1 **Dating *Alphaproteobacteria* evolution with eukaryotic fossils**

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17

18 **Abstract**

19 Elucidating the timescale of evolution of bacteria is key to testing hypotheses on their
20 co-evolution with eukaryotic hosts, which, however, is largely limited by the scarcity
21 of bacterial fossils. Here, we incorporate eukaryotic fossils to date the divergence
22 times of *Alphaproteobacteria*, based on the endosymbiosis theory that mitochondria
23 evolved from an alphaproteobacterial lineage. We estimate that *Alphaproteobacteria*
24 arose ~1900 million years (Ma) ago, followed by rapid divergence of their major
25 clades. We show that the origin of *Rickettsiales*, an order of obligate intracellular
26 bacteria whose hosts are mostly animals, predates the emergence of animals for ~700
27 Ma but coincides with that of eukaryotes. This, together with reconstruction of
28 ancestral hosts, strongly suggests that early *Rickettsiales* lineages had established
29 previously underappreciated interactions with unicellular eukaryotes. Our
30 mitochondria-based approach displays higher precision and robustness to
31 uncertainties compared with the traditional strategy using cyanobacterial fossils, and
32 suggests that previous applications using divergence times of the modern hosts of
33 symbiotic bacteria to date the bacterial tree of life may need to be revisited.

34

35 Main

36 The *Alphaproteobacteria* is one of the largest groups within bacteria (Ettema and
37 Andersson, 2009) and of great evolutionary significance for holding the origin of the
38 mitochondrion (Wang and Wu, 2015; Yang et al., 1985). The *Alphaproteobacteria* has
39 extensively diversified since its ancient origin, and comprised some of the most
40 environmentally abundant and metabolically diverse organisms on Earth (Boussau et
41 al., 2004; Ettema and Andersson, 2009; Luo et al., 2013). The intimate association
42 between some alphaproteobacterial lineages and eukaryotes is of central importance
43 for agricultural (e.g., rhizobia) and medical (e.g., rickettsia) applications. This makes
44 *Alphaproteobacteria* a promising system to study the timing of bacterial evolution and
45 their correlation with geological, ecological and evolutionary events (Luo et al., 2013;
46 Sun et al., 2017; Wang et al., 2020). However, the traditional way of divergence time
47 estimation has several limits when applied to *Alphaproteobacteria* and other
48 prokaryotes. First, fossil records of bacteria are extremely scarce and controversial in
49 terms of their estimated dates (Schirmer et al., 2016). Second, the most widely
50 used prokaryotic fossils are from cyanobacteria, but the long evolutionary distance
51 between cyanobacteria and other bacteria causes large uncertainties in dating (Nei et
52 al., 2001). Last, some studies assumed a strict relationship of bacteria-host evolution,
53 and calibrated the evolution of pathogenic/symbiotic bacteria based on the divergence

54 time of their modern hosts (mostly animals and plants). However, this precludes the
 55 possibility of host switching, which could occur frequently during evolutionary
 56 processes spanning millions of years (Wang et al., 2020). Due to these challenges, the
 57 origin time of *Alphaproteobacteria* estimated by previous studies varies from less
 58 than 600 million years (Ma) to more than 2000 Ma (Battistuzzi et al., 2004;
 59 Battistuzzi and Hedges, 2009; Chriki-Adeeb and Chriki, 2016; Luo et al., 2013),
 60 making any narratives based on its evolutionary timing contentious.

