# Phylogenomic evidence for the Origin of Obligately Anaerobic Anammox Bacteria around the Great Oxidation Event

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**Abstract**

The anaerobic ammonium oxidation (anammox) bacteria could transform ammonium and nitrite to dinitrogen gas, and this obligate anaerobic process accounts for up to half of the global nitrogen loss for surface environments. Yet its origin and evolution, which may give important insights into the biogeochemistry of early Earth, remains enigmatic. Here, we performed comprehensive phylogenomic analysis and showed a single origin of anammox bacteria within the phylum Planctomycetes. After accommodating the uncertainties and factors influencing time estimates with different statistical methods, we estimated that anammox bacteria originated at around the so-called Great Oxidation Event (GOE; 2.32 to 2.5 billion years ago [Ga]) which is thought to have fundamentally changed global biogeochemical cycles. We further showed that during the origin of anammox bacteria, genes involved in oxidative stress, bioenergetics and anammox granules formation were recruited, which might have contributed to their survival on an increasingly oxic Earth. Our findings suggest the rising levels of atmospheric oxygen, which made nitrite increasingly available, was a potential driving force for the emergence of anammox bacteria. This is one of the first studies that link the GOE to the evolution of obligate anaerobic bacteria.

## Introduction

Anaerobic ammonium oxidation (anammox, $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$), which usually occurs in anoxic marine, freshwater and wetland settings, accounts for up to 50% of the removal of fixed nitrogen (N) in nature. Along with denitrification, it is recognized as an important biological process that leads to N loss from the environment [1,2]. In wastewater treatment, anammox is more cost-effective and environmentally friendlier than denitrification due to its lower oxygen requirement for aeration (nitrite [N(+III)], instead of nitrate [N(+V)] is sufficient for the anammox metabolism), carbon-free cultivation (some denitrifying bacteria are heterotrophic) and its negligible emissions of greenhouse gases like $N_2O$. Consequently, anammox bacteria, the organisms that perform anammox, have been widely used in wastewater treatment plants [2-5]. Despite the environmental and industrial importance of these organisms, their evolutionary history and antiquity are poorly known, which hinders accurate reconstructions of the biogeochemical nitrogen cycle over geologic time.

Previous studies investigated the roles of key genes driving anammox, including *hzsABC* (hydrazine synthase)[6], *hdh* (hydrazine dehydrogenase), *hao* (hydroxylamine oxidoreductase) [7] and *nxr* (nitrite oxidoreductase) [8]. In general, the vital enzymes encoded by these genes either are directly involved in anammox or participate in replenishing electrons to the cyclic electron flow [9,10]. Further, all known anammox bacteria are found within the phylum Planctomycetes [11]. Six candidate genera of anammox bacteria, namely '*Candidatus* Brocadia', '*Candidatus* Kuenenia',

'*Candidatus* Jettenia', '*Candidatus* Scalindua', '*Candidatus* Anammoximicrobium' and '*Candidatus* Anammoxoglobus', have been proposed based on 16S ribosomal RNA (rRNA) gene sequences [12], but none of them have been successfully isolated into pure cultures. The habitat of anammox bacteria requires the simultaneous presence of reduced (ammonia) and oxidized (nitrite) inorganic N compounds. Such habitats are often found at the aerobic-anaerobic interface in aquatic ecosystems, including the margins of oxygen minimum zones (OMZs) in the ocean and sediment-water interfaces, where ammonium originates from the anaerobic degradation of organic matter and nitrite can be produced by aerobic ammonia oxidation [13]. Ammonium, one of the two substrates of the anammox metabolism, was thermodynamically stable and likely present in moderate concentrations in the deep ocean throughout the Archean [14] and probably extending well into the Proterozoic [15]. The availability of nitrite, the second important substrate, is less certain. Geochemical data from sedimentary rocks suggest that the early Earth (before 3 Ga) was fully anoxic and deficient in aerobic ecosystems that were able to generate nitrite/nitrate [16-18]. The first transient and/or localized occurrences of aerobic nitrogen cycling appear in the Mid- to Neoarchean (2.7 Ga [19-22]), but evidence of widespread nitrate availability does not appear until around 2.4 billion years ago (Ga), the so-called Great Oxidation Event (GOE) [23-25]. The GOE marks the rise of free $O_2$ in the atmosphere above $10^{-5}$ times modern levels, and was ultimately a result of the emergence of oxygenic cyanobacteria [26]. Hence it is conceivable that the appearance of anammox was linked to the appearance of aerobic ecosystems around the time of the GOE.

However, it is also possible that some nitrite was provided much earlier, through lightning reactions in the Archean atmosphere [27]. Lightning, even in the absence of $O_2$, can generate NO gas, which dissolves in water and converts into aqueous species, including nitrite [28]. Some anammox bacteria are even capable of using NO rather than nitrite directly as a substrate [9]. If these organisms capitalized on the lightning flux, then anammox might long pre-date the GOE.

Several tools exist for investigating the link between the evolution of metabolic pathways and geo-environmental transformations. One way to explore the evolutionary history of a specific metabolic pathway is based on organic biomarkers, which, however, are often affected by the poor preservation over geologic timescales [29]. For instance, ladderanes, a type of lipids that is unique to anammox bacteria [30], are rarely preserved to a level that can be used to date their evolutionary origin. Another approach is based on nitrogen isotope ratios of sedimentary records [14,31,32]. However, the isotopic fractionation factors for different metabolic pathways overlap widely (denitrification: -5 to -30‰; anammox: -16 or -24‰ [15]), making it difficult to single out and elucidate the evolution of the different redox reactions within the N cycle by this method [33]. Alternatively, molecular dating, which estimates the age of the last common ancestor (LCA) of analyzed lineages by comparing their sequences based on the molecular clock theory [34], provides an alternative strategy to investigate this issue. Briefly, it can estimate the divergence timescale of organisms using genetic data while accounting for issues like uncertainties in the calibrations and different evolutionary rates among lineages, thereby bypassing the paucity and uncertainty of

biomarkers and other biogeochemical records. On the one hand, because anammox is an anaerobic reaction, it is intuitive to assume that anammox should originate before the GOE, perhaps using nitrite or NO provided by lightning reactions in the atmosphere [35]. On the other hand, it is because of GOE that $O_2$ on Earth rose permanently to a concentration that is biologically meaningful, and as a result, (micro-)environments were created in the surface ocean that were rich in nitrate/nitrite produced by aerobic organisms. These redox-stratified conditions may have stimulated the anammox metabolism by providing both nitrite and ammonium at the redoxcline. In this regard, it could be hypothesized that anammox did not arise until the GOE. Here, we ask the question when anammox originated. To answer this question and to obtain more insights into the evolution of anammox bacteria, we compiled an up-to-date genomic data set of Planctomycetes (see Supplementary Text section 1; Dataset S1.1), placed the evolution of anammox bacteria into the context of geological events, and investigated genomic changes associated with the origin of this ecologically important bacterial lineage.

