

Reply to 'Evolutionary placement of Methanonatronarchaeia'

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1 **More genomes needed to resolve archaeal phylogeny**

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9 **Response to** Monique Aouad, Guillaume Borrel, Céline Brochier-Armanet, and Simonetta
10 Gribaldo

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12 “Methanonatronarchaeia are not evolutionary intermediates on the path from methanogens to
13 extreme halophiles”

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31 **Standfirst**

32 **Different phylogenetic methods applied to different gene sets yield alternative positions**
33 **for the proposed archaeal class “*Methanonatronoarchaeia*” in the archaeal tree. A more**
34 **representative sampling of archaeal genomes is essential to resolve this phylogenetic**
35 **impasse.**

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38 We appreciate the interest of Aouad and colleagues in our work on the proposed archaeal
39 class “*Methanonatronoarchaeia*”^{1,2} and their effort to clarify the phylogenetic position of
40 this unique group of extremely halophilic, methyl-reducing methanogens. In our analysis,
41 *Methanonatronoarchaeia* formed a clade with the class *Halobacteria*, the non-methanogenic
42 euryarchaeal extreme halophiles. Notably, this phylogenetic placement is 100% bootstrap-
43 supported in maximum-likelihood (ML) phylogenetic trees for both 16S rRNA and
44 concatenated alignments of ribosomal proteins¹. Given the congruence of the two trees, the
45 strong support for the *Methanonatronoarchaeia-Halobacteria* clade, the biological
46 plausibility of this affinity and the fact that these trees conformed with the currently favored
47 solutions for difficult problems in archaeal phylogeny (such as the monophyly of the DPANN
48 superphylum and the euryarchaeal assemblage including Class I methanogens and
49 Thermococci), we did not perform a more thorough phylogenetic analysis. Such an in-depth
50 analysis was undertaken by Aouad and colleagues³. Their results suggest a different position
51 for *Methanonatronoarchaeia*, much deeper in the archaeal tree, outside the branch that
52 consists of Methanomicrobia (formerly, Class II Methanogens), including *Halobacteria*
53 (denoted “Stenosarchaea” by Aouad et al.) and the class *Archaeoglobi*, and at the base of the
54 group which Aouad et al. denote the “superclass Methanotecta”. This difference between the
55 results of the two phylogenetic analyses stems primarily from the increasingly stringent
56 removal of fast-evolving sites from the alignment prior to the phylogenetic tree construction
57 that was applied by Aouad and colleagues. After a certain fraction of the fastest sites was
58 removed, the tree topology abruptly transitioned to the deep placement of
59 *Methanonatronoarchaeia*. This procedure is supposed to eliminate the false signal produced
60 by sites with multiple substitutions, and therefore, Aouad et al. conclude that the affinity of
61 *Methanonatronoarchaeia* with *Halobacteria* was an artifact caused by such sites. Aouad et al.
62 also obtained the “deeper” placement of *Methanonatronoarchaeia* with extended sets of
63 conserved protein families and expanded taxon sampling, in these cases, even without
64 removing the fast-evolving sites.

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66 In our view, the position of *Methanonatronoarchaeia* in the archaeal phylogeny remains an
67 open question. Removal of fast-evolving sites is a double-edged sword: it reduces the noise
68 introduced by multiple substitutions but phylogenetic information that is contained in
69 comparatively variable positions is lost as well ⁴. The most highly conserved sites are
70 phylogenetically uninformative and so are the most variable ones, whereas those with
71 intermediate variability carry the bulk of the phylogenetic signal ⁵. The loss of phylogenetic
72 signal can result in exactly what is observed for *Methanonatronoarchaeia*, namely, losing the
73 information on a specific affinity, in this case, with *Halobacteria*, and pushing a branch down
74 the tree, closer to the root. Inclusion of additional protein families, although potentially
75 enhancing the phylogenetic signal, also has its own caveats. Many of these families are less
76 strongly conserved during evolution than ribosomal proteins are, which leads to less reliable
77 alignments, and many are prone to horizontal gene transfer (HGT), which can dilute the
78 signal. Also, the observations on protein phylogenies cannot explain away the affinity between
79 *Methanonatronoarchaeia* and *Halobacteria* in the 16S RNA tree.