61 Recently, horizontal gene transfer (HGT) has been suggested to have great
 62 potential in dating the evolution of bacteria (Davín et al., 2018; Wolfe and Fournier,
 63 2018). Briefly, if the donor of an HGT event does not have fossil records while the
 64 recipient does, the temporal information recorded in the recipient can be transferred to
 65 date the evolution of the donor group (and vice versa), thereby bypassing the paucity
 66 of fossils in the donor lineage. Inspired by this idea, we developed a new strategy to
 67 date the divergence times of *Alphaproteobacteria* based on the endosymbiosis theory
 68 that the mitochondrion was transferred to a host nucleus from a bacterial lineage
 69 (Sagan, 1967), which was later shown to be within (Fan et al., 2020; Wang and Wu,
 70 2015; Yang et al., 1985) or closely related to (Martijn et al., 2018)
 71 *Alphaproteobacteria* by modern phylogenetic analysis. As mitochondria are
 72 characteristic of eukaryotes, we took advantages of eukaryotic fossils to anchor the

73 divergence time of *Alphaproteobacteria* in a tree integrating both alphaproteobacterial
74 and mitochondrial lineages.

75 We first reconstructed a phylogenomic tree of 80 carefully selected
76 *Alphaproteobacteria* and mitochondrial genomes using 24 conserved genes based on
77 prior phylogenomics studies (Martijn et al., 2018; Muñoz-Gómez et al., 2019) (see
78 Methods and Supplementary Note S1.1). We employed rigorous approaches to
79 delineate phylogenetic artefacts caused by long branch attraction and compositional
80 heterogeneity (see Methods), and obtained results consistent with recent studies where
81 i) *Rickettsiales*, *Holosporales* and *Pelagibacterales* (SAR11) had independent origins
82 (Muñoz-Gómez et al., 2019), and ii) mitochondria branched as a sister to
83 *Alphaproteobacteria* (Martijn et al., 2018) (Fig. S1A). We also tested the impact of
84 alternative topologies on dating (Fig. S1C; see below). We compiled two datasets to
85 estimate the time divergences within the *Alphaproteobacteria* calibrated by eukaryotic
86 fossils with relaxed molecular clock (Drummond et al., 2006), which accounts for
87 substitution rate variations among branches. The first dataset, which we referred to as
88 the mito-encoded dataset, was based on the aforementioned 24 conserved gene
89 encoded by mitochondrial genomes (Dataset S1), and the mitochondrial lineages
90 mainly comprised species of green plants, red algae and jakobids, whose
91 mitochondrial genomes are both gene-rich and relatively slowly-evolving (Martijn et

92 al., 2018; Rodríguez-Ezpeleta and Embley, 2012). Four high-confidence fossils from
 93 land plants and red algae were used as the calibration points (Supplementary Note
 94 S2.1; Fig. S2A). The second dataset, referred to as the nuclear-encoded dataset, was
 95 based on 22 mitochondria-derived genes that had been transferred to the nuclear
 96 genome identified by Wang and Wu, 2015 (Dataset S1; see Methods). This dataset not
 97 only circumvented the problem that in many eukaryotes genes encoded by
 98 mitochondrial genomes are few (e.g., apicomplexans and dinoflagellates) or
 99 fast-evolving (e.g., animals and fungi) (Roger et al., 2017), but integrated six
 100 additional eukaryotic fossils (Fig. S2A; Supplementary Note S2.1), allowing directly
 101 comparing the divergence time between symbiotic bacteria and their eukaryotic hosts
 102 on the same tree.

103 We selected a best-practiced scheme based on systematic comparisons of
 104 different combinations of parameters of MCMCTree for the mito- and
 105 nuclear-encoded datasets (see Methods and Supplementary Note S1.2). Similar
 106 divergence times were recovered for most nodes between the two datasets, although
 107 the mito-encoded dataset estimated older ages for deep nodes (Fig. 1). As shown in
 108 the infinite-sites plots (up- and bottom-left panels in Fig. 1), the posterior mean ages
 109 versus 95% HPD (highest posterior density) widths approached a straight line,
 110 suggesting that the uncertainty in time estimate was predominantly caused by the

111 uncertainty associated with fossil calibrations (Rannala and Yang, 2007). The
 112 estimated ages in the nuclear-encoded dataset exhibited smaller 95% HPD intervals,
 113 hence smaller uncertainties, likely due to their more calibration information compared
 114 with the mito-encoded dataset.

