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A site-and-branch-heterogeneous model on an expanded dataset favor mitochondria as sister to known Alphaproteobacteria

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Title: A site-and-branch-heterogeneous model on an expanded dataset favors mitochondria as sister to known *Alphaproteobacteria*

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Abstract

Determining the phylogenetic origin of mitochondria is key to understanding the ancestral mitochondrial symbiosis and its role in eukaryogenesis. However, the precise evolutionary relationship between mitochondria and their closest bacterial relatives remains hotly debated. The reasons include pervasive phylogenetic artefacts, as well as limited protein and taxon sampling. Here, we developed a new model of protein evolution that accommodates both across-site and across-branch compositional heterogeneity. We applied this site-and-branch-heterogeneous model (MAM60+GFmix) to a considerably expanded dataset that comprises 108 mitochondrial proteins of alphaproteobacterial origin, and novel metagenome-assembled genomes from microbial mats, microbialites, and sediments. The MAM60+GFmix model fits the data much better and agrees with analyses of compositionally homogenized datasets with conventional site-heterogenous models. The consilience of evidence thus suggests that mitochondria is sister to the *Alphaproteobacteria* to the exclusion of MarineProteo1 and *Magnetococcia*. We also show that the ancestral presence of a crista-developing MICOS complex (a Mitofilin domain-containing Mic60) supports this relationship.

Introduction

Mitochondria stem from an ancient endosymbiosis that occurred during the origin of eukaryotic cells[1]. As a result, all extant eukaryotes have mitochondria or evolved from mitochondrion-bearing ancestors[1–3]. Some hypotheses have it that mitochondria provided excess energy required for the origin of eukaryotic complexity[4], whereas others suggest that mitochondrial symbiosis brought efficient aerobic respiration into a more complex proto-eukaryote[5]. The nucleocytoplasm of eukaryotes is now known to be most closely related to Asgard archaea[6–8]. Mitochondria, on the other hand, have been known for decades to be phylogenetically associated with the *Alphaproteobacteria*[9,10,1]. However, the precise relationship between mitochondria and the *Alphaproteobacteria*, or any of its sub-groups, has been elusive and remains a matter of intense debate (e.g., see [11,12]). Settling this debate will provide insights into the nature of the mitochondrial ancestor and the ecological setting of its endosymbiosis with the host cell[1].

Mitochondria have been placed in various regions of the tree of the *Alphaproteobacteria*. Most early studies suggested that mitochondria were most closely related to the *Rickettsiales*[13–20] (*Rickettsiales*-sister hypothesis), a group classically known for comprising intracellular parasites. This led many to believe that mitochondria evolved from parasitic alphaproteobacteria[18,21]. However, relationships between mitochondria and the *Pelagibacterales*[22,23], *Rhizobiales*[24], or *Rhodospirillales*[25] have also been proposed. These alternative proposals suggested that mitochondria may have evolved from either streamlined or metabolically versatile free-living alphaproteobacteria[22–25]. Most recently, the phylogenetic placement of mitochondria has been vividly debated[11,12]. One study found mitochondria as a sister group to the entire *Alphaproteobacteria* (i.e., the *Alphaproteobacteria*-sister hypothesis)[11]. This conclusion was supported by the inclusion of novel alphaproteobacterial metagenome-assembled genomes (MAGs) from worldwide

oceans, and by decreasing compositional heterogeneity through site removal. However, a subsequent study argued that removing compositionally heterogeneous sites from alignments might lead to the loss of true historical signal[26,12].The authors of the latter study, instead, used a taxon-removal and -replacement approach, and concluded that mitochondria branch within the *Alphaprotoebacteria* as sister to the *Rickettsiales* and some environmental metagenome-assembled genomes (MAGs)[12].

There are several reasons why it is difficult to confidently place mitochondria among their alphaproteobacterial relatives. First, the evolutionary divergence between mitochondria and their closest bacterial relatives is estimated to have occurred >1.5 billion years ago[27,28]. This has erased the historical signal (e.g., through multiple amino acid replacements) that was originally present in the few genes that mitochondria and alphaproteobacteria still share. Second, the *Alphaproteobacteria* is under sampled and most of its diversity remains to be discovered, as suggested by recent metagenomic surveys[11]. Third, and perhaps most problematic, the genomes of some lineages in the *Alphaproteobacteria* and those of mitochondria have undergone convergent evolution. For example, the *Rickettsiales* and *Holosporaceae* (intracellular bacteria)[29], or the *Pelagibacterales* and '*Puniceispirillaceae*' (planktonic bacteria)[30], have reduced or streamlined genomes with compositionally biased genes similar to those of mitochondria. The genes and genomes of these taxa are biased towards A+T nucleotides (and their proteins towards F, I, M, N, K, and Y amino acids) in contrast to other groups that have not evolved reductively (which might be biased towards G+C nucleotides and G, A, R, and P amino acids)[29]. This sort of compositional heterogeneity is often the cause of artefactual attractions among lineages with similar compositional biases in phylogenetic inference[31].

To cope with the aforementioned sources of phylogenetic errors, we developed and implemented a new phylogenetic model of protein evolution that accounts for compositional heterogeneity across both alignment sites and tree branches. Moreover, we also gathered an expanded set of 108 proteins of alphaproteobacterial origin in eukaryotes (in comparison to <67 previously available) and assembled more than 150 non-marine alphaproteobacterial MAGs from microbial mat, microbialite, and lake sediment metagenomes. We combined these improvements to explore and dissect the phylogenetic signal for the origin of mitochondria present in both modern eukaryotes and alphaproteobacteria.

Results

To date, most studies that aimed to phylogenetically place the mitochondrial lineage have relied exclusively on mitochondrion-encoded protein datasets that range from 12 to 38 proteins[16–18,32,11,12]. These markers are not only few (e.g., 24 genes and 6,649 sites in [11]) but tend to be compositionally biased because most mitochondrial genomes are rich in A+T. The only set of nucleus-encoded proteins of mitochondrial origin published thus far comprises 29 proteins[19,20].

To expand the number of proteins for placing the mitochondrial lineage, we systematically surveyed both nuclear and mitochondrial proteomes. After a multi-step phylogenetic screening, we identified 108 marker proteins of alphaproteobacterial origin in eukaryotes. Of these, 64 are exclusively nucleus-encoded, 27 are both nucleus- and mitochondrion-encoded, and 17 are exclusively mitochondrion-encoded proteins (Fig. 1A, Fig. S1). Our expanded dataset comprises most marker proteins previously identified[11,19,20] and adds 56 new ones (Fig. S1). Functional annotations show that these proteins have diverse functions within mitochondria (Fig. 1B, Table S1). Most are involved in energy metabolism (e.g., respiratory chain complex subunits) and protein synthesis (e.g., ribosomal subunits) (Fig. 1B, Table S1). The fact that all these proteins have mitochondrial functions strengthens the view that the genes that encode them were transferred from (proto-)mitochondria to nuclear genomes and are therefore not secondary lateral transfers to eukaryotes. The new nucleus-encoded proteins also tend to have much less variable and biased amino acid compositions in comparison to those which are mitochondrion-encoded and some that are both nucleus- and mitochondrion-encoded (Fig. 1A). Similarly, nucleus-encoded proteins also have a broader range of G A R P/F I M N K Y amino acid ratios of 0.70–1.95, whereas mitochondrion-encoded proteins have a range of 0.25–0.77 which suggests that they are much more compositionally biased towards F I M N K Y amino acids (and their genes towards A+T). The expanded set of nucleus-encoded

genes are expected to increase phylogenetic signal by virtue of increasing the amount of data, and also introduce potentially less compositionally biased sequences that could otherwise cause phylogenetic artefacts.

