex in *Thermoanaerobacter kivui* was experimentally verified as a respiratory enzyme, we concluded that the energy-converting, ion-translocating hydrogenase (Ech) may be widespread in Asgard archaea (see Supplementary Figure 26a).

Issue 3. The Njordarchaeales chimerism issue. Focus on this crucial observation and how it impacts previous and future analyses, how it impacts metagenomic data processing pipelines, suggest ways to detect similar problems in future analyses (individual marker gene analyses and data availability), maybe present the problems surrounding the handling of these chimeras in the public databases.

**Response**: Some suggestions have been added in part 4 of Supplementary results and discussion.

Issue 4. The nickel narrative (Mcr) succumbs under the weight of the data showing Ni-dependent enzymes (CODH, ACS, three Ni hydrogenases) in these lineages. The oxygen narrative has a similar problem with FeS centers and 10 oxygen sensitive enzymes. Facultative anaerobes can have both kinds of enzymes in their genomes (O2 requiring and O2 sensitive), but differentially expressed (Martin and Müller, the hydrogen hypothesis, Nature 1998).

**Response:** In the revision, the nickel and oxygen narrative have been deleted. This part has been rewritten.

Other small matters:

In Figure 4, what is NR in the membrane, nitrate reductase? And where are the amino acid transporters listed (which goes back to the issue of Table S7 being incomplete)? From the figure it might seem that Hdr-Mvh complexes with Mcr? This is not reported for cultured methanogens. In addition, the common gene abbreviation is

Mvh/Hdr not MH.

**Response:** Corrected.

Some additional comments:

Line 49: probably facultative chemolithotroph and aerobic heterotroph. As explained above, this inference is problematic. The correct spelling would be "probably facultative chemolithotrophic and aerobic heterotrophic", the more likely inference would be "probably facultative chemolithoautotrophic and facultative anaerobic" (the figures show an autotroph with a WL pathway to pyruvate synthesis).

**Response**: Corrected.

Line 79: Heimdallarchaeota has been identified

Heimdallarchaeota have been proposed

**Response**: Corrected.

Line 146. dataset consisting of 57 proteins of archaeal origin that excluded ribosomal proteins, ….. [just a comment by this reviewer, this is a clear example of ad hoc data handling to obtain a desired result or avoid an undesired one. No proteins evolve more vertically than the conserved ribosomal proteins].

**Response**: We agreed with your viewpoint.

Figure 3. The higher order archaeal ("asgardal") taxa need to be indicated. When we read Heimdallarchaeia or similar in the text, we need to be able to see those categories in Figure 3 and Suppl figures as well.

**Response:** Corrected.

Line 158: and other archaea compared to the NM57 tree

and other archaea, in contrast to the NM57 topology

**Response:** Corrected.

Line 161: "…we integrated the aforesaid marker sets…" – which ones, do the authors mean the ribosomal and NM57 sets? It would be good to specify.

**Response:** Corrected.

Line 164. See Methods.

**Response:** Corrected.

Line 186: To uncover the mystery,

To identify the cause of this effect,

**Response:** Corrected.

Line 212: The inclusion of such chimeras in tree reconstruction could significantly impact the evolutionary position of eukaryotes in archaea.

The inclusion of such chimeras in tree reconstruction can be expected to impact the evolutionary position of eukaryotes within archaea.

**Response:** Corrected.

Line 274: The result indicated that the relationship between eukaryotes and Asgard archaea in the previous study was likely artifactual

The result indicated that the relationship between eukaryotes and Asgard archaea reported (ref. 3) was likely artifactual

**Response:** Corrected.

Line 279: Totally, our phylogenetic analyses indicate

Taken together, our phylogenetic analyses indicate

**Response:** Corrected.

Line 288 ff. See Issue 2 and Issue 4 above.