## Results and discussion

<u>Monophyletic origins of anammox bacteria and anammox genes</u>

The anammox bacteria form a monophyletic group within the phylum Planctomycetes in a comprehensive phylogenomic tree with 881 Planctomycetes genomes (Fig. S1). The anammox bacteria clade in this phylogenomic tree (Fig. S1) comprises four known genera including the

early-branching *Ca.* Scalindua and *Ca.* Kuenenia, and the late-branching *Ca.* Jettenia and *Ca.* Brocadia. It also includes two understudied lineages which we named 'basal lineage' and '*hzsCBA*-less lineage' (Fig. S1), both of which have relatively low genome completeness compared to the other anammox bacteria and are represented by metagenome-assembled genomes (MAGs) sampled from groundwater (see Supplementary Text section 2.1). Note that the absence of *hzsCBA* in several genomes within the '*hzsCBA*-less lineage' might be ascribed to the loss of these genes in evolution or their low genome completeness (Fig. S1). Because of their shallow (late-branching) phylogenetic position, this uncertainty should not affect the inference of the origin of anammox bacteria. Two described genera (*Ca.* Anammoximicrobium and *Ca.* Anammoxoglobus) do not have genomes available by the time of the present study (last accessed: April 2021), and are therefore not included in the phylogenomic analysis. Furthermore, using the last updated set of 2,077 Planctomycetes genomes (retrieved in April 2021) from NCBI, we obtained a consistent topology of anammox bacteria (Fig. S2).

To check whether important groups, particularly early-branching lineages, have been encompassed in our analyzed genome data sets, we built a 16S rRNA gene tree (Fig. S3) using the identified 16S rRNA genes from genomic sequences of anammox bacteria and the deposited 16S rRNA gene amplicons in SILVA from the class Brocadiae, to which anammox bacteria belong (see Supplementary Texts section 2.2). Since the anammox bacteria with genome sequences in this 16S rRNA gene tree (Fig. S3) showed a branching order congruent with the

topology of the phylogenomic tree (Fig. S1), and since the 16S rRNA gene tree included 913 (clustered from 20,142) sequences sampled from a wide array of habitats (marine, sediments, man-made reactor, freshwater and terrestrial ecosystems), the 16S rRNA gene phylogeny, which could better capture the diversity of anammox bacteria by including uncultured samples, likely encompasses the early-branching lineages of anammox bacteria. Taken together, our genome set, which encompasses the genomes of the early-branching lineages, is appropriate to be used to study the evolutionary origin of anammox bacteria (Dataset S1.3). We further analyzed the evolution (Fig. S4) of the genes (*hzsCBA*) critical to the anammox reaction [7,36]. Reconciliation (see Supplementary Texts section 2.3) of the gene and the genome-based species phylogenies pointed to a single origin of anammox metabolism at the LCA of anammox bacteria (Fig. S4).

Taken together, the above analyses suggest that our genome data sets have encompassed the earliest-branching lineages and deep phylogenetic diversity of anammox bacteria, and indicate its monophyletic origin, which agrees with previous studies [37-39]. This should provide a foundation for estimating the origin of anammox metabolism by estimating the origin time of anammox bacteria.

<u>The evolutionary origin of anammox bacteria coincided with the rising $O_2$</u>

We employed MCMCTree to estimate the divergence times of the anammox bacteria with i) soft bounds (time constraint allowing a small probability of violation) based on cyanobacteria

fossils, and ii) the relaxed molecular clock approaches allowing substitution rates to vary among branches. Using a best-practice dating scheme (Fig. 1; see Supplementary Text section 3), we dated the origin of anammox bacteria to 2,117 million years ago (Ma) (95% highest posterior density [HPD] interval, 2,002 - 2,226 Ma). Further, we repeated the analysis based on the expanded Planctomycetes genomes (Genome set 2; Dataset S1.1) and the constraint topology inferred with a profile mixture model (LG+C60+G; Genome set 1; Dataset S1.1). In general, both analyses based on different genome sets resolved consistent topologies (Fig. S5) as shown in Figure 1. Overall, they gave similar posterior times of the LCA of the anammox bacteria at 2,105 Ma (95% HPD: 1,961 - 2,235 Ma) or 2,005 Ma (95% HPD: 1,869 - 2,146 Ma).

Analyses with different combinations of calibrations and parameters broadly converge to similar time estimates (Fig. S6; Dataset S2.1). Specifically, assigning a more ancient time constraint to the total group of oxygenic cyanobacteria, which is based on the biogeochemical evidence of the presence of $O_2$ at nearly 3.0 Ga [40] instead of GOE (Dataset S2.1), shifted the posterior dates of the LCA of anammox bacteria into the past by ~300 million years (Myr) (e.g., C1 versus C10; Fig. S6). Varying the maximum bounds of the root by using 4.5 Ga, 3.8 Ga or 3.5 Ga had a minor impact (around 100 Myr) on the time estimates of anammox bacteria (e.g., C1 versus C3; Fig. S6). Though the auto-correlated rates (AR) model was less favored than the independent rates (IR) model based on the model comparison (see Supplementary Text section 3.3; Dataset S2.2), it is worth noting that the posterior ages estimated by the AR model were 1-12%

younger than estimated by the IR model (Dataset S2.1). Besides, there remains a possibility that there are unsampled or even extinct lineages of anammox bacteria that diverged earlier than all anammox bacteria analyzed in the present study. This scenario, if true, hints that the first anammox bacterium could have originated before the occurrence of the LCA of sequenced anammox bacteria but later than their split from the sister non-anammox lineage (Node X in Fig. 1; up to 2,600 Ma). Accommodating the above uncertainties, our analyses suggest that the origin of anammox bacteria, and hence the origin of anammox, most likely falls into the 2.6-2.0 Ga interval, or more generally, around the early Proterozoic. Running MCMCTree analysis with no sequence data showed different distributions of time estimates for both the clade of anammox bacteria and the four calibration points (Fig. S7), suggesting that sequence data are informative for our molecular clock analysis [41]. Besides, we estimated similar origin times of anammox at around 2.1 Ga (the AR model) or 2.3 Ga (the IR model; see Fig. S8) with the complete taxon sampling shown in Fig. S2 (Genome set 2; see Dataset S1.1). For this analysis, we used a likelihood-based dating algorithm since the Bayesian molecular clock analysis is known to be computationally intensive and not suitable to take a data set with a broad taxon sampling [42]. Note that unlike Bayesian molecular dating software, the likelihood-based method we used only makes point estimations for the node ages (no standard deviation), thereby not allowing for fully appreciating the uncertainties in age estimates and any inference based on that. In general, the above result suggests that taxon sampling may not affect our dating analysis.