Most studies have exclusively relied on genomes of cultured alphaproteobacteria (e.g., [18–20,32]). Only one recent study incorporated novel alphaproteobacterial MAGs from metagenomes sequenced by the Tara Oceans project[11]. So far, all of these alphaproteobacterial MAGs came from oceanic open waters and tend to be small and A+T-rich[11]. Moreover, none of them appeared to be most closely related to mitochondria to the exclusion of other alphaproteobacteria[11].

To further increase taxonomic sampling across the *Alphaproteobacteria*, we assembled MAGs from metagenomes sequenced from diverse microbial mats, microbialites, and lake sediments (see Table S2 for details). In addition, we also screened MAG collections released previously[11,33–39], as well as the GTDB r89 database[40], for potentially phylogenetically novel alphaproteobacteria—together, these databases comprise more than ~ 3,300 alphaproteobacterial genomes and MAGs. The newly assembled MAGs were considerably diverse and widely distributed across the tree of the *Alphaproteobacteria* (Fig. 1C). Despite considerably expanding the sampled diversity of the *Alphaproteobacteria*, however, most of these new MAGs appear to fall within previously sampled major clades (Fig. 1C, Fig. 1D, Table S3), including those recently reported[11,40] (Fig. 1D, Table S3). The most novel MAGs include new members of the 'early-diverging' MarineProteo1 clade whose genomes are relatively small (1.43–2.71 Mbp) and not heavily compositionally biased towards A+T (43.6–59.7%) (Fig. S2, Table S4). In addition, several novel MAGs for 'basal' members of the *Rickettsiales* were found to be larger (1.47–2.36 Mbp) and enriched in G+C (49.2–61.2 or ~49.3% on average) relative to previously sampled members of this group (>0.6–2.11 Mbp and 32.1–34.2% G+C on average in the *Rickettsiaceae*, *Anaplasmataceae*, and *Midichloriaceae*) (Fig. S2, Table S4). The new alphaproteobacterial MAGs have moderate-to-high quality (according to criteria by [39,40]; 53.41–100% completeness and 0–9.17 redundancy), a wide range of G+C content (30.3–73.5%) and sizes (0.88–4.85 Mbp), and varying degrees of phylogenetic novelty (0.99–0.56 Relative Evolutionary Divergence score[40]) (Fig. 1D, Table S3)—this suggests that the methods used here to recover MAGs were not biased toward those with certain features (e.g., small sizes or high A+T content). Most of the new MAGs, which are widely distributed across the *Alphaproteobacteria* tree, also appear to encode an almost-complete set of bacteriochlorophyll biosynthesis enzymes which suggest that they come from photosynthesizers in the diverse environments sampled (e.g., microbial mats; Fig. 1D, Table S3).