**Response:** Corrected. See the text.

Line 321: This is hydrogen dependent autotrophy (see Martin and Müller 1998)

**Response:** Corrected.

Line 326. the LAsCA was a chemolithotrophy. We

the LAsCA was a chemolithoautotroph. We

**Response:** Corrected.

Line 328: lack of genes encoding methyl-coenzyme M reductase and most of tetrahydromethanopterin (H4MPT) S-methyltransferase subunits, thus the LAsCA may originate from a methanogen

In that passage, the tetrahydromethanopterin (H4MPT) S-methyltransferase to which they refer is MtrA-H, is that correct? MtrA-H is explained in (Shima S et al. (2020) Structural basis of hydrogenotrophic methanogenesis. Annu Rev Microbiol. 74:713–733).

**Response:** The passage has been deleted.

Let us keep in mind that the genomes are not complete, some genes will be missing simply because of that.

Line 330: thus the LAsCA may originate from a methanogen.

thus the LAsCA may have been a methanogen.

**Response:** Corrected.

Line 334: decreased eruption of nickel-rich ultramafic rocks (27).

Reference 27 fails because methanogens thrive today (1 Gigatonne per year CH4) with much lower Ni than proposed in 27, and the Ni model fails here because of the 5 other Ni dependent enzymes, CODH, ACS, three hydrogenases (Issue 4 above). Simplest solution is to delete the Ni model. This paper does not need it, it only opens up broad flanks for easy attack that the paper does not need.

**Response:** The Ni model has been deleted. See the text.

Line 338: they did not abandon Ni dependent enzymes….

**Response:** Corrected.

Line 339: This reviewer is not familiar with a report of a Hdr-Fix complex. Please, provide a definition and reference. While Hdr is an enzyme of methanogenesis, Fix is not, it is an electron carrier often found in syntrophs.

**Response:** The sentence has been deleted. For the Hdr-Fix complex, the *fixABCX* operon comprises genes encoding electron transfer flavoprotein (ETF) subunits (*fixAB*), ETF dehydrogenase (*fixC*), and ferredoxin (*fixX*). The *fixABCX* operon is found in many syntrophic metabolizer genomes and is thought to perform quinone-mediated reverse electron transport from ETFred to a membrane-bound hydrogenase (possibly is HdrABC) for $H_2$ generation (Sieber et al., 2010, Environ Microbiol 12: 2289-2301; Nobu et al., Environ Microbiol, 2015, 17:4861-4872).

Line 341: Do we now have two asgard archaea as the host for mitochondrial symbiosis?

**Response:** Corrected.

Line 345, The LAsCA genome encoded various NiFe/FeFe hydrogenases (Fig. 4a, Supplementary Table 7, Supplementary Fig. 15), suggesting that it was a hydrogen-dependent autotroph.

Now the authors are saying H2 dependent autotrophy. In other passages it is H2 production. This is going to get readers lost fast.

**Response:** Corrected.

Line 350: The evidence for pre-GOE whiffs has been debunked: The oxidation of the sediments in question occurred post-deposition [Slotznick SP, Johnson JE, Rasmussen B, Raub TD, Webb SM, Zi JW, Kirschvink JL, Fischer WW. 2022 Reexamination of 2.5-Ga "whiff" of oxygen interval points to anoxic ocean before GOE. Sci. Adv. 8, eabj7190].

**Response:** This part has been deleted.

Line 351: This is naked speculation.

**Response:** This part has been deleted.

Line 358: Contrary to the result by Eme et al., our inference revealed that the Heimdallarchaeia and Hodarchaeales ancestors also possessed a complete WLP pathway (Fig. 4b), which is consistent with their geological ages because H2 was still intermittently available at that time.

This passage is not logical. First, the data here show two WL pathways, not one. Second, H2 is produced today in massive amounts by serpentinization (cubic kilometers of H2 per year). It always has been since there was water on Earth. [Tamblyn, R and Hermann, J 2023. Geological evidence for high H2 production from komatiites in the Archaean. Nature Geoscience 16:1194-1199. Sleep, N. H., Meibom, A., Fridriksson, T., Coleman, R. G., and Bird, D. K. (2004). H2-rich fluids from serpentinization: Geochemical and biotic implications. PNAS 101, 12818–12823]. It is also produced by fermentations. What H2 famine?