115 Most alphaproteobacterial orders diverged 1500-1000 Mya, and *Rickettsiales* and
 116 *Pelagibacterales* appeared to be the oldest and youngest alphaproteobacterial orders,
 117 respectively (Fig. 1). The origin times of *Rickettsiales*, an obligate endosymbiont
 118 lineage whose hosts cover diverse eukaryotes but mostly animals (Merhej and Raoult,
 119 2011), were estimated to be 1771 Ma (95% HPD 1972-1565 Ma) and 1523 Ma (95%
 120 HPD 1677-1368 Ma) using the mito- and nuclear-encoded datasets, respectively (Fig.
 121 1). A merit of our mitochondria-based method is that divergence times of the
 122 eukaryotic hosts and of the host-associated bacteria can be simultaneously estimated.
 123 As shown in Fig. 1, we dated the origin of animals to be 803 Ma (95% HPD 844-752
 124 Ma), consistent with previous dating analyses (Betts et al., 2018; Dos Reis et al., 2015;
 125 Parfrey et al., 2011), but not others (Hedges et al., 2004). We also estimated that
 126 mitochondrial lineages diverged from *Alphaproteobacteria* ~1900 Ma ago and that the
 127 last common ancestor (LCA) of mitochondria occurred ~1550 Ma ago (Fig. 1). Thus,
 128 the origin of *Rickettsiales* likely predated the evolutionary emergence of animals for
 129 ~700 Ma but coincided with the mitochondrial endosymbiosis process and the

130 occurrence of the LCA of eukaryotes, according to our (Fig. 1) and others' estimates
 131 (Betts et al., 2018; Eme et al., 2014; Parfrey et al., 2011), and fossil records (reviewed
 132 in Butterfield, 2015). This agrees with recent findings of an increasingly broad range
 133 of protistan hosts of *Rickettsiales* (reviewed in Castelli *et al.*, 2016), and suggests that
 134 host switches to animals from protists occurred later in the evolution of *Rickettsiales*.
 135 The origin time of *Holosporales*, another important endosymbiotic lineage in
 136 *Alphaproteobacteria* whose extant members are mostly endonuclear parasites of the
 137 ciliate *Paramecium* (Schrallhammer et al., 2018), dated back to 1379 Ma (95% HPD
 138 1559-1201 Ma) or 1212 Ma (95% HPD 1353-1067 Ma), respectively, based on the
 139 mito- or nuclear-encoded datasets. This implies that the origin of *Holosporales*
 140 roughly coincided with the that of ciliates, which dated back to ~1150 Ma according
 141 to other estimates (Fernandes and Schrago, 2019; Parfrey et al., 2011). While the
 142 above analyses were based on amino acid sequences using MCMCTree, the basic
 143 patterns held similar with PhyloBayes or using coding sequences (Fig. S3).

144 We assessed the impact of uncertainties in Bayesian relaxed molecular clock
 145 time estimation, including the disparity between fossil evidence and molecular clock
 146 estimates, root age, across-branch rate variation, sequence partitioning, and clock
 147 model, on the posterior ages (Dataset S2). When we used only Phanerozoic fossils
 148 and excluded all Proterozoic fossils (which are thought controversial by some), the

149 estimated ages of most nodes were shifted towards the present for the mito-encoded
150 dataset but not for the nuclear-encoded dataset (*Phan* in Figs. 2A, S4). Removing
151 potentially controversial maximum age constraints led to minor changes in the
152 posterior ages (*Max-1* in Figs. 2A, S4). Using more conservative calibrations of the
153 root pushed the time estimates to be slightly older for the mito-encoded dataset
154 (*Root-1* in Figs. 2A, S4). Allowing larger rate variation among branches resulted in
155 highly consistent results with the best-practiced scheme (*Sigma* in Fig. S5).
156 Decreasing the number of partitions resulted in decreased precision, as indicated by
157 the increase of the slope in infinite-sites plots (Fig. S6), but the estimated dates
158 remained similar (*Single partition* in Fig. 2A). The largest changes in the posterior
159 ages were obtained when the independent rates (IR) instead of autocorrelated rates
160 (AR) clock model was used: the divergence times of most alphaproteobacterial orders
161 were shifted towards the present by ~20% (*IR* in Figs. 2A, S4). Collectively, the
162 composite of the ages estimated from six different analyses shows that
163 *Alphaproteobacteria* originated 2012 Ma (95% HPD 2284-1750 Ma) and 1786 Ma
164 (95% HPD 2004-1574 Ma), based on the mito- and nuclear-encoded datasets,
165 respectively (Fig. S7), and diversified soon thereafter.