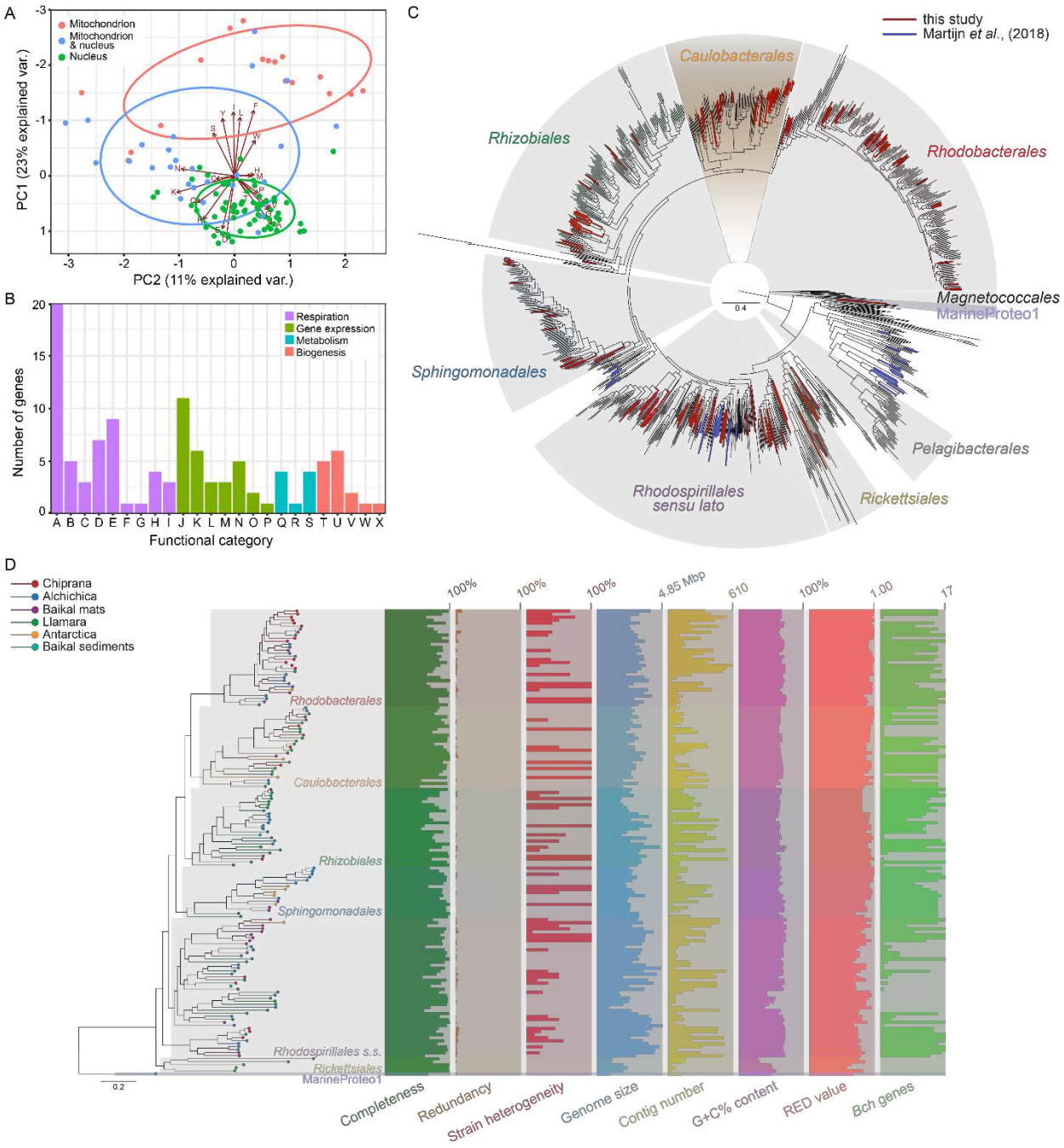


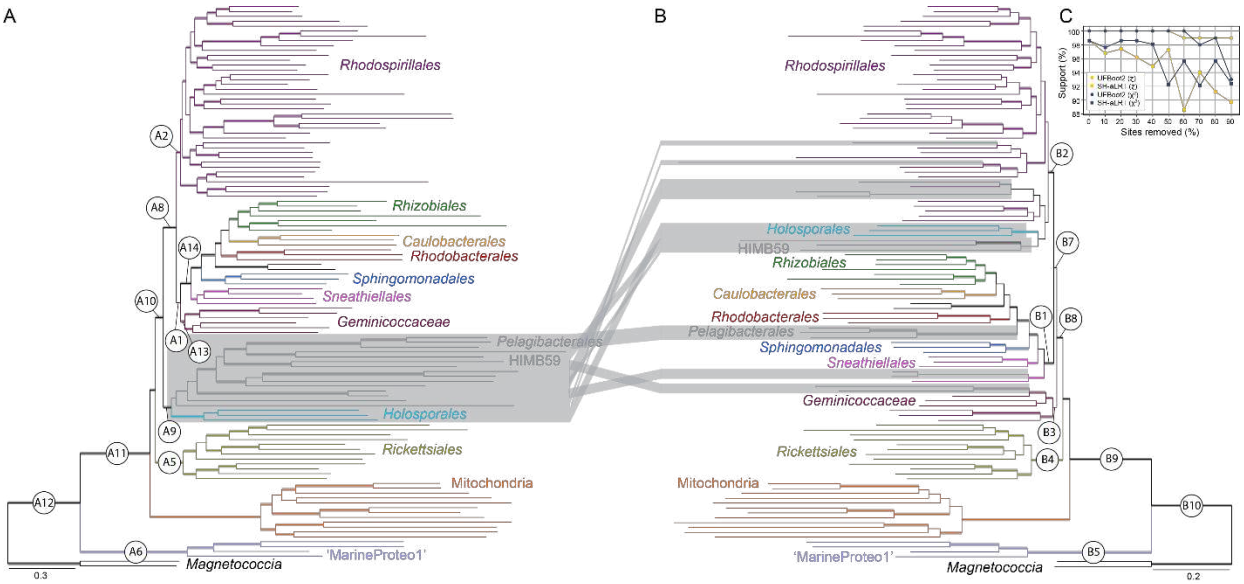
Figure 1. An expanded gene set and novel alphaproteobacterial MAGs from diverse environments. (**A**) Principal Component Analysis (PCA) of amino acid compositions for each one of the 108 mitochondrial genes of alphaproteobacterial origin used in this study. Mitochondrion-encoded genes (light red); Mitochondrion- and nucleus-encoded genes (light blue); nucleus-encoded genes (green); 95% confidence ellipses follow the same color code as genes. This PCA was inferred from alignments that contain only eukaryotes. (**B**) Functional classification of the marker genes of alphaproteobacterial origin in eukaryotes used for multi-gene phylogenetic analyses in this study. All these functions take place inside mitochondria. A: Complex I subunit/assembly factor; B: Complex II subunit/assembly factor; C: Complex III subunit/assembly factor; D: Complex IV subunit/assembly factor; E: Complex V subunit/assembly factor; F: Cytochrome c biogenesis; G: D-lactate dehydrogenase (respiratory chain); H: Pyruvate

139 dehydrogenase complex subunit; I: Krebs cycle; J: Ribosome large subunit; K: Ribosome small subunit;
140 L: Ribosome translational factor; M: rRNA modification/maturation; N: tRNA modification/maturation; O:
141 Aminoacyl-tRNA synthetase; P: RNA polymerase; Q: Branched-chain amino acid/fatty acid metabolism,
142 R: Pyrimidine biosynthesis; S: Ubiquinone biosynthesis; T: Protein import/export; U: Iron-sulfur cluster
143 biogenesis; V: Clp protease complex subunit; W: Proteasome-like complex subunit; X: Mitochondrial
144 division (see also Table S1). (**C**) Phylogenetic tree of 154 novel MAGs reported here, the 45 MAGs
145 reported by Martijn *et al*. (2018), and 1,188 of maximally diverse alphaproteobacterial genomes in GTDB
146 r89 database. Taxon sample reduction was done with Treemmer[41] and phylogenetic inference with IQ-
147 TREE (-fast mode) and the LG4X model (120 GTDB-Tk marker genes; 14,048 amino acid sites). (**D**).
148 Phylogenetic tree for the 154 alphaproteobacterial MAGs reconstructed from diverse metagenomes
149 sequenced in this study and summary of major features for each MAG. Tree was inferred with IQ-TREE (-
150 fast mode) and the LG4X model after having removed 50% of most compositionally heterogeneous ʒ
151 sites (120 GTDB-Tk marker genes; 7,024 amino acid sites) (see also Table S3).

152 To address recent controversies[11,26,12], we first assembled the largest dataset to date that includes a new
153 set of 64 nucleus-encoded and 44 mitochondrion-encoded proteins (108 genes in total and 33,704 amino
154 acid sites; see above). Our dataset also comprised a wide taxon sampling with twelve mitochondria from
155 diverse eukaryotes (from most 'supergroups'), and a broad set of 104 alphaproteobacteria that covered
156 all major known lineages and maximized phylogenetic diversity (subsampled from a set of more than
157 3,300 genomes; see Methods). Importantly, our dataset incorporated several *Rickettsiales* species that
158 have short branches and are less compositionally biased (Fig. 1D, Fig. S2, Table S4), as well as novel
159 representatives of the MarineProteo1 clade (Fig. 1D, Fig. 2A, Table S4). Instead of relying on *Beta*-, and
160 *Gammaproteobacteria* as outgroups (as in [11,12]), we used the much closer *Magnetococcia* which has
161 been consistently found to be sister to all other alphaproteobacteria (e.g., [11,12,20]). This was done to
162 decrease potential artefactual attractions between the long mitochondrial branch and distant outgroups, a
163 concern raised before[11,26,12]. Furthermore, we also removed sites estimated to have undergone functional
164 divergence at the origin of mitochondria (these represented only 5.2% of all sites) using the FunDi mixture
165 model[42]. This was done to reduce potential artefacts from model misspecification as no phylogenetic
166 model currently available adequately captures such patterns of functional divergence in proteins.

167 We first analyzed our dataset using the MAM60 site-heterogeneous model that was specifically inferred
168 from our own dataset—this model has been shown to have a better fit than generic site-heterogenous
169 models (e.g., C10-60)[43]. Analyses on the untreated dataset (i.e., without compositionally heterogeneous
170 sites removed) placed mitochondria as sister to all of the *Alphaproteobacteria* with maximum support, i.e.,
171 both the monophyly of the *Alphaproteobacteria* and the *Alphaproteobacteria*-mitochondria clade were
172 fully supported (Fig. 2A). However, these analyses also recovered the grouping between the
173 *Pelagibacterales*, *Holosporaceae*, and other long-branching species (Fig. 2C, Mendeley Data) that, in
174 previous work[29], were shown to artefactually attract each other because of similar amino acid
175 compositional biases. A common strategy for dealing with compositional heterogeneity in the absence of
176 site-and-branch-heterogeneous models is to remove alignment sites based on metrics that quantify their
177 compositional heterogeneity[11,12,29]. The progressive removal of the compositionally most heterogeneous
178 sites according to the z and χ^2 metrics[11,29,44] disrupted compositional attractions and showed clear support
179 for the *Alphaproteobacteria*-sister hypothesis (Fig. 2B, Fig. 2C).