**Response:** The reasons for the existence of the two WL pathways in Asgard archaea have been explained above (the bacterial WLP is incomplete in most of genomes). Other sentences have been deleted.

Line 369: besides heterotrophically

**Response:** Corrected.

Line 371: This is exactly what the hydrogen hypothesis said (Martin and Müller, 1998). Autotrophy and methanogenesis were lost, the glycolytic pathway became

established in the cytosol via gene transfer from endosymbiont to host. The first eukaryote was a facultative anaerobe.

**Response:** Corrected. We basically agreed with your point.

Figure S15. Mvh/hdr is indicated twice. Again, is mtr at lower left MtrA-H? Methylamine degradation is shown, do we see pyrrolysine synthesis in the genomes?

**Response:** This is an error and this figure has been updated. The mtr at lower left does not exist and no reaction occurs here. Given the absence of an MCR enzyme related to methanogenesis, methylamine degradation cannot occur.

Line 617, Figure S14 and one row in Table S4: an S98 dataset is mentioned, but not described anywhere

**Response:** This is an editorial error; the S98 dataset does not exist in the current article.

Some typos:

Fig. 1 legend, line 874: “Only Asgard archaeal lineages are shown…”

Fig. 3c x-axis label: “Removal”

Line 306: “improving>improve”

Line 317: “based on comparative genomics…”

**Response:** These typographical errors have been corrected.

In summary, this is an excellent analysis of massive new data that speak to the essence of the issue and present a major advance on a crucial evolutionary question (the heritage of our first microbial ancestors). The comments are long because the paper is interesting. The recommended revisions are short because it should go forward without delay.

## Responses to reviewer's comments

**Referee #1 (Remarks to the Author):**

The authors have performed extensive re-analysis on marker gene selection, alternative data sets, chimerisms/taxonomic assignment, and ESP definitions. In addition they have recontextualized their findings. All this work is massively appreciated.

I have three short comments, one semantic and two optional remarks / discussion points.

1. The authors perform removal of fast evolving sites to evaluate the robustness of phylogenetic signal. Thereby -for example- showing that for the NM57 (?) inference of eukaryotes within heimdallarchaeia disappears and instead eukaryotes sister to heimdallarchaeia is favored for more slowly evolving sites. This is a very insightful analysis, but the main text and rebuttal seem to refer to this as removing (highly compositionally) heterogeneous sites. Compositional heterogeneity is its "own" class of potential issues in phylogenetics (with its own set of tools see e.g. https://doi.org/10.1016/j.ympev.2013.09.011 https://doi.org/10.1016/j.isci.2022.105594) and distinct (albeit in practice likely somewhat overlapping) from homoplasies that result from fast evolving sites. I would suggest to refer to these analyses as fast site removal or something similar rather than removal of heterogeneous sites.

**Response**: Thank you for your valuable suggestion. We agreed with your perspective and have revised the manuscript accordingly. Specifically, we have replaced "heterogeneous sites" with "fast-evolving sites" and substituted "compositional heterogeneity" with "rate heterogeneity" throughout the main text.

2. In the rebuttal/revision, the authors very graciously reanalysed the eme et al. 2023 data and markers in different contexts to try and arrive at an explanation for the discrepancy in the precise placement of eukaryotes. I am still somewhat perplexed that using more or less exactly the same marker set on more or less the exact same species using more or less the same phylogenetic methods and models, a different

result is obtained. Upon closer reading the literature, the only thing that I can still think of that would explain the discrepancies between various inferences, is that the phylogenetic affinities inferred in previous studies were obtained through fast site removal combined with recoding and then phylobayes with CAT model variants. I am just mentioning this because I would like to get to the bottom of why different results are obtained so take this as FYI.