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81 The highly conserved ribosomal-based phylogeny is not the only line of evidence that links
82 *Methanonatronoarchaeia* with *Halobacteria*. The two groups share a variety of genes that are
83 not commonly found in other archaea, in particular, those encoding multiple membrane ion
84 transport systems involved in halophily and uncharacterized membrane proteins (see
85 Supplementary Table 3 in Ref. 1). Especially conspicuous is the UspA family of stress
86 response proteins ⁶ that is dramatically expanded in both *Methanonatronoarchaeia* and
87 *Halobacteria* (see Supplementary Figure 8 in Ref. 1). It appears most likely that these
88 proteins contribute to the extreme salt tolerance. Phylogenetic analysis of the UspA family
89 shows a complex picture, but for a number of branches, inheritance of the respective genes
90 from a common ancestor of *Methanonatronoarchaeia* and *Halobacteria* appears to be the
91 most likely scenario (Supplementary File 1). The two sequenced genomes of
92 *Methanonatronoarchaeia* encompass integrated virus-like elements (His2-like proviruses)
93 that closely resemble viruses of *Halobacteria* (see Table 1 in Ref. 1). Given the generally
94 narrow host range of archaeal viruses ⁷, the presence of these elements in
95 *Methanonatronoarchaeia* seems to suggest a common evolutionary history with
96 *Halobacteria*. Together, these observations appear to be compatible with a common ancestor
97 of *Methanonatronoarchaeia* and *Halobacteria* that was already adapted to hypersalinity
98 including the expansion of the UspA family. Admittedly, none of this is incontrovertible

99 evidence, and in particular, HGT always offers an alternative. However, in cases like the
100 UspA family and His2-like elements, the HGT scenario seems less parsimonious than
101 common ancestry.

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103 As Aouad and colleagues point out ³, repositioning *Methanonatronoarchaeia* in the archaeal
104 phylogenetic tree would have distinct biological implications, in particular, indicating
105 independent origins of the adaptations to hypersalinity in *Methanonatronoarchaeia* and
106 *Halobacteria*. The problem runs even deeper because another recent study by Aouad and
107 colleagues ⁸ suggests also the relocation of the candidate division Nanohaloarchaea from the
108 DPANN superphylum to "Stenosararchaea", suggesting two independent origins of non-
109 methanogenic extreme halophiles from different lineages of Methanomicrobia and putting
110 into question the monophyly of DPANN. A recent comprehensive phylogenetic modeling
111 study has yielded a clear support for a monophyletic DPANN ⁹. These phylogenetic travails
112 also resemble the long debate on the position of Nanoarchaea ¹⁰⁻¹² that, with the discovery of
113 many other archaea with miniature genomes, seemed to have been settled on the DPANN
114 superphylum. The impending changes to the archaeal phylogeny and taxonomy could be quite
115 profound. A phylogenetic tree of archaea generated from a set of 122 marker proteins using a
116 recently developed methodology for genome phylogenies ¹³ has led to the proposal of the
117 phylum *Halobacterota* that is placed outside the Euryarchaeota and unites *Archaeoglobi*,
118 *Halobacteria*, *Methanomicrobia*, *Methanonatronoarchaeia*, *Methanosarcini*, and NRA6, with
119 deeply placed *Methanonatronoarchaeia* (<http://gtdb.ecogenomic.org/tree>).

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121 Deep phylogenies are fraught with uncertainty, so that definitive solutions might be out of
122 reach. However, one remedy seems to be consistently efficient, namely, improved taxon
123 sampling ^{14,15} which, indeed, has been attempted by Aouad and colleagues ³. However, the
124 representation of *Methanonatronoarchaeia* remains obviously insufficient to reach
125 compelling conclusions, with the current sample including only two genomes (but, notably,
126 two additional sequences clustering with *Methanonatronoarchaeia* in the 16S RNA tree).
127 Further progress in microbial genome sequencing, in particular, by methods of metagenomics
128 and single-cell genomics, will substantially expand the diversity of archaea available for
129 phylogenomic analysis, providing for more robust phylogenies in the near future. Indeed, a
130 high quality draft single-cell genome corresponding to one of these additional 16S RNA
131 sequences (SA1) has recently become available ¹⁶. There is no doubt that, within a few years,

132 more genomes will follow, likely, providing for the resolution of the current phylogenetic
133 impasse.

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192 **Supplementary File 1. Phylogenetic tree of the UspA family.**

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The tree (Newick format) is constructed from an alignment of 4,550 UspA domain sequences from 4,184 distinct loci in 427 archaeal genomes using the FastTree program (WAG evolutionary model, gamma-distributed site rates)¹⁷. Sites with more than 50% of gap characters and homogeneity less than 0.1 were removed; both the raw (<https://ftp.ncbi.nlm.nih.gov/pub/wolf/suppl/archtre/UspA.raw.afa>) and the filtered (<https://ftp.ncbi.nlm.nih.gov/pub/wolf/suppl/archtre/UspA.tre.afa>) alignments are available.

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The sequences of the following genes of Methanonatronarchaeia are included in the tree: BTN85_0146, BTN85_0312, BTN85_1038, BTN85_1108 (two UspA domains), BTN85_1119, BTN85_1447 (two domains), BTN85_1704, BTN85_1755. BTN85_1868, BTN85_1870 from Candidatus Methanohalarchaeum thermophilum and AMET1_RS00685, AMET1_RS00685, AMET1_RS01465 (2 domains), AMET1_RS02155 (2 domains), AMET1_RS02155, AMET1_RS03320, AMET1_RS03675, AMET1_RS03980 (2 domains), AMET1_RS05120, AMET1_RS06595 from Methanonatronarchaeum thermophilum AMET1.

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