180 Because nucleus-encoded and mitochondrion-encoded proteins display different amino acid
181 compositional patterns (Fig. 1A), we also analyzed these two protein sets separately. Whereas nucleus-
182 encoded proteins unambiguously supported the *Alphaproteobacteria*-sister hypothesis across all
183 analyses (Mendeley Data), the mitochondrion-encoded proteins showed decreased support for this
184 hypothesis as compositionally heterogeneous sites are removed (Fig. S3, Mendeley Data). However, no
185 alternative hypothesis was favored and any placement of mitochondria among the *Alphaproteobacteria*
186 was unsupported for mitochondrion-encoded proteins (Fig. S3; Mendeley Data). This suggests that
187 mitochondrion-encoded proteins may have a more equivocal phylogenetic signal. Unlike in many previous
188 studies[19,20,12,11], we did not find support for the *Rickettsiales*-sister hypothesis in any of our analyses
189 (Mendeley Data).



D

Model \ Tree	B10	B5	A12	A6	B2	B1	B3	B7	B4	B8	B9	A2	A14	A13	A1	A9	A8	A5	A10	A11
MAM60 (no partitions)	1470 (-3003129)	1469 (-3003127)	842 (-3002501)	841 (-3002499)	792 (-3002450)	768 (-3002426)	743 (-3002401)	743 (-3002401)	653 (-3002311)	649 (-3002307)	626 (-3002284)	182 (-3001840)	176 (-3001834)	166 (-3001824)	159 (-3001817)	89 (-3001747)	63 (-3001721)	33 (-3001691)	25 (-3001684)	0* (-3001658)*
Model \ Tree	A12	A6	B10	B5	A2	A14	A13	A1	A9	A8	B2	A5	A10	B1	A11	B7	B3	B4	B8	B9
MAM60 (10 partitions)	1136 (-2990187)	1132 (-2990182)	834 (-2989884)	830 (-2989880)	495 (-2989545)	477 (-2989528)	446 (-2989497)	444 (-2989494)	339 (-2989389)	331 (-2989381)	294 (-2989344)	233 (-2989283)	220 (-2989271)	214 (-2989265)	207 (-2989257)	119 (-2989170)	87 (-2989138)	50 (-2989100)	44 (-2989094)	0* (-2989050)*
Model \ Tree	B10	B5	A12	A6	B2	B1	B7	B3	B4	B8	B9	A2	A14	A13	A1	A9	A8	A5	A10	A11
MAM60+GFmix (no partitions)	956 (-2983168)	954 (-2983166)	830 (-2983042)	828 (-2983040)	315 (-2982527)	306 (-2982518)	303 (-2982515)	293 (-2982505)	229 (-2982441)	215 (-2982426)	166 (-2982378)	161 (-2982373)	154 (-2982366)	141 (-2982353)	131 (-2982343)	120 (-2982332)	114 (-2982326)	32 (-2982244)	27 (-2982239)	0* (-2982212)*
Model \ Tree	A12	A6	B10	B5	A2	A14	A13	A1	A9	A8	A5	A10	A11	B2	B1	B7	B3	B4	B8	B9
MAM60+GFmix (10 partitions)	1382 (-2965660)	1381 (-2965659)	836 (-2965113)	830 (-2965108)	811 (-2965089)	785 (-2965063)	780 (-2965058)	779 (-2965057)	697 (-2964974)	685 (-2964962)	611 (-2964888)	605 (-2964882)	559 (-2964836)	216 (-2964494)	196 (-2964474)	161 (-2964438)	160 (-2964437)	51 (-2964328)	46 (-2964323)	0* (-2964278)*

lnL

lnL

Figure 2. Phylogenetic tree of the *Alphaproteobacteria* and mitochondria, and support from our new site-and-branch-heterogeneous model. (**A**) Phylogenetic tree for the *Alphaproteobacteria* and mitochondria derived from a site-heterogeneous analyses of an untreated dataset. (**B**) Phylogenetic tree for the *Alphaproteobacteria* and mitochondria derived from a site-heterogeneous analysis of a dataset from which 50% of the most compositionally heterogeneous sites according to the ζ metric had been removed. The removal of this amount of ζ sites minimizes the variation of G A R P/F I M N K Y amino acid ratios across taxa (Table S5). The taxonomic labels follow the higher-level taxonomy outlined in [29]. Thickened branches represent branch support values of >90% SH-aLRT and >90% UFBoot2+NNI. (**C**) Variation in support values for the placement of mitochondria outside of the *Alphaproteobacteria* (SH-aLRT and UFBoot2+NNI) throughout the progressive removal of compositionally heterogenous sites according to the ζ and χ^2 metrics. Support for the branch that groups mitochondria with all alphaproteobacteria (but excludes MarineProteo1 and the *Magnetococcia*) is always maximum (i.e., 100% SH-aLRT /100% UFBoot2+NNI; Mendeley Data). (**D**) Heatmap table summarizing the differences in log-likelihoods (lnL) relative to the highest log-likelihood for several alterative placements of mitochondria (A1-14 and B1-B12 in (**A**) and (**B**); see Table S6 and Fig. S4 for all tree topologies) under a conventional site-heterogeneous model (MAM60) and our new site-and-branch-heterogeneous model (MAM60+GFmix). Models (rows) are arranged in increasing order (from top to bottom) according to lnL values. For each model (row), tree topologies (columns) are arranged in increasing order (from left to right) according to lnL values. Absolute log-likelihood values for each tree (A-T) under the different models tested are reported within parentheses. For all four models, all topologies other than the maximum-likelihood tree were rejected with *p*-values of < 0.0001 according to Bonferroni-corrected χ^2 tests. See Table S6 for all tree topologies and datasets tested.

All studies to date have exclusively relied on either site-homogenous or purely site-heterogeneous models (e.g., CAT in PhyloBayes or C60 in IQ-TREE)[11,12,14–20,22,23,32]. Indeed, no tractable model that accounts for compositional heterogeneity across branches and sites simultaneously is available; current branch-heterogeneous models cannot be combined with site-heterogenous models[31], or are too computationally intensive and suffer from convergence problems[45,46]. To overcome these shortcomings, we developed a model that captures the most important compositional heterogeneity in alphaproteobacterial genomes— namely the variation in the G A R P/F I M N K Y amino acid ratio that is driven by variation in G+C vs. A+T nucleotide content (see [29]). Our new branch-heterogeneous model, GFmix, models the variation in the ratio of G A R P/F I M N K Y amino acid frequencies across the phylogenetic tree in combination with conventional site-heterogeneous models (e.g., C10-60, MAM and UDM models). Briefly, this model requires a rooted tree, and introduces a new parameter that represents the G A R P/F I M N K Y ratio for every branch in a tree that is based on the amino acid compositions of all taxa that descend from that branch (see Materials and Methods for details). These parameters, in turn, adjust the frequencies of each site class in the site-profile mixture model resulting in a new transition rate matrix, Q_c, for each mixture class for the given branch. We developed and implemented the new GFmix model in a maximum likelihood framework.