**Response**: This is a good question. In the study by Eme et al.(2023), maximum likelihood (ML) phylogenomic analysis of the untreated NM57 dataset--including 175 Asgard archaea (hereafter referred to as NM-A175)--placed eukaryotes at the base of Heimdallarchaeia, forming a sister clade to Njordarchaeales (Supplementary Fig. 3 in their study). To further investigate this, we reanalyzed the NM-A175 dataset and replicated the topology reported by Eme et al. (2023) using the same genome sampling, gene selection, phylogenetic tools, and parameters. However, upon excluding Njordarchaeales and the outgroup, we observed a distinct shift in topology, with eukaryotes instead branching within Heimdallarchaeia as a sister group to the Hodarchaeales (Fig. 1R2). We inferred that the presence of the outgroup (including TACK archaea) in Eme et al.'s analysis may have attracted TACK archaea-related sequences within the Njordarchaeales genomes, thereby likely biasing the placement of eukaryotes towards the base of Heimdallarchaeia. To explore this further, we applied a site-by-site desaturation strategy to the NM-A175 supermatrix, removing Njordarchaeales and the outgroup (Fig. 2R2). Our findings revealed that after excluding 30% of the fastest-evolving sites, support for the Hodarchaeals-sister topology significantly decreased, while support for the Heimdallarchaeia-sister topology began to rise. However, further site removal resulted in fluctuations between these two topologies, likely suggesting an intricate interplay between phylogenetic signals and fast-evolving sites.

By comparison, phylogenomic analyses of site-exclusion subsets of the NM57 dataset using an expanded Asgard archaea sampling (411 Asgard archaea) consistently supported the Heimdallarchaeia-sister topology (Fig. 3c in our study) after removing the fastest-evolving sites. This finding suggests that the dataset with expanded Asgard

archaeal sampling may retain stronger phylogenetic signals, thereby improving the resolution of the evolutionary relationship between eukaryotes and Asgard archaea. Moreover, in Eme et al.'s study, the NM-A175 dataset—already limited in phylogenetic information—was analyzed using a combination of SR4-recoding and fast-evolving site removal, which may have led to the loss of genuine phylogenetic signals supporting the placement of eukaryotes. A key indication of this issue appears in Supplementary Figure 24 of Eme et al.' study, where support for the Hodarchaeales-sister topology was highly unstable and sharply declined after the removal of 40% of the fastest-evolving sites, with fluctuations even before this threshold was reached). In this context, the complex sequence composition in Njordarchaeales genomes may have resulted in a transient clustering of eukaryotes with Hodarchaeales.