The geochemical context of the origin of anammox bacteria

We speculate that the timing of the origin of the anammox metabolism is linked to the increasing availability of nitrite in surface environments, because ammonium was likely present in the deep anoxic ocean throughout the Precambrian [14,15] and therefore presumably not a limiting substrate. The first abiotic source of nitrogen oxides on the early Earth, including NO and nitrite, would have been lightning reactions between $N_2$ and $CO_2$ in the Archean atmosphere [43,44]. A prior study suggested that this process led to micromolar levels of nitrite in seawater [43], and the supply of NO may have been even higher, considering that it is the initial reaction product of lighting [43,44]. This implies the possibility of an earlier origin of anammox bacteria. In fact, an intriguing hypothesis is that NO-dependent anammox [12] is ancestral to all anammox bacteria and arose before the appearance of significant nitrite levels with the GOE, perhaps driven by the lightning-source of NO. However, the original estimates of the lightning-derived flux of nitrogen oxides may have been based on perhaps unrealistically high amounts of $CO_2$ [45]. Furthermore, to our knowledge, there has been no isotopic evidence in the early Archean rock record prior to 3 Ga for the presence of a significant nitrite or nitrate reservoir in the early ocean [16-18], and it is relatively conceivable that any nitrogen oxides that were supplied to the ocean by lightning were rapidly reduced to ammonium or $N_2$ by ferrous iron, possibly even abiotically [46,47]. Hence, although a background lightning flux of nitrogen oxides almost certainly existed, to our

knowledge, there seems little evidence that it could supply a reliable metabolic substrate for early life. Furthermore, it is important to note that our phylogenetic results are not supportive of this hypothesis since the NO-utilizing anammox bacteria discovered to date are affiliated with phylogenetically shallow lineages from *Ca.* Kuenenia and *Ca.* Brocadia (Fig. 1). To our knowledge, there have been no studies reporting the ability of using NO as the sole electron acceptor in any other lineages of anammox bacteria. Hence, if NO-dependent anammox would have been present in the LCA of anammox bacteria, it was not clear why it was lost in all other anammox bacteria which resulted from multiple independent losses of the trait, a scenario apparently not favored from an evolutionary perspective (as shown by the arrows in Fig. S2). There are also anammox bacteria that are capable of using hydroxylamine as an oxidant; however, this molecule is a highly reactive compound and is usually considered as an intermediate in the nitrogen cycle, thereby rendering it a less important compound from the perspective of geological timescales. We are not aware of any non-biological sources of hydroxylamine. Taken together, our results are inconsistent with an Archean emergence of the anammox metabolism driven by lightning-derived nitrogen oxides. Instead, our results are most parsimoniously explained by the appearance of more significant nitrite/nitrate reservoirs around the time of the GOE.

The first transient appearances of nitrite/nitrate-dependent metabolisms are captured by the sedimentary nitrogen isotope record in late Mesoarchean soils at 3.2 Ga [20] and in Neoarchean

shallow-marine settings at 2.7 Ga and 2.5 Ga [19,21,22]. These observations may reflect local and/or temporally restricted oxygen oases [48,49]. Widespread nitrite/nitrate availability is inferred for the Paleoproterozoic (2.4-1.8 Ga), i.e., in the immediate aftermath of the GOE [23-25]. Importantly, $O_2$ is necessary, although a trace amount is feasible, to extant nitrifying organisms for the oxidation of ammonium to nitrite and nitrate [50-52]. We noticed that a recent study [53] reports the ability of the model ammonia-oxidizing archaeal species (*Nitrosopumilus maritimus*) to continue ammonia oxidation after consuming all supplied $O_2$ by nitric oxide disproportionation to generate $O_2$, but the underlying genetic pathway is not known, making it difficult to extrapolate this mechanism to any other ammonia-oxidizing organisms. Hence the rise of oxygen almost certainly triggered the growth of the nitrite/nitrate reservoir, and therefore potentially provided one of the key substrates for anammox bacteria. We note that also NO would have become more abundant after the GOE, because it is an intermediate product in nitrification and denitrification [54]. This would put the possibility of NO-driven anammox back on the table. However, NO levels in seawater are highly variable, and so nitrite would likely have been a more reliable substrate. In any case, it would not violate our conclusion that the appearance of anammox in the Paleoproterozoic was linked to the GOE and directly or indirectly to the rise of marine nitrite. It has been shown that a low concentration of nitrite significantly decreases the rate of N removal by anammox bacteria in reactors [55,56]. In modern OMZ in the Bay of Bengal, which may to some extent serve as analogues for the Paleoproterozoic redox-stratified ocean, the concentration of nitrite, instead of

ammonium, was proposed as the rate-limiting factor to the anammox metabolism [57]. These imply that the rise of nitrite, which is driven by the rise of $O_2$, could have facilitated the appearance of anammox bacteria in the early Proterozoic.

In modern environments, the majority of nitrite used in anammox is derived from nitrate reduction or aerobic ammonia oxidation [58], the latter performed by ammonia-oxidizing archaea (AOA) or bacteria (AOB). As noted above, nitrification could have appeared by at least 2.7 Ga, as indicated by nitrogen-isotope evidence for local accumulation of nitrate in surface ocean waters [15,59,60]. A recent study inferred that the LCA of AOA dates back to ~2.3 Ga and first appeared on land, driven by increasing $O_2$ concentrations in the atmosphere at that time, and that the expansion of AOA from land to the ocean did not occur until nearly 1.0 Ga [61]. If true, this hints at a dominant role of AOB in early ocean, before 2.3 Ga. Consistent with this idea, the vast majority of the early-branching lineages of anammox bacteria (*Ca.* Scalindua) and the sister Planctomycetes lineages of the anammox clade in the phylogenomic tree (Fig. 1) are found in marine environments. Furthermore, in the 16S rRNA gene tree which represents a greater diversity than the phylogenomic tree, marine lineages still account for the majority of the earliest-branching lineages of anammox bacteria (Fig. S3). Thus, it seems likely that the LCA of anammox bacteria originated in the marine realm where AOB thrived first. The above arguments, although speculative, tentatively suggest that the nitrite demand for anammox had been readily met by GOE by taking advantage of the significant amount of $O_2$ newly available, which is

broadly consistent with our molecular dating results suggesting an origin of anammox shortly before or after the GOE.