To further test the phylogenetic placement of mitochondria, we used the MAM60+GFmix model to estimate log-likelihoods on two sets of fixed trees (Fig. 2A, Fig. 2B, Fig. S4). The first tree set was inferred from the untreated dataset (108 genes, 33,704 sites), whereas the second tree set was inferred from a compositionally homogenized dataset through site removal (108 genes, 16,029 sites); the latter dataset minimized the differences of G A R P/F I M N K Y amino acid ratios among taxa (Table S5). (Both tree sets were inferred using the MAM60 site-heterogeneous model; see above.) We then varied the position of mitochondria along all backbone branches on each fixed tree (Fig. 2A, Fig. 2B, Fig. S4). Furthermore, we also grouped proteins into partitions according to distances calculated based on their G A R P/F I M N K Y compositional disparity (Fig. S5). Our analyses show that likelihoods estimated under the MAM60+GFmix model improved significantly when compared to conventional site-heterogeneous models (Fig. 2D, Table S6, likelihood ratio test (LRT) p-value = 0); model fit was improved even more when the proteins were grouped into ten separate partitions according to G A R P/F I M N K Y compositional disparity (Fig. 2D, Table S6, LRT p-value = 0). Importantly, the partitioned MAM60+GFmix model clearly favours trees that display the *Alphaproteobacteria*-sister relationship and where the grouping of long-branching and compositionally biased taxa (e.g., *Pelagibacterales*, *Holosporaceae*) is disrupted (i.e., those trees recovered from compositionally homogenized datasets through ζ site removal; Fig. 2D, Table S6). This suggests that the removal of ζ sites effectively decreases overall compositional heterogeneity and potential artefacts.

The top three trees often favored by the MAM60+GFmix model (i.e., those with the highest likelihoods) have mitochondria in adjacent branches: *Alphaproteobacteria*-sister (trees A11 and B9 in Fig. 2A and Fig. 2B), *Rickettsiales*-sister (trees A5 and B4 in Fig. 2A and Fig. 2B), and mitochondria as sister to all alphaproteobacteria except the *Rickettsiales* (or *Caulobacteridae*-sister; trees A10 and B8 in Fig. 2A and Fig. 2B)[29,47]. However, Bonferroni-corrected χ^2 topology tests show that the optimal trees that display the *Alphaproteobacteria*-sister relationship are significantly better than all trees with other positions for mitochondria (see Fig. 2D). Even though the *Alphaproteobacteria*-sister relationship is also favored by the MAM60+GFmix model for the mitochondrion-encoded protein dataset, the *Caulobacteridae*-sister relationship cannot be rejected by the Bonferroni-corrected χ^2 tests (i.e., p-values > 0.05; Table S6). This further supports the notion that the phylogenetic signal for the placement of mitochondria is weaker in mitochondrion-encoded proteins (see above). The *Rickettsiales*-sister relationship is rejected for all datasets and models (p-value < 0.005; Table S6). Overall, most of our distinct phylogenetic approaches show support for the *Alphaproteobacteria*-sister hypothesis.

Discussion

We have found significant support for the *Alphaproteobacteria*-sister hypothesis that has the mitochondrial lineage as the closest sister to all currently sampled alphaproteobacteria. Our findings thus conflict with the recent suggestion that mitochondria may branch within the *Alphaproteobacteria* as sister to the *Rickettsiales*[12]. Indeed, we believe that the design of the study by Fan *et al*., (2020) was particularly prone to artefacts. In an effort to choose less compositionally biased (i.e., G+C-rich) species for mitochondria and the *Rickettsiales*, these authors inadvertently selected species that are more divergent than most members of their respective groups. For example, the inclusion of mitochondria of flowering plants led to a considerably long stem branch for the mitochondrial lineage (see their Fig. S31-48). Similarly, *Anaplasma*, *Neorickettsia*, and *Wolbachia* (*Anaplasmataceae*) are among the longest branches in the *Rickettsiales* (see their Fig. S50; see also our Fig. S2). All these species are secondarily, and not ancestrally, less compositionally biased, i.e., they evolved from species with A+T-rich genomes. Moreover, their analyses were based on a rather small dataset that comprised only 18 or 24 mitochondrion-encoded genes (5,583 and 6,643 sites, respectively) and fewer than 41 taxa. These factors may, in combination, have led to the inference of poorly supported trees (e.g., see their Figs. S31-40), and an artefactual attraction between mitochondria, the *Rickettsiales*, and the FEMAG I and II groups (i.e., Fast-Evolving MAG I and II; see their Fig. 4).

Several previous studies have suggested that mitochondria were either sister to the *Rickettsiales*[18–20] or phylogenetically embedded in a larger group comprised of both the *Rickettsiales* and the *Holosporaceae*[20]. These hypotheses implied that the mitochondrial ancestor may have been an intracellular parasite: throughout its early evolution, the ancestor of mitochondria changed its function from an energy parasite to an ATP-producing respiratory organelle[18–21]. The finding that mitochondria are no longer phylogenetically associated to the *Rickettsiales* and are instead sister to the entire *Alphaproteobacteria* clade makes a parasitic origin of mitochondria less plausible. However, the nature of the mitochondrial ancestor remains poorly constrained. Future studies on species of the MarineProteo1 clade might shed some light on the early evolution of the *Alphaproteobacteria*, and possibly also on the mitochondrial ancestor. However, we note that the MarineProteo1 clade is separated by a long branch from the *Alphaproteobacteria* and mitochondria. Currently available genomes for the MarineProteo1 clade are relatively small, but not necessarily compositionally biased, and suggest that these alphaproteobacteria might be reduced and physiologically specialized (Fig. S2, Table S4).

Unravelling the deep evolutionary history of mitochondria is an inherently hard phylogenetic problem. One of the main challenges is to properly account for the drastically different compositional biases across anciently diversified lineages[29]. Here, we have moved towards overcoming this major obstacle. Our newly developed and implemented site-and-branch-heterogenous model allowed us, for the first time, to test different phylogenetic placements for mitochondria relative to the *Alphaproteobacteria* while accounting for the drastic amino acid compositional changes that alphaproteobacterial and mitochondrial proteins have undergone. A consilient view emerges from the combination of modelling and reducing compositional heterogeneity: the *Alphaproteobacteria*-sister hypothesis is robust and unlikely to be artefactual. However, we caution that the phylogenetic signal preserved in mitochondrion-encoded proteins is weak and ambiguous. The recovery of the *Rickettsiales*-sister relationship in previous studies[11,12] may thus be result of ambiguous phylogenetic signal and long-branch attraction. Therefore, we suggest that it is currently best to view mitochondria as an early offshoot of the alphaproteobacterial lineage that diverged just prior to the diversification of known extant groups. This is suggested by the short internal branch lengths between mitochondria and *Alphaproteobacteria* (see Fig. 2A, Fig. 2B) and is supported by the shared presence of the Mitochondrial Contact Site and Cristae Organizing System (i.e., a Mitofilin domain-containing Mic60) in only mitochondria and the *Alphaproteobacteria*, but not in members of the *Magnetococcia* and MarineProteo1[48,49] (Fig. S2, Table S4). Future efforts should focus on exploring diverse environments for unknown and extant alphaproteobacterial lineages that may be more closely related to mitochondria.