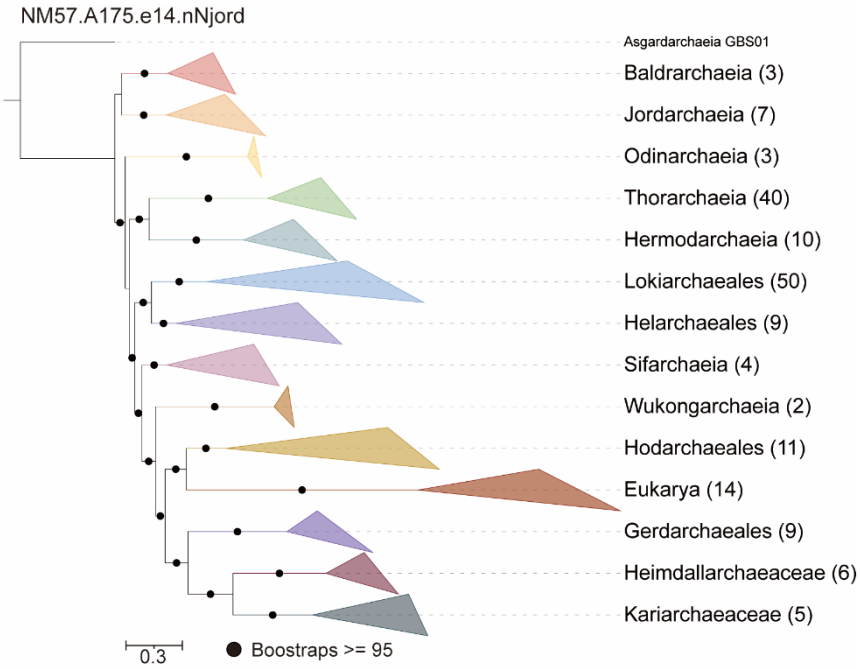


Fig. 1R2.The maximum likelihood phylogenomic analysis based on the NM-A175 dataset (including 175 Asgard archaea), with exclusion of Njordarchaeales and the outgroup, using IQ-TREE under the LG+C60+F+G+PMSF model. Bootstrap support values ≥ 95% are represented by black dots. The scale bar denotes the average expected number of substitutions per site.

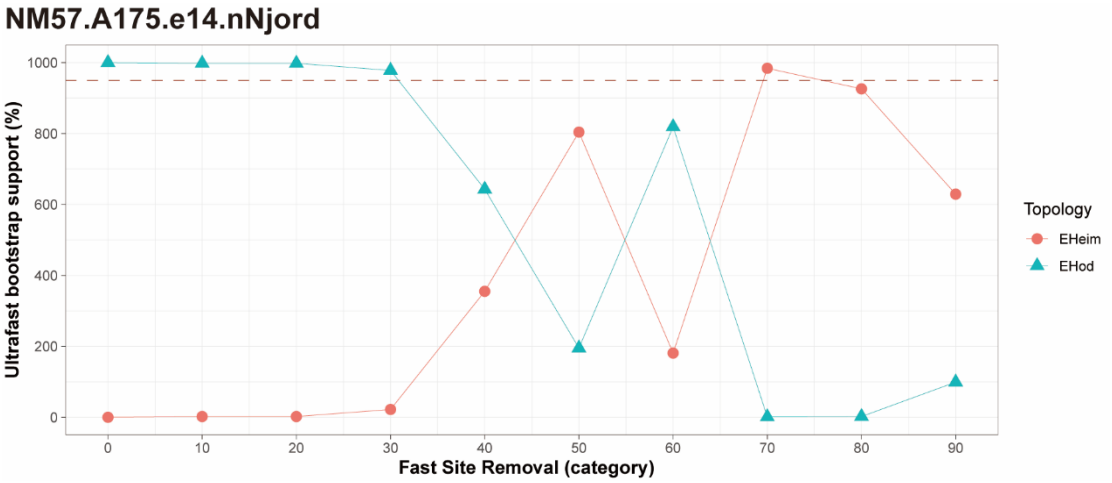


Fig. 2R2. Evolution of ultrafast bootstrap support for the monophyly of either eukaryotes and Heimdallarchaeia (EHeim, red line) or the monophyly of eukaryotes and Hodarchaeales (EHod, blue line), in the phylogenies inferred from the NM-A175 dataset (including 175 Asgard archaea), with exclusion of Njordarchaeales and the outgroup, as the fastest-evolving sites were progressively removed. The tree was inferred using IQ-TREE under the LG + C60 + F + G + PMSF model.

3. In the rebuttal the authors indicate correctly that ribosomal proteins are much less prone to horizontal gene transfer and other non-vertical processes than enzymes. And that this is an argument for one marker set over another. I would caution to go overboard in this line of thinking for two reasons. First the marker selection and inspection of individual trees that the authors perform should supersede (or could contradict) such general statements. Second, it is precisely the ribosomal proteins that give this problematically long branch at the base of eukaryotes that is so difficult for phylogenetic reconstructions to accommodate. And attempts at finding other markers that are vertical (within the part of the ToL here concerned with) but do not show this extremely long branch should be explored and at least somewhat appreciated rather than so easily dismissed.