Genomic changes potentially related to anammox metabolism and habitat adaptation upon the origin of anammox bacteria

We further explored the genomic changes characterizing the origin of anammox bacteria (see Supplementary Text section 4). This includes the gains of the aforementioned genes that directly participate in anammox, viz., *hzsABC*, *hdh*, and *hao*. The current view of the anammox metabolism posits that electrons consumed by anammox for carbon fixation are replenished by the oxidation of nitrite to nitrate by nitrite oxidoreductase (NXR) [10], which was inferred to be acquired upon the origin of anammox bacteria (Fig. 2). Moreover, multiple auxiliary genes for N assimilatory pathways including N regulatory protein (*glnB*), assimilatory nitrite reductase large subunit (*nirB*), transporters like *amt* (ammonium), *nirC* (nitrite) and NRT family proteins (nitrate/nitrite), were also gained at the origin of anammox bacteria (Fig. 2). However, genes for nitrogen-related dissimilatory pathways encoding nitrite reduction to nitric oxide (*nirK* or *nirS*) and to ammonium (*nrfAH*), respectively, were likely acquired after the origin of anammox bacteria (Fig. 2). Anammox occurs at the membrane of anammoxosome, an organelle predominantly composed of a special type of lipid called ladderane. The ladderane membranes show low proton permeability, which helps maintain the proton motive force during the

anammox metabolism [30]. Although the biosynthetic pathway of ladderane is yet to be characterized, a previous study [62] predicted 34 candidate genes responsible for the synthesis of ladderane lipids, four of which were potentially acquired during the origin of anammox bacteria (see Supplementary Text section 4.2; Fig. 2), hinting at their potential roles in ladderane synthesis and providing clues to future experimental investigations.

Anammox bacteria occur in anoxic habitats and exhibit low oxygen tolerance [12]. Specifically, the $O_2$-sensitive intermediates generated by anammox, e.g., hydrazine, a powerful reductant, requires anoxic conditions. Accordingly, the likely acquired peroxidases include cytochrome c peroxidase (Ccp), which could scavenge hydrogen peroxide in the periplasm [63], and the most prevalent peroxidase [64], thioredoxin-dependent peroxiredoxin (AhpC). Besides peroxide scavengers, the desulfoferrodoxin (Dfx), which functions as superoxide reductase (SOR) to reduce superoxide to hydrogen peroxide and which is broadly distributed among anaerobic bacteria [64], were likely acquired during the origin of anammox bacteria (Fig. 2). Another acquired gene is *fprA*, which encodes flavo-diiron proteins that scavenge $O_2$.

Investigating other metabolisms that were also acquired upon the origin of anammox bacteria allows reconstructing the coeval ecology. Our data show that *sat* (Fig. 2), which encodes sulfate adenylyltransferase for assimilatory incorporation of sulfate into bioavailable adenylyl sulfate, as well as *aprAB* and *fsr* (Fig. 2) for encoding dissimilatory sulfite reductase, were acquired upon the origin of anammox bacteria (Fig. 2). This acquired capability of sulfate

metabolism is coincident with the increased concentrations of seawater sulfate from ~100 μM or less throughout much of the Archean [65,66] to over 1mM after the rise of atmospheric $O_2$ [67,68]. Unlike other Planctomycetes, anammox bacteria are generally autotrophs that use the Wood-Ljungdahl pathway for carbon assimilation [69]. Consequently, they potentially lost many genes involved in carbohydrate utilization (Fig. 2). The metabolic loss is further strengthened by the enrichment analysis where the genes predicted to be lost by the LCA of anammox bacteria are enriched in pathways involving hydrolase activity, intramolecular oxidoreductase activity and carbohydrate kinase activity (Fig. 2; see Data and Code availability), hinting at a decreased ability of anammox bacteria to degrade organics.

Additionally, iron is a vital element for HZS [6] and HDH [70], and *in vitro* studies revealed that increased iron concentrations can promote the growth of anammox bacteria [71]. The ancient oceans are thought to have been ferruginous (high iron availability) according to sedimentary records of iron speciation across several coeval marine settings from the Archean through most of the Proterozoic [72], meaning that large amounts of soluble iron would have been available for anammox bacteria. Interestingly, a series of iron-related genes to make use of the abundant iron from the environment were likely acquired upon the origin of anammox bacteria. First, anammox bacteria likely acquired the gene *fur*, which encodes a ferric uptake regulator for iron assimilation. Moreover, the iron-containing proteins, specifically cytochrome, are encoded by the acquired genes *petBC*, which potentially encodes Rieske-heme b [10], and *CcsAB*, which encodes a

protein involved in cytochrome c maturation systems (Fig. 2). Notably, *ahbABCD*, which encodes proteins-synthesizing *b*-type hemes in anammox bacteria [73], were likely acquired before the origin of anammox bacteria (Fig. 2). Furthermore, we identified that the gene *Bfr* that encodes the oligomeric protein, bacterioferritin involved in the uptake and storage of iron [74] was likely acquired during the origin of anammox bacteria (Fig. 2), which may help to hoard iron in settings where iron became locally sparse, such as along euxinic (sulfide-rich) or oxic marine margins in the Paleoproterozoic [75]. A recent study highlights the vital role of bacterioferritin to the anammox regulation [76]. It is thought that the appearance of euxinic margins around the time of the GOE trapped iron that upwelled from the deep ocean, leading to the demise of iron oxide deposits (i.e., banded iron formations) [77]. Our results tentatively support this model, if the microenvironments that those primordial anammox bacteria colonized were located near the euxinic-oxic interface where nitrite and ammonium were available while upwelled iron was titrated out of the water column by freely dissolved hydrogen sulfide.

Taken together, the timelines and corresponding genomic changes of anammox bacteria provide implications for the physiological characteristics of descendant anammox bacteria and the origin of other nitrogen-transforming pathways in the context of an estimated evolutionary timeline of anammox bacteria. For example, the earlier branching lineage *Ca.* Scalindua exhibit high nitrite affinity (0.45 μM) compared to other anammox genera (up to 370 μM) [12,78], which may reflect a progressive growth of the marine nitrite reservoir, linked to the protracted

oxygenation of the surface environments over geologic timescales [26]. Likewise, genomic data reveal that also canonical denitrification radiated across the tree of life after the GOE [79], and the gains (Fig. S9; see Supplementary Text section 4.4) of genes *nrfAH* for dissimilatory nitrate reduction to ammonium (DNRA) at the late-branching lineages (*Ca.* Brocadia and *Ca.* Kuenenia) at around 800 Ma are consistent with the time when the nitrite/nitrate availability was increased by the Neoproterozoic Oxygenation Event [80]. The time estimate and genomic changes of the anammox bacteria have gone some way towards enhancing the understanding of the physiological characteristics of modern anammox bacteria and the historical N cycle.

## Caveats and concluding remarks

In the present study, we link an estimated evolutionary timeline of anammox and geological context of early Earth on a simplified view that nitrite-dependent anammox is ancestral to anammox bacteria. However, there are several limitations. First, dating the bacterial evolution has many challenges, which, among others, include the paucity of appropriate fossils, and a long evolutionary distance between anammox bacteria and cyanobacteria [81]. Hence, the date estimates should be read as potential ranges of time (indicated by the 95% HPD interval), instead of accurate time points, under the molecular clock model. Moreover, it is worth pointing out the metabolism called feammox, a process where ferric instead of nitrite is taken as the electron acceptor for ammonia oxidation. Both

feammox and anammox can anaerobically convert ammonia into dinitrogen [82], but the former process is supposed to be carried out by Acidimicrobiaceae from the Actinobacteria phylum[83,84] and under anaerobic environments without the presence of anammox bacteria [85]. Likewise, a recent experimental study [86] proposed extant anammox bacteria as electroactive organisms and suggested the feasibility to utilize graphene oxides and man-made metallic electrodes mimicking metal oxides as the electron acceptor. However, the lack of detailed molecular mechanism hampers the inference of its evolutionary history. In any case, metal oxides such as $Fe_2O_3$ or $MnO_2$ also became more abundant in the Neoarchean to Paleoproterozoic, i.e. around the time of the GOE [77,87], and hence even if these oxides were used as substrates for anammox, it would not violate our overall conclusion that the GOE played a primary role in driving the evolution of this metabolic pathway. However, it is also worth noting that metal oxides are solids and thus require extra-cellular electron transfer [86]. An entirely intra-cellular metabolism, using dissolved nitrite, may be more likely to evolve first, especially given that nitrate became bioavailable in the ocean around the same time (see above).