Materials and Methods

310 Metagenomic sequencing and MAG assembly

311 Samples collected from (1) microbial mats in the Salada de Chiprana (Spain, December 2013), Salar de
312 Llamara[50], Lakes Bezymyannoe and Reid (Antarctica, January 2017) and several hot springs around
313 Lake Baikal (Southern Siberia, July 2017), (2) microbialites in Lake Alchichica[51], and (3) sediments in
314 Lake Baikal, were fixed in ethanol (>70%) *in situ* and stored at -20°C as previously described[50]. Total
315 DNA was purified from samples using the DNeasy PowerBiofilm Kit (QIAGEN, Germany) by following the
316 manufacturer's guidelines. DNA extracted from microbialite fragments was further cleaned using the
317 DNeasy PowerClean Cleanup Kit (QIAGEN, Germany) as previously described[52]. DNA was quantified
318 using Qubit®. DNA library preparation and sequencing were performed with an Illumina HiSeq2000 v3
319 (2x100 bp paired-end reads) by Beckman Coulter Genomics (Danvers, MA, USA), and with an Illumina
320 HiSeq2500 (2x125 bp paired-end reads) by Eurofins Genomics (Ebersberg, Germany). A summary of the
321 metagenomic libraries sequenced can be found in Table S2.

322 Raw Illumina short reads from all sequenced Illumina paired-end libraries were quality-assessed with
323 FastQC v.0.11.7 and quality-filtered with Trimmomatic v.0.36[53]. Libraries made from samples from Lake
324 Alchichica and the Llamara saltern were processed with the following workflow. Libraries were individually
325 assembled, and technical replicates co-assembled (Table S2), with metaSPAdes v.3.10.0[54]. Contigs
326 smaller than 2,500 bp in the (co-)assemblies were removed. Filtered reads were then individually mapped
327 onto each assembly with Bowtie2 to obtain contig coverages[55]. Contigs were binned using MaxBin v.2.2.2
328 which relies on differential coverage across samples, tetranucleotide composition and single-copy marker
329 genes[56]. The completeness and contamination of the bins reported by MaxBin v.2.2.2 were assessed with
330 CheckM v.1.0.12[57]. Genome bins that were phylogenetically affiliated to the *Alphaproteobacteria* based
331 on the manual examination of the CheckM reference genome tree (itself based on the concatenation of
332 43 marker genes) were retained. Reads were then individually mapped onto each alphaproteobacterial
333 genome bin with Bowtie2. All paired and unpaired reads that successfully mapped to the
334 alphaproteobacterial bins were subsequently co-assembled with metaSPAdes. The resulting co-assembly
335 was processed through the Anvi'o metagenomic workflow[58]. In brief, reads were mapped to the final
336 metaSPAdes co-assembly with Bowtie2 to obtain contig coverage values. DIAMOND searches[59] of
337 predicted proteins against the NCBI GenBank nr database were done to assign taxonomic affiliations to
338 each contig. CONCOCT2[60], implemented in the Anvi'o suite, was used to bin the resulting metagenome.
339 Contigs were organized according to the composition and coverage by anvi-interactive. The predicted
340 CONCOCT2 bins were visualized and manually refined based on their composition, coverage, taxonomy
341 and completeness/redundancy. Libraries made from samples from Antarctica, the Chiprana saltern and
342 Lake Baikal were processed with the following workflow. Libraries from the same location or environment
343 type were co-assembled with MEGAHIT v.1.1.1[61]. Contigs smaller than 2,500 bp in the co-assemblies
344 were removed. Filtered reads were then individually mapped onto each co-assembly with Bowtie2 to
345 obtain contig coverages. Contigs were binned using three different binners (MetaBAT v.2.12.1[62], MaxBin
346 2.2.4[56], CONCOCT2[60]) and their results were combined into consensus contigs bins with DAS Tool
347 v.1.1.0[63].

348 Marker protein selection

349 We built an expanded dataset of mitochondrion- and nucleus-encoded proteins of alphaproteobacterial
350 origin in eukaryotes. For the nucleus-encoded proteins, BLAST[64] similarity searches of all proteins
351 contained in the predicted proteomes of 13 representative eukaryotes were conducted against a
352 database of 176 prokaryotes (136 bacteria and 40 archaea). BLAST hits were clustered into homologous
353 families with a custom Perl script, aligned with MAFFT and the L-INS-I method[65], and then trimmed with
354 BMGE[66]. Phylogenetic trees for each homologous gene family were inferred under the LG model in
355 RAxML v.8[67]. These trees were then sorted based on the criterion that eukaryotes form a clade with
356 alphaproteobacteria. Manual inspection of the trees then followed to remove paralogs and contaminants.
357 For mitochondrion-encoded genes, mitochondrial clusters of orthologous genes (MitoCOGs)[68] that are
358 widespread among eukaryotes were used.

359 Both mitochondrion-, and nucleus-encoded candidate marker proteins were then compared through
360 BLAST searches against those reported previously by Wang and Wu (2015)[20] and Martijn *et al.*, (2018)[11].
361 Our dataset encompassed most proteins from these other datasets, with few exceptions. The non-
362 redundant and remaining candidate marker proteins comprising the union of these five datasets, were
363 then further screened phylogenetically. Using a representative eukaryotic (mitochondrial) query for each
364 marker gene, BLAST searches were done against a database that comprises 107 diverse bacteria
365 (representing 27 cultured phyla) and 23 diverse eukaryotes (representing 6 major groups); eukaryotes
366 were selected based on the availability of both mitochondrial and nuclear genomes or transcriptomes
367 (see Table S7). Homologues were aligned with MAFFT, alignments trimmed with TrimAl[69] and single-
368 protein trees inferred with IQ-TREE[70]. The single-protein trees were inspected visually to remove
369 duplicates, paralogues, and any other visual outlier such as extremely divergent sequences. Single-
370 protein trees were then re-inferred from the curated alignments and visually inspected. Proteins for which
371 trees showed a sister relationship between eukaryotes and alphaproteobacteria were kept for further
372 analyses. Finally, these candidate marker proteins were annotated and further refined using the EggNOG
373 database and BLASTp searches. The final marker proteins set comprised 108 genes, 64 of which are
374 exclusively nucleus-encoded, 17 are exclusively mitochondrion-encoded, and 27 are both mitochondrion-
375 and nucleus-encoded (Fig. S1). The annotations confirm that all marker proteins are predicted to be
376 localized to mitochondria in eukaryotes (Table S1).

377 Dataset assembly

378 To increase taxon sampling as much as possible, MAGs reported in Anantharaman *et al.*, (2016)[33],
379 Graham *et al.*, (2018)[34], Delmont *et al.*, (2018)[35], Martijn *et al.*, (2018)[11], Mehrshad *et al.*, (2016)[36], Tully *et*
380 *al.*, (2017)[37], Tully *et al.*, (2018)[38] and Parks *et al.*, (2017)[39] were added to those reconstructed here (see
381 Metagenomic analyses). To improve the quality of our MAG selection, MAGs were analyzed with the
382 CheckM lineage workflow and those with quality values (completeness – 5x contamination) lower than 50
383 were discarded, just as done before by Parks *et al.*, (2017, 2018)[39,40]. MAGs were then filtered according
384 to their taxonomic affiliation to the *Alphaproteobacteria*. A phylogenetic tree for all MAGs and all
385 *Proteobacteria* taxa in the GTDB r89 database[40] was inferred from 120 marker proteins, built-in in the
386 GTDB-Tk software, using IQ-TREE v.1.6.10[70] and the LG4X+F model. To increase phylogenetic
387 accuracy, a second tree was inferred with the LG+PMSF(C60)+G4+F using the LG4X tree as guide. All
388 MAGs that fell within the *Alphaproteobacteria* clade in the GTDB-Tk tree were chosen for subsequent
389 analyses. Together, these added up to more than 3,300 alphaproteobacteria. In order to reduce
390 computational burden, Treemmer v.0.1b was then used to reduce the number of alphaproteobacterial
391 taxa from the GTDB-TK tree while maximizing phylogenetic diversity[41]. The Treemmer analysis was
392 constrained so representatives from major clades, as visually identified, were retained. Finally, a set of
393 reference alphaproteobacteria (formally described species) were added, and long-branching
394 alphaproteobacteria were replaced by short-branching relatives.

