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Evolutionary placement of Methanonatronarchaeia

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***Methanonatronarchaeia*, a newly discovered archaeal lineage of extremely halophilic methanogens, were proposed to represent an evolutionary intermediate between archaeal methanogens and the extremely halophilic *Halobacteria*. Here, we show that the sistership between *Methanonatronarchaeia* and *Halobacteria* results from a tree reconstruction artefact and that the divergence of *Methanonatronarchaeia* is in fact much deeper. This sheds a new light on the adaptation to extreme halophilic lifestyle in archaea and on the evolution of methanogenesis.**

Sorokin and colleagues recently reported the identification of *Methanonatronarchaeia*, a fascinating archaeal lineage of extremely halophilic, moderately thermophilic, methyl-reducing methanogens^{1,2}. Similar to most recently discovered methanogens, *Methanonatronarchaeia* perform methanogenesis based on H₂ and methyl compounds, a metabolism not previously reported from hypersaline environments. Together with *Halobacteria* and *Nanohaloarchaea*³, *Methanonatronarchaeia* represent the third discovered lineage of extreme halophilic archaea and the most halophilic methanogens ever found. They have likely adapted to this lifestyle by employing a salt-in osmoprotection strategy¹, unlike previously known halophilic methanogens and similarly to the two other extreme halophilic archaeal lineages⁴. Moreover, *Methanonatronarchaeia* rely on cytochromes for methanogenesis¹, a characteristic previously thought to be restricted to the *Methanosarcinales*⁵. A maximum Likelihood (ML) phylogenetic analysis of a supermatrix gathering ribosomal proteins indicated *Methanonatronarchaeia* as the closest relatives to *Halobacteria* (Fig. 1A, red branches)¹. They were therefore proposed to be evolutionary intermediates on the path from methanogens to extreme halophiles¹. However, multiple substitutions occurring at the same site in sequences can mask the original phylogenetic signal and provoke tree reconstruction artefacts⁶, a phenomenon particularly evident in lineages that adapted to extreme salinity⁷.

To test the phylogenetic position of *Methanonatronarchaeia*, we reanalyzed the original supermatrix of ribosomal proteins used by Sorokin et al.¹, through the progressive removal of the fastest evolving sites, a method that is frequently used to reduce artefacts linked to multiple substitutions⁷. This analysis, both by ML and Bayesian approaches including non-homogeneous evolutionary models, shows that the clustering of *Halobacteria* and *Methanonatronarchaeia* (Fig. 1B-C, red line) was recovered only when the fastest evolving-sites are included in the analysis, while the progressive removal of these sites shifted the position of *Methanonatronarchaeia* away from *Halobacteria* and to a deeper branching position at the base of the superclass 'Methanotecta'⁸ (Fig. 1B-C, green line). This placement is also consistently and robustly recovered when *Methanonatronarchaeia* were included in two recently published supermatrices comprising a larger number of markers⁶ (over 250 conserved protein families) or a larger taxonomic sampling of the Methanotecta⁹ (including ANME1, Syntrophoarchaeales, Methanoliparia, and a third *Methanonatronarchaeia* member). In contrast with the dataset of Sorokin et al.¹, the new placement of *Methanonatronarchaeia* was robust to the removal of the fastest-evolving sites for both these supermatrices (Fig. 1D-G).

Our analyses indicate that the placement of *Methanonatronarchaeia* as the methanogenic closest relatives of *Halobacteria* proposed in Sorokin et al.¹ is likely the consequence of a tree reconstruction artefact induced by a multiple substitution-bias which is particularly strong in their ribosomal protein dataset, but not in the other two datasets. The alternative position of the *Methanonatronarchaeia* disclosed here provides a new perspective on the evolution of this fascinating lineage. For example, it indicates that their adaptation to extreme halophily would have occurred independently from the *Halobacteria*. Moreover, following the recent proposal for the placement of *Nanohaloarchaea* as sister to the *Methanocellales*⁶, the salt-in strategy used for thriving in hypersaline environments would have emerged three times independently in the *Archaea*, a remarkable example of convergent evolution for adaptation to similar environments. Finally, the new placement of *Methanonatronarchaeia* is highly relevant for the evolution and diversity of methanogenesis, as their characteristics may reflect those of the methanogenic ancestor of the whole 'Methanotecta' superclass. For example, the fact that *Methanonatronarchaeia* rely on cytochromes for methanogenesis³ raises the question of whether this feature may be ancestral to all Class II methanogens and was retained only in *Methanosarcinales* while *Methanomicrobiales*, *Methanocellales* and *Methanoflorentaceae* shifted secondarily to methanogenesis without cytochromes, or if instead it emerged twice independently.

The current pace in the acquisition of genomic data and the discovery of new lineages^{8,10} will certainly allow to tackle these fundamental questions in the evolution and ecology of methanogens and of *Archaea* in general.

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Author contributions

S.G. and C.B.A. supervised the study. M.A. and G.B. assembled the datasets and performed all analyses. All authors analysed the data and wrote the manuscript.

Competing interests

The authors declare no competing interest.

Legend of Fig. 1

(A) Schematic phylogeny of the *Archaea*, with a focus on the ‘Methanotecta’ superclass⁸. Dotted lines indicate two alternative branchings of *Methanonatronarchaeia*: as the sister-lineage of *Halobacteria* (red) or at the base of ‘Methanotecta’ (green).

(B-G): Impact on the placement of *Methanonatronarchaeia* of the progressive removal of the fastest-evolving sites from the three analysed supermatrices (see Supplementary Information (SI) for details). **(B-C)**: the supermatrix of ribosomal proteins (8,072 amino acid positions) derived from Sorokin et al.¹, **(D-E)**: the supermatrix, derived from Adam et al.⁸ (40 conserved protein families, 9,228 amino acid positions), and **(F-G)**: the supermatrix derived from Aouad et al.⁶ (258 conserved protein families, 62,398 amino acid positions).

The x-axis indicates the percentage of amino acid positions of the supermatrices that were kept for phylogenetic analyses during the progressive removal of the fastest evolving sites. The y-axis corresponds to bootstrap values associated to the ML trees inferred using the LG+G4 evolutionary model **(B, D, and F)** or the PMSF+LG+G4 evolutionary model **(G)**, or to posterior probabilities associated to the Bayesian trees inferred with the CAT+GTR+G4 evolutionary model **(C, and E)**. The green and red lines shown on these graphs correspond to the bootstrap values and posterior probabilities supporting the two alternative placements of *Methanonatronarchaeia* as illustrated in Figure 1A. In all trees, the clustering of *Methanonatronarchaeia* with ‘Methanotecta’ was strongly supported, excluding the branching of *Methanonatronarchaeia* elsewhere in the archaeal phylogeny. For two supermatrices on panel C (86, 82, indicated by an asterisk), *Methanonatronarchaeia* branched in-between *Archaeoglobales* and ‘Ca. Methanophagales’ (ANME-1). All trees and corresponding supermatrices are provided in Supplementary Information (SI).

A **B** **C** **D** **E** **F** **G**