Another important aspect to highlight about our approach is that, our analysis began with inferring the LCA of anammox bacteria based on the presence of *hzsCBA*, a common strategy in phylogenetic studies. It is important to note that such an enzyme complex encoded by multiple genes might originate in different genomic backgrounds and were presented together in a suitable genomic background later in evolution. This scenario, if true, indicates that the appearance of

any single subunit of *hzsCBA* genes, which could not assembly a functional hydrazine synthase, should predate the LCA of anammox bacteria. Nevertheless, the alternative hypothesis would not affect our inference and following analysis since we only focus on the inferred ancestor of extant anammox bacteria, a strategy commonly used in modern phylogenetic analysis. These alternative possibilities, although not affecting the molecular clock analysis, should be carefully considered when making inference based on our estimated evolutionary timeline of anammox bacteria.

Here, using molecular dating and comparative genomics approaches, we link the emergence of an obligate anaerobic bacterial group, which drives the loss of fixed N in many environments, to the rise of $O_2$, and highlight their evolutionary responses to major environmental disturbances. Apparently, the GOE opened novel niches for the origin and subsequent expansion of diverse aerobic prokaryotes such as AOA and AOB, which in turn facilitated the origin of other bacterial lineages, including some anaerobes like the anammox bacteria, by providing the resources for their energy conservation. Our results open up the possibility that a significant proportion of the Proterozoic nitrite budget was consumed by anammox bacteria, and that the sedimentary nitrogen isotope record might be influenced by their activity. Our findings may have implications for Proterozoic climate, because $N_2O$ has previously been invoked as an important greenhouse gas at that time [88], but a relatively higher contribution of anammox bacteria to the marine nitrogen cycle may have hampered $N_2O$ production [3]. Lastly, our study suggests that the impacts of the GOE go well beyond aerobic

prokaryotes. We therefore conclude that molecular dating is a likely feasible approach to complement isotopic evidence for resolving the timeline of biological evolution and for providing additional constraints on climate models of the distant past.

## Materials and Methods

Overall, we compiled two genome sets and one 16S rRNA gene set in our study (Dataset S1.1). A phylogenomic tree (Fig. S1) was generated based on the concatenated alignment of 120 ubiquitous proteins (bac120; Dataset S1.3) proposed for tree inference by the Genome Taxonomy Database [89]. All published genomic sequences (952 in total) affiliated with the phylum Planctomycetes in NCBI Genbank up to December 2019 were retrieved (Dataset S1.2). To further examine whether the patterns obtained with this Planctomycetes dataset hold the same, we further constructed an expanded set of Planctomycetes genomes by retrieving a total of 2,077 Planctomycetes genomes released by April 2021 at Genbank (Fig. S2; see Data and Code availability). Key anammox genes *hzsCBA* and *hdh* were identified against manually curated reference sequences by BLASTP. For each gene, identified protein sequences were aligned using MAFFT (v7.222) [90] and the alignments were refined by trimAl (v1.4) [91]. All phylogenies were constructed by IQ-tree (v1.6.2) [92] with substitution models automatically selected by ModelFinder [93] and branch support assessed with 1,000 ultrafast bootstrap replicates. Note that the constraint topologies for dating analysis (Fig. 1; Fig. S5) were inferred with the profile

mixture model (LG+C60+G) which better accommodates across-site heterogeneity in deep-time evolution. Following manual curation, four well-recognized genera and two separate lineages of anammox bacteria were highlighted with different colours, and the presence of key genes were annotated with filled symbols beside the labels (Fig. S1). Furthermore, the phylogenomic tree was generated with similar methods using 16S rRNA genes identified from downloaded genomes and those retrieved from the SILVA database (Fig. S3; Dataset S1.3). All trees (including phylogenomic and 16S trees) in our study were visualized with iTOL [94]. More details are shown in supplementary text, section 2.

Molecular dating analysis was carried out using the program MCMCTree from the PAML package (4.9j) [95]. In our study, 13 calibration sets constructed with different time constraints of the root and three calibration nodes within the cyanobacteria lineage were used (see Supplemental Texts section 3.2). The topology constraint for dating analysis was generated using 85 genomes, which were sampled from the phylogenomic tree of phylum Planctomycetes (see Supplemental Texts section 3.1), and the model LG+C20+F+G under posterior mean site frequency (PMSF) approximation [96]. To perform dating analysis, clock models and different calibration sets were compared in 26 schemes (see Supplemental Texts section 3; Dataset S2.1) [97,98]. For each scheme, the approximate likelihood method [99] of MCMCTree were conducted in duplicate with identical iteration parameters (burn-in: 10,000; sample frequency: 20; number of sample: 20,000). The convergence of each scheme was evaluated by comparing the posterior

dates of two independent runs (see Data and Code availability). With the updated genome set 2 (Dataset S1.1), we repeated the taxon sampling process and generated an alternative phylogeny for dating analysis (Fig. S5) using LG+C20+F+G model (C20: 20 classes of site-specific amino acid profiles) under PMSF approximations. Similarly, we also generated another phylogeny for dating analysis using the same genome set as used in Fig. 1 but with the profile mixture model (LG+C60+G) which uses 60 classes of amino acid profiles [100]. Note that the missing parameter +F would not significantly change the results since it only adds another profile amino acid calculated from the original data. These two repeat dating analyses were conducted with the best practice dating scheme (IR model and calibration C1). Moreover, we performed likelihood based dating analyses [101] (Fig. S8) using the phylogenomic tree (Fig. S2) and the calibration C1 (see Data and Code availability) implemented in ape [42].

The phylogenetic differences between gene trees and the species tree were reconciliated by GeneRax [102] with unrooted gene tree as input and automatically optimized duplication, transfer, loss (DTL) rates. For each gene identified from the genome set 2 (Dataset S1.1), we used a species tree comprised by all anammox bacteria pruned from the phylogenomic tree (Fig. S2) as the reference. We used recommended parameters including SPR strategy, undated DTL model and a maximum radius of five.