166 The topology of *Alphaproteobacteria* is another much-debated issue (Fan et al.,
167 2020; Luo, 2015; Muñoz-Gómez et al., 2019; Wang and Wu, 2015). We repeated the

168 MCMCTree analysis by fixing the species phylogeny to 11 alternative topologies (Fig.
169 S1B). Most alphaproteobacterial orders showed highly consistent estimated ages (Fig.
170 2B). However, the posterior mean ages of *Holosporales* varied from ~1800 to ~1200
171 Ma across different topologies. This was because the alternative phylogenetic position
172 of *Holosporales* was a sister to *Rickettsiales*, in contrast to the topology used in the
173 main analysis where *Holosporales* branched within the *Rhodospirillales* (Fig. S1B).
174 Likewise, the origin time of *Pelagibacterales* showed considerable variations
175 depending on whether it formed a monophyletic group with the *Rickettsiales*. On the
176 other side, the impact of the topology of the eukaryotic tree (mitochondria subtree)
177 was relatively minor, as different topologies showed similar estimates of
178 alphaproteobacterial lineages at the order level (Fig. S8).

179 Traditionally, constructing the evolutionary timeline for bacteria is based on
180 fossils of cyanobacteria since bacterial fossils that can be accurately assigned to a
181 taxonomic group are only available for cyanobacteria (Schirrmeister et al., 2016).
182 Based on a best-selected scheme, the dates estimated using calibrations within
183 cyanobacteria were generally comparable to using the mitochondria-based strategy
184 (Fig. S9). However, with a slope of 0.251 in the infinite-sites plot (Fig. 2C), the
185 cyanobacteria-based strategy exhibited less precision compared with the
186 nuclear-encoded dataset (slope of 0.214 in the infinite-sites plot in Fig. 1). Further,

187 analyses with different calibration schemes within cyanobacteria revealed larger
188 variations in the estimated ages for some clades (Fig. S9). For example, by using
189 different calibration schemes, we observed a spread of 444 Ma for the posterior mean
190 age of the *Rickettsiales* crown group with the cyanobacteria-based strategy, in contrast
191 to 103 and 82 Ma as estimated by the mito-encoded and nuclear-encoded datasets of
192 the mitochondria-based method, respectively (Fig. S9), likely underlined by the
193 abundant high-confidence fossils in eukaryotes and the shorter phylogenetic distance
194 between the mitochondrial lineage and *Alphaproteobacteria*.

195 The abundant eukaryotic fossils in our mitochondria-based strategy greatly
196 reduced dating uncertainty compared with the cyanobacteria-based approach. By
197 co-estimating the divergence times of both bacteria and their eukaryotic hosts, it
198 allows directly analyzing their co-evolution with the same dataset on the same tree,
199 thereby avoiding methodological artefacts stemming from comparing age estimates
200 from different studies. In addition, the mitochondria-based approach to date evolution
201 is conceptually distinct from the HGT-based method (Davín et al., 2018; Wolfe and
202 Fournier, 2018), as mitochondrial endosymbiosis is far more complex than simply an
203 HGT event (Roger et al., 2017; Sagan, 1967). Consequently, thanks to the many
204 high-confidence orthologs shared by mitochondrial and alphaproteobacterial lineages,
205 our taxon- and gene-rich multigene data mitigate the challenges faced by others (Shih

et al., 2017) (see Supplementary Note S3.1). Nevertheless, our tentative analysis is subject to other challenges such as the lack of internal fossils within the *Alphaproteobacteria* and violation to molecular clock caused by the fast-evolving nature of mitochondrial genomes. Although its impact should be attenuated by the relaxed molecular clock model, it is important to keep this limit in mind when interpreting the results. Further, the mitochondria-based strategy works well for *Alphaproteobacteria*, but calibrating the evolution of related bacteria like *Beta*- and *Gammaproteobacteria* with this strategy requires additional testing and benchmarking.