395 To retrieve homologues, PSI-BLAST searches with either one, two, or three iterations using
396 representative mitochondrial (eukaryotic) query sequences for each marker protein were done against a
397 database that comprised all carefully selected predicted proteomes. To remove non-orthologous
398 sequences, homologous protein sets were retrieved for each marker protein, aligned with MAFFT,
399 trimmed with TrimAl and trees inferred with IQ-TREE. The single-protein trees were visually inspected to
400 remove duplicates, paralogues, and any other visual outlier such as extremely divergent sequences. The
401 curated homologous protein sets were finally aligned again with MAFFT v.7.3.10 and the L-INS-I method.
402 To increase phylogenetic signal by removing poorly aligned and non-homologous aligned regions, Divvier
403 v.1.0 was used with the -partial and -mincol options[71]. Only sites with more than 10% of data were
404 retained. To reduce incongruency among proteins due to, for example, lateral gene transfer, Phylo-MCOA
405 v.1.4[72] was employed on single-protein trees with UFBoot2+NNI as branch support which were inferred
406 with IQ-TREE v.1.6.10 and the best-fitting model as identified by Model-Finder[70,73]. Single-protein
407 alignments were concatenated with SequenceMatrix v.1.8[74].

408 Phylogenetic analyses using site-heterogeneous models

409 For multi-protein phylogenetic analyses on the supermatrix, trees were first inferred in IQ-TREE v.1.6.10
410 under the LG4X+F model. The resulting site-homogenous tree was then used as a guide tree to infer a
411 new phylogenetic tree under the LG+PMSF(C60)+F+G4 model[75]. Consequently, the resulting site-
412 heterogenous tree was used as a guide tree to infer a new phylogenetic tree under the dataset-specific
413 LG+PMSF(MAM60)+F+G4 model. The dataset-specific MAM60 model was estimated using the MAMMaL
414 software[43]. This site-heterogeneous mixture model is directly inferred from the dataset analyzed and
415 therefore is more specific than the general C10-60 mixture models. To account for more than 60 (e.g.,
416 C60 or MAM60) amino-acid composition profiles across the data, we used the general UDM128 mixture
417 model as LG+UDM128+G4+F that allows for 128 amino acid composition profiles[76]. The software FunDi
418 was used to estimate functionally divergent sites in the branch that separates the mitochondrial lineage
419 from all other taxa[42]. Sites with a probability of being functionally divergent > 0.5 were removed.
420 Progressive removal of compositionally heterogeneous sites was performed according to the ζ and the χ^2
421 metrics/methods as described before[11,29,44]. Both metrics are designed to estimate compositional
422 heterogeneity per site based on different criteria.

423 Bayesian analyses were conducted with PhyloBayes MPI v1.8 using the CAT-GTR+G4 model[77,78].
424 PhyloBayes MCMC chains were run for >20,000 cycles or until convergence between the chains was
425 achieved and the largest discrepancy in posterior probabilities for splits between chains ('max-diff') was
426 <0.1. Individual chains were summarized into a Bayesian consensus tree using a burn-in of 500 trees and
427 subsampling every 10 trees. However, most chains did not reach convergence or resolve the
428 phylogenetic placement of mitochondria relative to alphaproteobacterial lineages (Mendeley Data).

429 Phylogenetic analyses using the site-and-branch-heterogeneous GFmix model

430 The site profile mixture models discussed above have C site frequency profiles and a K-class discretized
431 gamma mixture model for site rates. Under these models, the likelihood of site pattern $\mathbf{x_i}$ at site *i* is given
432 by:

433 $$P(\mathbf{x_i}; w_c, \boldsymbol{\theta}) = \sum_{c=1}^{C} w_c \sum_{k=1}^{K} P\left(\mathbf{x_i} \mid r_k, \boldsymbol{\pi}^{(c)}; \boldsymbol{\theta}\right)/K$$

434 Where r_k is the site rate of gamma-rates class *k*, $\boldsymbol{\pi}^{(c)}$ is the vector of amino acid frequencies in class *c* of
435 the site-profile mixture model, w_c is the class weight and θ is the vector of other adjustable parameters
436 (branch lengths, α shape parameter and tree topology) in the model. In order to model shifts in the
437 relative frequencies of the amino acids G A R P (specified by G+C-rich codons) and F I M N K Y
438 (specified by A+T-rich codons) in different branches of the tree, the foregoing vectors of amino acid
439 frequencies, $\boldsymbol{\pi}^{(c)}$, are modified in a branch-specific manner in the following way.

440 Let *b* denote the ratio of aggregate frequencies of G A R P to F I M N K Y amino acids; i.e., $b := \pi_G/\pi_F$
441 for $\pi_G = \sum_{j \in \{G,A,R,P\}} \pi_j$ and $\pi_F = \sum_{j \in \{F,Y,M,I,N,K\}} \pi_j$ where π_j is the frequency of amino acid *j*. For every
442 branch *e* in the phylogenetic tree under consideration, we can obtain estimates by a hierarchical
443 procedure where b_e is obtained from the GARP/FIMNKY ratio of all of the sequences at the tips of the
444 tree that descend from branch *e*. Using these estimates, the values in the class frequency vectors, $\boldsymbol{\pi}^{(c)}$,
445 for any site profile class are modified in the following way to be branch-*e*-specific class frequencies, $\boldsymbol{\pi}^{(ce)}$.
446 The modified class frequencies have to satisfy a number of constraints including:

447 $$\pi_j^{(ce)} = \begin{cases} \mu^{(ce)} S_G^{(e)} \pi_i^{(c)} & j \in \{G, A, R, P\} \\ \mu^{(ce)} S_F^{(e)} \pi_j^{(c)} & j \in \{F, Y, M, I, N, K\} \\ \mu^{(ce)} \pi_j^{(c)} & otherwise \end{cases}$$

448 and $\sum_j \pi_j^{(ce)} = 1$ and

$$\frac{\sum_{c=1,j\in\{G,A,R,P\}}^{C} w_c \pi_j^{(ce)}}{\sum_{c=1,j\in\{F,Y,M,I,N,K\}}^{C} w_c \pi_j^{(ce)}} = b_e$$

This leads to non-linear equations for $\mu^{(ce)}$, $S_G^{(e)}$ and $S_F^{(e)}$ that are solved numerically for each branch *e* to generate the modified class frequencies. For each branch and site class *c*, $\pi_j^{(ce)}$ values are used to create a new transition $Q^{(ce)}$ matrix for likelihood calculations for all site patterns over that branch. The same approach is used with frequencies coming all extant taxa to obtain the root frequencies. A software implementation of GFmix is available at https://www.mathstat.dal.ca/~tsusko/software.html.