For comparative genomics analysis, the protein-coding sequences were annotated against KEGG [103], CDD [104], InterPro [105], Pfam [106], TIGRFAM [107] and TCDB [108], individually (see

Supplemental Texts section 4). Following annotations, the potentially gained/lost genes between groups (anammox bacteria versus non-anammox bacteria; an anammox bacterial genus versus all other anammox bacteria) were statistically tested using two-sided fisher's exact tests. Finally, the resulting *p*-values were corrected with the Benjamini-Hochberg FDR (false discovery rate) procedure. Moreover, genes with corrected *p*-values smaller than 0.05 and with larger ratio in the study group were defined as gained genes, and genes with corrected *p*-values smaller than 0.05 and with smaller ratio in the study group were defined as potentially lost genes. Those potentially gained/lost genes were summarized for their detailed functions (see Data and Code availability).

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**Data and Code availability**

All used genomic sequences, generated phylogenetic trees, estimated divergence times and the used python codes are deposited in the online repository https://github.com/luolab-cuhk/anammox-origin.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Figure legends

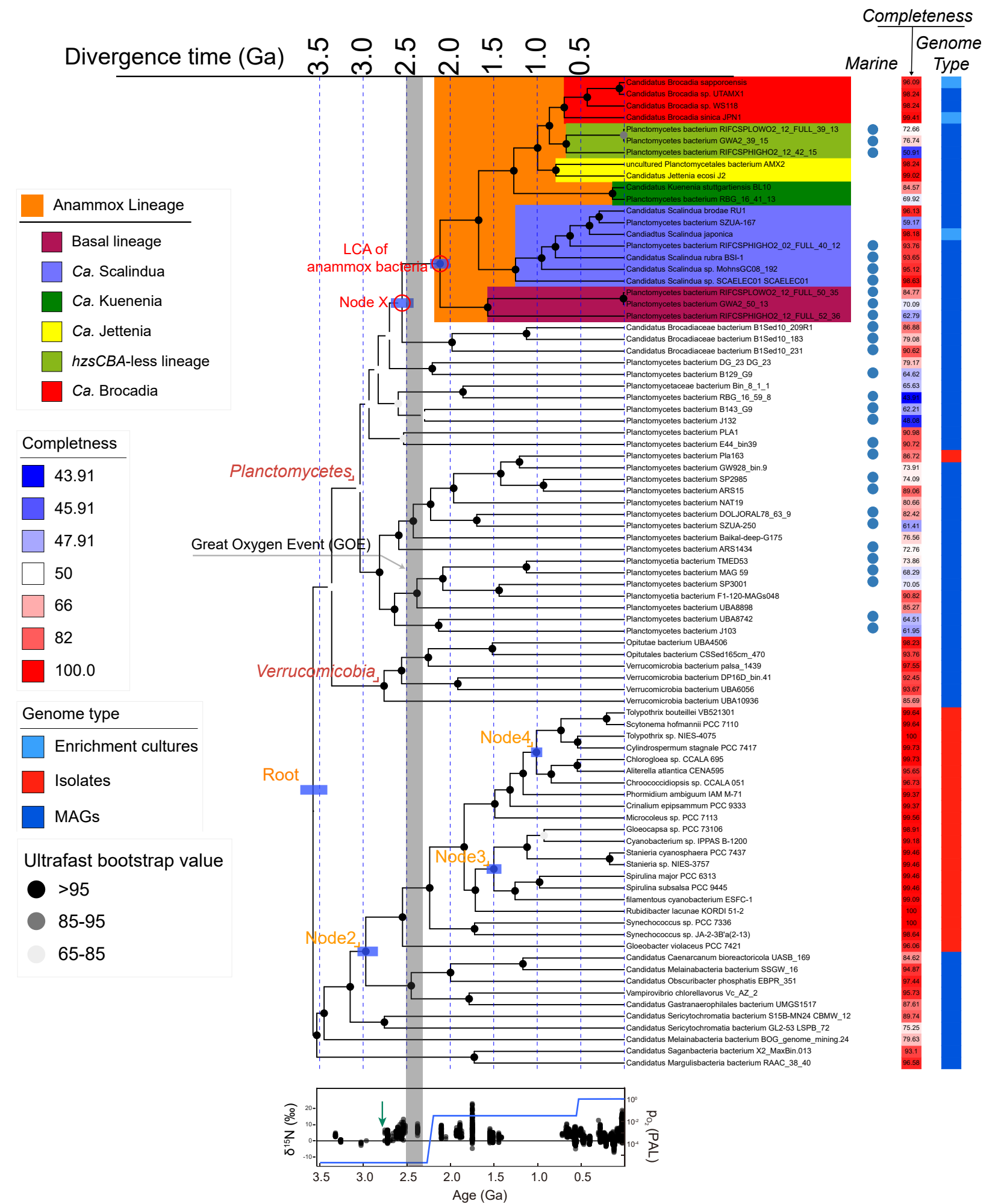
**Figure 1.** The evolutionary timeline of anammox bacteria using MCMCTree. The chronogram was estimated based on the calibration set C1 (see Supplementary Text section 3) and sequence alignments of the 25 orthologs conserved in bacteria (20040 sites). The blue bars on the four calibration nodes and the last common ancestor (LCA) of anammox bacteria represent the posterior 95% highest probability density (HPD) interval of the posterior time estimates. These alternative calibration sets were selected by choosing the three that accommodate different calibration constraints (see Supplementary Texts section 3.3). More alternative time estimates are provided in Figure S4. The vertical grey bar represents the period of the GOE from 2,500 to 2,320 Ma. The calibration constraints used within the phylum Cyanobacteria are marked with orange texts: the LCA of Planctomycetes and Cyanobacteria (Root), the total group of oxygenic Cyanobacteria (Node 2), the total group of *Nostocales* (Node 3), and the total group of *Pleurocapsales* (Node 4). The genome completeness estimated by checkM is visualized with a gradient color strip. The right next color strip indicates the genome type of genomic sequences used in our study including metagenome-assembled genomes (MAGs) and whole-genome sequencing (WGS) from either enriched culture sample or isolate. The diagram below the dated tree illustrates the change of atmospheric partial pressure of $O_2$ ($P_{O_2}$) and nitrogen isotope fractionations. The blue line shows the proposed model according to Lyons, et al. [26]. The green arrow suggests the earliest evidence for aerobic nitrogen cycling at around 2.7 Ga. PAL on right

axis means $P_{O_2}$ relative to the present atmospheric level. The black dots denote the change of nitrogen $\delta^{15}N$ isotope values according to Kipp, et al. [25].