**Response:** We concurred with your perspective that selecting markers with stronger vertical evolutionary signals and validating them through single-gene trees is essential

for mitigating artifacts caused by long-branch attraction in phylogenetic reconstruction. We therefore appreciate the efforts of Eme et al. (2023) and Petitjean et al. (2015, Mol. Biol. Evol. 32, 1242–1254) in moving beyond ribosomal proteins and exploring the use of enzymes for phylogenetic tree reconstruction. In light of this, we have revised our statement in the Supplementary Discussion—originally adapted from the literature—which suggested that non-ribosomal markers exhibit higher frequencies of horizontal gene transfer and mixed paralogs than ribosomal proteins. For further details, please refer to the last paragraph of the second section in the Supplementary Discussion.

**Referee #2 (Remarks to the Author):**

The revised version of the manuscript is very much improved and the authors addressed all of mine and the other reviewers comments. In the manuscript, besides generating 223 nearly complete metagenome-assembled genomes (MAGs) of Asgard archaea (16 previously unknown lineages) the authors show the chimeric nature of Njordarchaeales assemblies. By excluding problematic datasets and performing careful phylogenomic reconstructions, the authors concluded the ancestry of eukaryotes prior to the diversification of Heimdallarchaeia lineages sampled so far. By ancestral state reconstruction and molecular dating, the authors proposed that the last common ancestor of Asgard archaea and eukaryotes likely existed before the Great Oxidation Event (~2.3 billion years ago) and was an anaerobic, hydrogen-dependent acetogen. The major improvement of the current version is the improved metabolic reconstructions and I think, the authors do not need to perform any additional analyses to support their conclusions.

Minor:

Line 128 -130 – at least Ca. Lossiferum has a closed genome, so does it make sense to talk about completeness? Not all gene markers are present in all lineages…

**Response:** Here, we simply aim to reflect the overall quality of all MAGs we used, demonstrating that the vast majority are high-quality genomes.

**Referee #3 (Remarks to the Author):**

The manuscript by Zhang et al. “Phylogenomic analyses with expanded taxon sampling…” has been extensively revised and is significantly improved with respect to all criticisms of referees in the first round. The amount of new data for asgard related lineages remains an undeniable strength of the paper. Everyone will want to be working with that data moving forward. The phylogenetic analyses have been flanked by new experiments that undescore the problems of earlier reports, problem which were identified by the authors in the first version. The case for the assembly-artefact nature of the Njordarchaeales has been strengthened. The nickel dependence narrative has been removed, increasing the focus on the phylogenetic findings. The impact of these data on the new placement of the eukaryote host lineage within archaeal diversification adds significantly to developments in the field, as do the metabolic reconstructions, which now clearly point to a chemolithoautotrophic ancestry of the host lineage at eukaryote origin. These are findings about which all biologists need to know, and they shed vast amounts of new light on the humble symbiotic beginnings of our own eukaryotic lineage.

Some minor corrections remain, listed in order of reading, most are editorial. The line numbers correspond to the numbering in the manuscript version with changes tracked.

Line 42. Delete “could”. The analyses show that this is what occurs.

**Response:** Out of cautious consideration, we did not remove "could".

Line 113: “which is significantly distinct” —> which significantly differ

**Response:** Corrected. See line 102.

Line 143: “it is vital to unveil…” —> it is essential to identify the factors underlying the conflicting…

**Response:** Corrected. See line 144-145.

Line 158: “not necessarily related to” —> not caused by

**Response:** Out of cautious consideration, this sentence has not been modified.

Line 159: “the number” —> the number and nature

**Response:** Corrected. See line 160-161.

Line 165. Delete “aforesaid”. The context is clear.

**Response:** Corrected. See line 167.

Line 192: “outputted” —> generated

**Response:** Corrected. See line 193.

Line 198: “owing to” —> stemming from

**Response:** Corrected. See line 199.

Line 200: “archaea.” —> archaea, specifically into the Nordarchaeales.