The divergence times estimated here agree with some previous studies (Battistuzzi et al., 2004; Battistuzzi and Hedges, 2009), but are much older than in other studies that constrained the origin time of symbiotic or pathogenic bacteria to be the same as that of their dominant modern hosts, thus ignoring the possibility of host shifts (Chriki-Adeeb and Chriki, 2016; Luo et al., 2013; Weinert et al., 2009) (Supplementary Note S3.2). Our results suggest that the origin of *Rickettsiales*, where most extant members adapt to an animal-associated lifestyle, predated animals' emergence for ~700 Ma but coincided with the origin of eukaryotes (Fig. 1). Moreover, ancestral lifestyle reconstruction using BayesTraits (Meade and Pagel, 2016) with a much broader taxon sampling of *Rickettsiales* suggests the LCA of

225 *Rickettsiales* to be symbionts of protists and that the transition rate from
 226 protist-associated to animal-associated lifestyle was more than ten times higher than
 227 that of the reverse process (Fig. 2D-E). This strongly challenges the view of the
 228 concurrence of *Rickettsiales* and animals (Weinert et al., 2009), and instead suggests
 229 frequent host transitions from unicellular eukaryotes to animals during *Rickettsiales*
 230 evolution. Presumably, the predatory nature of early eukaryotes like amoebae or
 231 ciliates might have helped forming primitive association with ancestral *Rickettsiales*
 232 (Vannini et al., 2005). Later, animals could have acquired *Rickettsiales* parasites early
 233 in their evolution by filter-feeding on infected protists (Supplementary Note S3.3).
 234 This scenario is also supported by recent findings of the association between
 235 *Rickettsiales* members with diverse protists (Castelli et al., 2016; Montagna et al.,
 236 2013) and by the presence of amoebae-derived genes in some *Rickettsiales* genomes
 237 (Ogata et al., 2006). *Holosporales* are mostly intracellular parasites of the ciliate
 238 *Paramecium*, but are increasingly found associated with a diverse range of protists
 239 including amoebae, rhizarians and *Euglenozoa* (Muñoz-Gómez et al., 2019),
 240 consistent with their deep divergence. Overall, our results are not contradictory to the
 241 idea of host-bacteria co-evolution, but suggest more frequent host transitions than
 242 previously understood and that caution should be exercised when assigning the
 243 divergence time of bacteria based on that of their modern hosts. Besides, the ubiquity

244 of microbial eukaryotes like amoebae in modern environments reminds people to pay
245 more attention to them as potential reservoirs of emerging human diseases.

246

247 **Methods**

248 **Phylogenomic reconstruction**

249 We followed Martijn *et al.*, 2018, and used the 24 conserved genes encoded by both
250 the mitochondrial and alphaproteobacterial genomes annotated by MitoCOGs
251 (Kannan *et al.*, 2014) to determine their phylogenetic relationship. Eighty genomes
252 were carefully selected for phylogenomic reconstruction based on prior studies
253 (Supplementary Note S1.1; Table S1). Genes were aligned using MAFFT v7.222
254 (Katoh and Standley, 2013) and trimmed with TrimAl v1.4 (“-st 0.001”)
255 (Capella-Gutiérrez *et al.*, 2009). Because *Alphaproteobacteria* is subjected to strong
256 compositional heterogeneity across lineages (Luo, 2015; Martijn *et al.*, 2018;
257 Muñoz-Gómez *et al.*, 2019), which might confound phylogenetic signals and cause
258 phylogenetically unrelated species with similar GC content to cluster together, we
259 recoded the 20 amino acids into four nucleic acid characters according to their
260 physicochemical properties with the dayhoff4 and SR4 recoding scheme, respectively
261 (Luo, 2015; Martijn *et al.*, 2018; Muñoz-Gómez *et al.*, 2019). Phylogenomic
262 reconstruction was performed under the empirical profile mixture model