**Figure 2**. The phyletic pattern of ecologically relevant genes in the comparison between anammox bacteria and non-anammox bacteria. Solid circles at the nodes indicate the ultrafast bootstrap values in 1,000 bootstrapped replicates. Note that copy number difference is not indicated since most genes displayed here are present as a single copy in genome except a few exceptions like *hao* (see Dataset S1.5 for the table summarizing the copy number of each gene). The phylogenomic tree on the left was constructed with 887 genomic sequences described in Supplementary Texts section 2.1. Those Planctomycetes genomes not used for comparative genomics analyses are collapsed into grey triangles, and the numbers of collapsed genomes are labelled next to the triangles. The target group and reference group for comparative genomic analysis are within an orange or blue box, separately. For each genome, the genome completeness estimated by CheckM is visualized with a color strip and labelled besides leaf nodes. The right next color strip represents the type of genomic sequences used in our study including metagenome-assembled genomes (MAGs) and whole-genome sequencing (WGS) from either enriched culture sample or isolate. The filled and empty circles, respectively, represent the presence and absence of particular genes in corresponding genomes. For gene clusters, only genomes with at least half of the members of the gene cluster are indicated by a

filled circle. The classifications of annotated genes are labelled above the gene names. *hzsCBA*, hydrazine synthase subunit C, B and A; *hdh*, hydrazine dehydrogenase; *hao*, hydroxylamine dehydrogenase; *nxrAB*, nitrite oxidoreductase subunit A and B; *nirK*, copper-containing and NO-forming nitrite reductase; *nirS*, cytochrome NO-forming nitrite reductase; *nrfAH*, ammonia-forming nitrite reductase subunit A and H; *glnB*, nitrogen regulatory protein P-II; *nirC*, nitrite transporter; NRT, nitrate/nitrite transporter; *amt*, ammonium transporter; kuste2805, 3603, 3605-3606, proposed genes relative to the synthetic pathways for ladderane at Rattray, et al. [62]; *cbiG*, cobalt-precorrin 5A hydrolase; *cbiD*, cobalt-precorrin-5B(C1)-methyltransferase; *cbiOMQ*, cobalt/nickel transport system; *AhpC*, peroxiredoxin; *Ccp*, cytochrome *c* peroxidase; *dfx*, superoxide reductase; *fprA*, $H_2O$-forming enzyme flavoprotein; *CcsAB*, cytochrome c maturation systems; *petB*, Cytochrome b subunit of the bc complex; *petC*, Rieske Fe-S protein; *ahbABCD*, heme biosynthesis; *sat*, sulfate adenylyltransferase; *aprAB*, adenylylsulfate reductase, subunit A and B; *fsr*, sulfite reductase (coenzyme F420); *higB-1*, toxin; *higA-1*, antitoxin; *mnhABCDEG*, multicomponent $Na^+/H^+$ antiporter; *fdhAB*, formate dehydrogenase subunit A and B; *nuo*(A-N), NADH-quinone oxidoreductase; *ndh*(A-N), NAD(P)H-quinone oxidoreductase; *nqrABCDEF*, $Na^+$-transporting NADH:ubiquinone oxidoreductase; *rnfABCDEG*, $Na^+$-translocating ferredoxin:$NAD^+$ oxidoreductase; *atp*(A-H), F-type $H^+$-transporting ATPase; *lacAZ*, beta-galactosidase; *melA*, galA, alpha-galactosidase; *ebgA*, beta-galactosidase; *galK*, galactokinase; , fructokinase; *fruK*, 1-phosphofructokinase; *rhaB*, rhamnulokinase; rbsK,

ribokinase; *araB*, L-ribulokinase; *xylB*, xylulokinase; *kdgK*, 2-dehydro-3-deoxygluconokinase; GALK2, N-acetylgalactosamine kinase; *cah*, cephalosporin-C deacetylase; *argE*, acetylornithine deacetylase; *nagA*; N-acetylglucosamine-6-phosphate deacetylase; *HDAC11*, histone deacetylase 11.