**Response:** Corrected. See line 201.

Line 210: delete “may”. This is what the results indicate.

**Response:** Out of cautious consideration, we did not remove "may".

Line 211: “datasets, The NM57 dataset may harbour” —> datasets, the NM57 dataset harbours

**Response:** Out of cautious consideration, we did not remove "may".

Line 213: “are selected” —> were selected

**Response:** Corrected. See line 215.

Line 221: help to resolve the placement of eukaryotes among archaea. To this end, we added

**Response:** Corrected. See line 224-225.

Line 224: ML trees …. within the TACK superphylum

**Response:** Corrected. See line 226.

Line 229: mitigate the effect

**Response:** Corrected. See line 230.

Line 307: delete “significantly”. The 2.26 value overlaps with the GOE.

**Response:** Corrected. See line 307.

Line 309: documented —> reported

**Response:** Corrected. See line 309.

Line 320: a gradual increase in ancestral gene content was observed

**Response:** Corrected. See line 320-321.

Line 330: it was inferred

**Response:** Corrected. See line 329.

Line 344: “abundant CO2 and H2 in the Archean ocean41 would” …

Please add a reference because H2 in the atmosphere [41] is not important, it diffuses to space; in the ocean, H2 produced by serpentinization fuels deep sea microbial communities locally it its sites of synthesis. Adjust to: —> …abundant CO2 in the Archean ocean[41] and marine H2 from serpentinization [41a] would…

New reference, perhaps: [41a] Tamblyn, R and Hermann, J. 2023. Geological evidence for high H2 production from komatiites in the Archaean. Nature Geoscience 16: 1194–1199.

**Response:** Corrected. See line 343.

Line 370: Something is wrong here, ref 44 is not about Heimdahlarchaeia at all or inference of archaeal acetogens, it is about Ech. The only archaea that actually grow as acetogens are generated in the laboratory through extensive genetic manipulation [Schöne et al. (2022). Deconstructing Methanosarcina acetivorans into an acetogenic archaeon. PNAS 119, e2113853119.] Maybe ref 46 was meant here. Ref 44 would fit well on line 501.

**Response:** You are correct. This reference has been modified. See line 369.

Line 511: Ref 46 in this context should be replaced with the original reference: Martin W and Müller M. 1998. The hydrogen hypothesis for the first eukaryote. Nature 392: 37–41.

**Response:** Corrected. See line 375.

Line 561: “may represent chimeric assemblies” —> likely represent chimeric assemblies

**Response:** Corrected. See line 392.

Line 567: “eukaryote common ancestor was an anaerobic H2-dependent acetogen”

Perhaps it is adviseable to be more general —> “the host lineage at eukaryotic origin was an anaerobic, H2-dependent chemolithoautotroph” (this would include the possibility of acetogens)

**Response:** Thank you for the valuable suggestion. The sentence has been revised accordingly. See line 398-399.

Line 570: “will help to refining scenarios of eukaryogenesis” —> will help to refine evolutionary reconstructions [new reference] of eukaryogenesis. The new reference might be: Giger et. al. 2024. Inducing novel endosymbioses by implanting bacteria in fungi. Nature 635: 415–422. This would point to the new area of research where people are generating mitochondrion-like symbiotic relationships using technology.
**Response:** Corrected. See line 402.

This referee is satisfied that they have highlighted quality as a parameter of importance.The phylogenetic methods are sufficiently documented, the alignments are available on Figshare. Phylogeny buffs will think of a thousand new analyses that could be done with this data, and those analyses can be done by anyone who wants to — once these data are published. The authors are to be commended for hard work and straightforward phylogenetic reporting.

Overall the authors have undertaken earnest and effective efforts to improve the paper in the few areas needing attention. They present a very significant contribution to the topic of eukaryogenesis. These new genomes significantly sharpen the focus on which lineages are the sisters of eukaryotes and which genes were likely present in those genomes. The data fit well with current views about Earth history and move the field forward in many ways.