263 GTR+G+F+C30 with the PMSF approximation (guide tree GTR+G+I+F) and 1,000
264 ultrafast bootstraps using IQ-Tree v1.6.11 (Nguyen et al., 2015). As the trees obtained
265 by dayhoff4 and SR4 recoding schemes showed similar topologies (Fig. S1), we used
266 the one obtained by the dayhoff4 recoding scheme for dating (and we subsequently
267 included more eukaryotic taxa as mitochondrial lineages based on the general
268 consensus understanding of the eukaryotic phylogeny for both the mito- and
269 nuclear-encoded datasets in dating [see Fig. S2A and Supplementary Note S1.1]).

270

271 **Calibration information**

272 Four and ten calibration points within the eukaryote clade were selected for the mito-
273 and nuclear-encoded datasets, respectively (Fig. S2). We based the lower limit of a
274 calibration point upon the most ancient uncontroversial fossil from within the clade.
275 Since fossil records only tell the time that the group of interest had already appeared,
276 the actual origin time of a clade could be more ancient than these minima. Maximum
277 time constraints were determined from the youngest geological formation or
278 stratigraphic range without any members of the clade of interest, as used and
279 recommended by many studies (Betts et al., 2018; Donoghue and Benton, 2007; Dos
280 Reis et al., 2015). Several alternative time constraints were also considered to
281 accommodate the uncertainties in calibration. The full details of calibrations are given

282 in Supplementary Note S2.

283

284 **Divergence time estimation**

285 We compiled two datasets for the mitochondria-based divergence time estimation of

286 *Alphaproteobacteria*, the mito-encoded dataset and the nuclear-encoded dataset. The

287 mito-encoded set was based on the aforementioned 24 orthologs conserved in

288 *Alphaproteobacteria* and mitochondrial lineages. For the nuclear-encoded set, we

289 retrieved the 29 genes that are likely transferred from the mitochondrial genome to the

290 nuclear genome during the early evolution of eukaryotes from Wang and Wu, 2015,

291 and excluded seven genes involving putative paralogs (Dataset S1). Wrongly

292 annotated sequences in each alignment were removed by manually checking the

293 alignment and gene phylogeny.

294 To alleviate the impacts of mutational saturation, we used amino acids in the

295 main analysis but also repeated the analysis with nucleotide sequences (only the first

296 two codon positions). Dating analyses were predominantly carried out with the

297 approximate likelihood calculation with MCMCTree 4.9j (Yang, 2007), and we also

298 examined the consistency of the results with PhyloBayes v4.1b (Lartillot et al., 2009).

299 A constraint tree constructed using the dayhoff4 recoding scheme described above

300 was applied as both MCMCTree and PhyloBayes require a fixed phylogeny topology.

301 Because previous studies often came to different topologies of the
302 *Alphaproteobacteria* phylogeny, which are mainly associated with the positions of
303 mitochondria, *Holospirales* and *Pelagibacterales* (Fan et al., 2020; Luo, 2015;
304 Martijn et al., 2018; Muñoz-Gómez et al., 2019), we considered two, two, and three
305 distinct topologies for these three orders respectively, totaling $2 \times 2 \times 3 = 12$
306 topologies (Fig. S1B; Supplementary Note S1.2). We addressed the topology
307 uncertainty by repeating MCMCTree analysis with each alternative topology. We
308 further selected a best-practiced dating scheme by investigating the impact of the
309 calibration information, clock model, number of partitions, and cross-lineage rate
310 variation on the estimated posterior ages (see Supplementary Note S1.2 for details).