Divergence time (Ga)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Completeness
Marine
Genome Type
Anammox Lineage
Basal lineage
Ca. Scalindua
Ca. Kuenenia
Ca. Jettenia
hzsCBA-less lineage
Ca. Brocadia
Completness
43.91
45.91
47.91
50
66
82
100.0
Genome type
Enrichment cultures
Isolates
MAGs
Ultrafast bootstrap value
>95
85-95
65-85
LCA of anammox bacteria
Node X
Planctomycetes
Great Oxygen Event (GOE)
Verrucomicobia
Root
Node4
Node3
Node2
Candidatus Brocadia sapporoensis
Candidatus Brocadia sp. UTAMX1
Candidatus Brocadia sp. WS118
Candidatus Brocadia sinica JPN1
Planctomycetes bacterium RIFCSPLOWO2_12_FULL_39_13
Planctomycetes bacterium GWA2_39_15
Planctomycetes bacterium RIFCSPHIGHO2_12_42_15
uncultured Planctomycetales bacterium AMX2
Candidatus Jettenia ecosi J2
Candidatus Kuenenia stuttgartiensis BL10
Planctomycetes bacterium RBG_16_41_13
Candidatus Scalindua brodae RU1
Planctomycetes bacterium SZUA-167
Candiadtus Scalindua japonica
Planctomycetes bacterium RIFCSPHIGHO2_02_FULL_40_12
Candidatus Scalindua rubra BSI-1
Candidatus Scalindua sp. MohnsGC08_192
Candidatus Scalindua sp. SCAELEC01 SCAELEC01
Planctomycetes bacterium RIFCSPLOWO2_12_FULL_50_35
Planctomycetes bacterium GWA2_50_13
Planctomycetes bacterium RIFCSPHIGHO2_12_FULL_52_36
Candidatus Brocadiaceae bacterium B1Sed10_209R1
Candidatus Brocadiaceae bacterium B1Sed10_183
Candidatus Brocadiaceae bacterium B1Sed10_231
Planctomycetes bacterium DG_23 DG_23
Planctomycetes bacterium B129_G9
Planctomycetaceae bacterium Bin_8_1_1
Planctomycetes bacterium RBG_16_59_8
Planctomycetes bacterium B143_G9
Planctomycetes bacterium J132
Planctomycetes bacterium PLA1
Planctomycetes bacterium E44_bin39
Planctomycetes bacterium Pla163
Planctomycetes bacterium GW928_bin.9
Planctomycetes bacterium SP2985
Planctomycetes bacterium ARS15
Planctomycetes bacterium NAT19
Planctomycetes bacterium DOLJORAL78_63_9
Planctomycetes bacterium SZUA-250
Planctomycetes bacterium Baikal-deep-G175
Planctomycetes bacterium ARS1434
Planctomycetia bacterium TMED53
Planctomycetes bacterium MAG 59
Planctomycetes bacterium SP3001
Planctomycetia bacterium F1-120-MAGs048
Planctomycetes bacterium UBA8898
Planctomycetes bacterium UBA8742
Planctomycetes bacterium J103
Opitutae bacterium UBA4506
Opitutales bacterium CSSed165cm_470
Verrucomicrobia bacterium palsa_1439
Verrucomicrobia bacterium DP16D_bin.41
Verrucomicrobia bacterium UBA6056
Verrucomicrobia bacterium UBA10936
Tolypothrix bouteillei VB521301
Scytonema hofmannii PCC 7110
Tolypothrix sp. NIES-4075
Cylindrospermum stagnale PCC 7417
Chlorogloea sp. CCALA 695
Aliterella atlantica CENA595
Chroococcidiopsis sp. CCALA 051
Phormidium ambiguum IAM M-71
Crinalium epipsammum PCC 9333
Microcoleus sp. PCC 7113
Gloeocapsa sp. PCC 73106
Cyanobacterium sp. IPPAS B-1200
Stanieria cyanosphaera PCC 7437
Stanieria sp. NIES-3757
Spirulina major PCC 6313
Spirulina subsalsa PCC 9445
filamentous cyanobacterium ESFC-1
Rubidibacter lacunae KORDI 51-2
Synechococcus sp. PCC 7336
Synechococcus sp. JA-2-3B'a(2-13)
Gloeobacter violaceus PCC 7421
Candidatus Caenarcanum bioreactoricola UASB_169
Candidatus Melainabacteria bacterium SSGW_16
Candidatus Obscuribacter phosphatis EBPR_351
Vampirovibrio chlorellavorus Vc_AZ_2
Candidatus Gastranaerophilales bacterium UMGS1517
Candidatus Sericytochromatia bacterium S15B-MN24 CBMW_12
Candidatus Sericytochromatia bacterium GL2-53 LSPB_72
Candidatus Melainabacteria bacterium BOG_genome_mining.24
Candidatus Saganbacteria bacterium X2_MaxBin.013
Candidatus Margulisbacteria bacterium RAAC_38_40
96.09
98.24
98.24
99.41
72.66
76.74
50.91
98.24
99.02
84.57
69.92
96.13
59.17
98.18
93.76
93.65
95.12
98.63
84.77
70.09
62.79
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79.08
90.62
79.17
64.62
65.63
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62.21
48.08
90.98
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97.55
92.45
93.67
85.69
99.64
99.64
100
99.73
99.73
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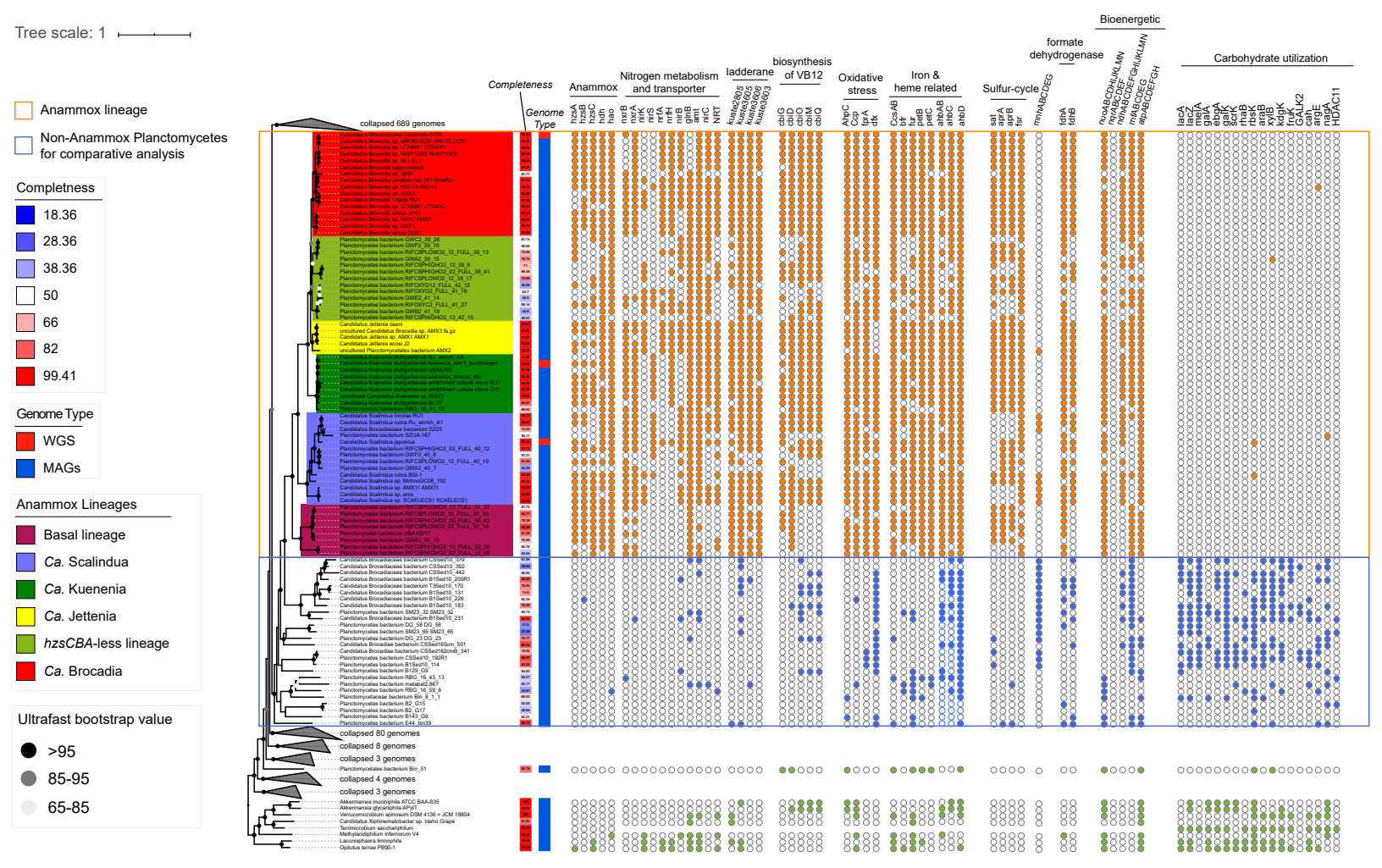
96.73
99.37
99.37
99.56
98.91
99.18
99.46
99.46
99.46
99.46
99.09
100
100
98.64
96.06
84.62
94.87
97.44
95.73
87.61
89.74
75.25
79.63
93.1
96.58
δ15N (‰)
20
10
0
-10
PO2 (PAL)
10⁰
10⁻²
10⁻⁴
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Age (Ga)



Tree scale: 1
Anammox lineage
Non-Anammox Planctomycetes for comparative analysis
Completness
18.36
28.36
38.36
50
66
82
99.41
Genome Type
WGS
MAGs
Anammox Lineages
Basal lineage
Ca. Scalindua
Ca. Kuenenia
Ca. Jettenia
hzsCBA-less lineage
Ca. Brocadia
Ultrafast bootstrap value
>95
85-95
65-85
Completeness
Genome Type
Anammox
Nitrogen metabolism and transporter
ladderane
biosynthesis of VB12
Oxidative stress
Iron & heme related
Sulfur-cycle
formate dehydrogenase
Bioenergetic
Carbohydrate utilization
collapsed 689 genomes
collapsed 80 genomes
collapsed 8 genomes
collapsed 3 genomes
collapsed 4 genomes
collapsed 3 genomes