Partitioning the data matrix for GFmix calculations.

The foregoing framework assumes that for each aligned protein in a given concatenated dataset, the GARP/FIMNKY ratios (b_e's) for every branch in the tree will be similar. However, for our data matrix this assumption is not true as different proteins show different degrees of GARP/FIMNKY variation across taxa depending on the location of the corresponding gene (e.g., nucleus-encoded vs. mitochondrial-encoded) and degree of conservation. For this reason, we clustered the proteins in our dataset into groups in the following way. For each protein *v* and each taxon *t* we calculated the GARP/FIMINKY ratio, $b_v^{(t)} = \pi_G^{(t)}/\pi_F^{(t)}$. Then, we calculated the overall distance between these ratios for every pair of proteins *u* and *v* in the data matrix as $d_{u,v} = \sum_t \left|b_v^{(t)} - b_u^{(t)}\right| / N_{u,v}$ where $N_{u,v}$ is the total number of taxa for which sequences were available for both proteins (this normalization accounts for the differing amounts of missing data for different proteins). The proteins were then clustered based on $d_{u,v}$ distances using the UPGMA algorithm in MEGA-X[79] and 10 clusters were chosen as a computationally tractable number of partitions for further analysis. The GFmix model was then applied to these 10 partitions allowing for separate b_e values and branch lengths for each partition. The overall log-likelihoods for topologies were obtained as the sum of log-likelihoods of that topology over all partitions.

To test the relative fits of the foregoing phylogenetic models to the data we used likelihood ratio tests (LRTs). Briefly, the log-likelihood of a given mixture model (e.g., MAM60) under its optimal tree was compared to the log-likelihood of the corresponding mixture-GFmix model. The former model is a special case of the latter where all the b_e parameters are equal to the overall GARP/FIMNKY ratio. The likelihood ratio statistic LRS, which is defined as twice the difference in these log-likelihoods, was calculated and a *p*-value was determined as $P[\chi_d^2 > LRS]$ where *d* is the difference the number of additional parameters in the more complex model (i.e., the b_e parameters); here *d*=2t-2 where t is the number of taxa. A similar approach is taken to compare the partitioned models to the non-partitioned models. In this case there were additional branch lengths and b_e parameters for each partition and so for 10 partitions, *d*=9(2t-2)+9(2t-3). We note that this test is conservative because b_e estimates were not determined by maximum likelihood. Therefore, the true *p*-values for the LRTs are less than $P[\chi_d^2 > LRS]$. If the LRT rejects the null hypothesis under these conditions, then the correct test would also reject.

Topology testing using the Bonferroni-corrected χ^2 test.

The topology test is a variation of the chi-squared test presented in Susko (2014)[80] that corrects for selection bias. The chi-squared test is a test of two trees. The null hypothesis $H_0: \tau = \tau_0$ is tested against $H_A: \tau = \tau_A$ where τ is the true topology. As a test statistic, it uses the likelihood ratio statistic, *LRS*, which is defined as twice the difference between the maximized log likelihood when the true topology is τ_A and the maximized log likelihood for τ_0. It gives a *p*-value $p(\tau_A) = P[\chi_d^2 > LRS]$, the probability that a chi-squared random variable with *d* degrees of freedom is greater than the observed *LRS.* Here the degrees of freedom*, d,* are determined as the number of branches that are 0 in the consensus tree representing both τ_0 and τ_A.

In the absence of a particular τ_A of interest, to test whether $H_0: \tau = \tau_0$ can be rejected, we consider the alternative $H_A: \tau = \hat{\tau}$, where $\hat{\tau}$ is the maximum likelihood (ML) topology. Because the topology under the

alternative hypothesis was selected based on the data rather than being fixed *a priori*, this can induce a selection bias[81]. The Bonferroni approach uses a input set of trees and approximates the *p*-value when $H_A: \tau = \hat{\tau}$ by the Bonferroni-corrected *p*-value one would obtain testing $H_0: \tau = \tau_0$ against $H_i: \tau = \tau_i$, $i \in A$ where A is the set of input trees that are compatible with the consensus tree of τ_0 and $\hat{\tau}$.

The approximation is based on probability calculations treating the consensus tree of $\hat{\tau}$ and τ_0 as the true tree. This is consistent with what is done in the chi-square test and in testing more generally, where one often calculates *p*-values under parameters on the boundary between the null and alternative hypotheses spaces (see [80] for additional discussion). If the true tree is the consensus tree, then it is likely that the ML topology will be in A. Because the largest likelihood is the one corresponding to $\hat{\tau}$, the smallest *p*-value among the $n(A)$ *p*-values obtained by testing $H_0: \tau = \tau_0$ against $H_i: \tau = \tau_i$ is likely to be $p(\hat{\tau})$; there is some possibility that a tree with a smaller degrees of freedom would give the smallest *p*-value, so this is an approximation. In summary, $p(\hat{\tau})$ is approximately the same as the minimum *p*-value obtained by testing $H_0: \tau = \tau_0$ against $H_i: \tau = \tau_i$.

Rephrasing the test as approximately the same as the result of multiple tests $H_0: \tau = \tau_0$ against $H_i: \tau = \tau_i$, $i \in A$ lays bare that multiple testing is the source of selection bias. Bonferroni correction is a widely used approach to adjusting for multiple testing. As one final approximation, rather than using the usual Bonferroni-corrected *p*-value, $\mathrm{n(A)\ p(\hat{\tau})}$, we use the exact correction had the *p*-values coming from the tests been independent,

$$1 - [1 - p(\hat{\tau})]^{n(A)}.$$

This *p*-value is approximately the same as the usual Bonferroni correction when $n(A)\, p(\hat{\tau})$ is small, which is the case of greatest interest, but has the advantage of always being between 0 and 1. Additional information about the Bonferroni correction is available in [82].

Profile Hidden Markov Model (pHMM) searches

To search for bacteriochlorophyll enzymes, a set of 17 custom-made pHMMs for the genes *bchB, bchC, bchD, bchE, bchF, bchG, bchH, bchI, bchJ, bchL, bchM, bchN, bchO, bchP, bchX, bchY, bchZ* was used against predicted proteomes from the MAGs reconstructed in this study. These pHMMs were created from manually curated sets of *bch* genes from diverse proteobacteria. The searches were done with the program hmmsearch of the HMMER suite using an E-value cut-off of 1E-25. To search for mitofilin-domain containing *mic60* genes, the Pfam pHMM for Mitofilin (PF09731) was used with its own GA cut-off value.

Data Availability

Sequencing data were deposited in NCBI GenBank under the BioProjects PRJNA315555, PRJNA438773, PRJNAXXXXXX, PRJNAXXXXXX, PRJNAXXXXXX, and PRJNA703749. Assembled metagenomes, novel alphaproteobacterial MAGs, and gene files (unaligned, aligned, and aligned and trimmed) are available at: DOI: 10.6084/m9.figshare.14355845. Datasets and phylogenetic trees inferred in this study are available at: DOI: http://dx.doi.org/10.17632/dnbdzmjjkp.1. The GFmix model software is available at: https://www.mathstat.dal.ca/~tsusko/software.html.

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715

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