311 The burn-in, sampling frequency, and number of the iterations were adjusted to
312 100,000, 100, and 20,000, respectively, based on the results of testing runs. This
313 ensured that the effective sample size for all parameters were above 200, as
314 commonly recommended for MCMC-based Bayesian phylogenetic inference
315 (Nascimento et al., 2017). Convergence was assessed by comparing the posterior
316 means from two independent chains and with Tracer v1.6
317 (<http://tree.bio.ed.ac.uk/software/tracer/>). The posterior ages were compared with
318 effective priors (“usedata = 0”) to ensure that their distributions were different and
319 thereby the sequences used in MCMCTree analysis were informative (Table S2).

Further, we followed the above procedure to date the divergence time of *Alphaproteobacteria* using the traditional strategy where all calibration points were placed within cyanobacteria for comparison (see Supplementary Note S2.2).

Data availability

All of the sequences, phylogenetic trees, molecular dating analysis results, and the Ruby (Goto et al., 2010) codes generating them, are available at <https://figshare.com/s/ad430d1f5bace9eb2000>.

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341

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480

481 **Figure legends**

482 **Figure 1. Divergence time estimate using the mitochondria-based strategy**

483 The evolutionary timeline of the *Alphaproteobacteria* estimated using the
 484 best-practiced dating scheme for the mito-encoded (the top) and nuclear-encoded (the
 485 bottom) datasets. Node bars denote the 95% HPD interval of estimated dates. Nodes
 486 with red circles denote calibration points. The cartoon graphs above the timeline
 487 indicate the origin times of eukaryotic lineages that *Rickettsiales* and *Holosporales*
 488 are mostly associated with, inferred simultaneously with *Alphaproteobacteria* using
 489 the same dataset. The potential time range of mitochondrial endosymbiosis is also
 490 indicated. The panels in the upper and lower left corners are the infinite-sites plots,
 491 where the uncertainty in the divergence time (measured as the 95% HPD width) is
 492 plotted against the posterior mean of estimated times for each node. A lower value of
 493 the slope indicates less changes in the 95% HPD width, hence higher precision in
 494 dating. The sources and credits of the cartoon graphs are provided in the online open
 495 access repository FigShare (see Data availability).

496

497 **Figure 2.** Comparison of the estimated times with alternative dating strategies using
 498 MCMCTree and ancestral lifestyle reconstruction of *Rickettsiales*. (A) The divergence
 499 times estimated using alternative schemes (y-axis; see Dataset S2) versus using the

best-practiced scheme (x-axis). The best-practiced scheme used a full partition and autocorrelated rates clock model (Dataset S2). The bars in blue indicate the 95% HPDs. *Phan*: only calibration points with Phanerozoic fossils considered; *Max-I*: maximum constraints for nodes with controversial maximum ages removed; *Root-I*: a more conservative root age; *Single partition*: all sequences considered as a single partition; *IR*: independent rates clock model (Dataset S2). (B) Changes in the estimated times (95% HPD) that result from using different species tree topologies (Fig. S1C). The detailed posterior dates for each clade are shown in Table S2. (C) The infinite-sites plot using the cyanobacteria-based strategy. (D) Inferred ancestral hosts of *Rickettsiales*. The pie charts on the nodes show the estimated probabilities of the hosts, and the branch colors indicate the hosts with the higher probability at the corresponding node. Tips represent the randomly selected representative of each OTU (defined by 97% identity of 16S rRNA gene). (E) The transition rates from animal-associated to protist-associated (q_{AP}) and from protist-associated to animal-associated (q_{PA}) estimated by the MCMC method in BayesTraits multistate. The log-transformed Bayes factor (logBF) is indicated, where values above 10 are considered very strong evidence for support (Meade and Pagel, 2016). The sources and credits of the cartoon graphs are provided in the online open access repository FigShare (see Data availability).

Fig. 1

Fig. 2

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