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Short sequence-paper

A phylogenetic analysis of the cytochrome *b* and cytochrome *c* oxidase I genes supports an origin of mitochondria from within the Rickettsiaceae[1]

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Abstract

We have cloned and sequenced the genes encoding cytochrome *b* (*cob*) and cytochrome *c* oxidase subunit I (*cox1*) from *Rickettsia prowazekii*, a member of the α-proteobacteria. The phylogenetic analysis supports the hypothesis that mitochondria are derived from the α-proteobacteria and more specifically from within the Rickettsiaceae. We have estimated that the common ancestor of mitochondria and Rickettsiaceae dates back to more than 1500 million years ago.

Keywords: Mitochondrion; Cytochrome *c* oxidase 1; Cytochrome *b*; (*Rickettsia prowazekii*)

It is of general interest to understand how mitochondrial gene functions such as oxidative phosphorylation and ATP synthesis have originated. Phylogenetic analyses based on ribosomal RNA gene sequences and heat shock protein sequences suggest that mitochondria are derived from the α-proteobacteria, and more specifically from members of the Rickettsiaceae [1–4]. Genomic studies are in progress for one member of this family, *Rickettsia prowazekii* [5], an obligate intracellular parasite with a genome size of 1.1 Mb [6]. Genes encoding components that are characteristic of mitochondria such as for example enzymes of the tricarboxylic acid cycle and the electron transport system have been identified in the *R. prowazekii* genome [5]. In addition to aerobic respiration, *R. prowazekii* is able to obtain energy in the form of ATP directly from the host cell environment via an unusual ATP/ADP transport system [7,8].

For some mitochondrial genes horizontal transfer events may have blurred the traces of the original endosymbiotic event [9–11]. In order to distinguish phylogenetic relatedness caused by vertical descent from relatedness caused by horizontal transfer events, it is necessary to integrate information from phylogenetic inferences based on a variety of mitochondrial genes [12]. We have selected for a phylogenetic analysis the genes encoding cytochrome *b* and cytochrome *c* oxidase subunit I, which are two of the most highly conserved proteins of the electron transport system. In such an analysis it is important to include sequences from those bacterial species that are thought to represent the closest modern relatives of mitochondria. Unfortunately, very limited mitochondrial gene homologs are available within the α-proteobacteria. In this study, we discuss the results of phylogenetic reconstructions based on cytochrome

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[1] The nucleotide sequences in this paper have been deposited in the EMBL sequence database under accession Nos. Y13854 (*cob*) and Y13855 (*cox1*).

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c oxidase 1 and cytochrome *b*, including our own recently determined sequences from the α-proteobacterium *R. prowazekii*.

Isolation and DNA sequencing. PCR-amplified sequences of the *Reclinomonas americana* cytochrome *c* oxidase 1 (*cox1*) and cytochrome *b* (*cob*) genes were used to identify the *R. prowazekii* homologs. To serve as a hybridization probe, 15 ng of the mitochondrial PCR product was labeled with [^{32}P]dATP and Klenow DNA polymerase, at standard conditions [13]. Hybridizations were performed against a set of 1000 randomly isolated, in vivo excised clones [4] obtained from a *R. prowazekii* genomic library constructed in λZap II [14]. Hybridizations were carried out under standard conditions [13]. Plasmid DNAs with *R. prowazekii* inserts were isolated by Qiagen large-scale plasmid preparations (KEBO, Stockholm, Sweden) and the nucleotide sequences on both strands of the inserts were determined by double-stranded dideoxy sequencing using modified T7 DNA polymerase (Sequenase) and fluorescent dATP (Pharmacia, Uppsala, Sweden). The products of the sequencing reactions were separated and analyzed with the aid of an A.L.F. Sequencer (Pharmacia, Uppsala, Sweden).

Sequence analysis. Alignments of the protein sequences were performed using the CLUSTALW multiple sequences alignment program [15] and were further improved by visual inspection. K_a values (nonsynonymous substitutions per site) were calculated with Li's method [16]. Phylogenetic relationships among these aligned sequences were estimated with the help of the graphical program Phylo_win [17] using the neighbor-joining [18] and the maximum parsimony methods. Maximum likelihood analysis was performed using the program PUZZLE [19]. To assess the level of statistical confidence in the tree, 1000 bootstraps were performed.

Characterization of the cox1 and cob genes from R. prowazekii. The *R. prowazekii* cytochrome *b* gene (*cob*) and cytochrome *c* oxidase subunit 1 (*cox1*) genes were isolated from a genomic library constructed in λZap II [14]. First, 1000 previously isolated and in vivo excised clones [5] were screened for the *cob* gene using a probe prepared by PCR amplification of mitochondrial DNA from *R. americana*. In this search, one positive plasmid clone (A925) was identified by the *cob* probe, and a second clone containing the *cob* gene (A289) was identified during systematic sequencing of the end-terminal fragments of the same set of clones [5]. Clone A925 was selected for sequence analysis. In our second search, more than 2000 phage plaques from the λZap II library (corresponding to roughly six *R. prowazekii* genome equivalents) were screened with the *cox1* probe. Four clones hybridizing to both the 5′ and the 3′ terminal

Table 1
Base composition, nucleotide and amino acid bias

Species	Cytochrome *b*						Cytochrome *c* oxidase subunit 1					
	L[a]	G+C[b]	$GC3_S$ [c]	AT/GC[d]	AC[e]	Ref.	L[a]	G+C[b]	$GC3_S$ [c]	AT/GC[d]	AC[e]	Ref.
Marchantia polymorpha	404	0.38	0.24	1.30	M68929	[40]	522	0.39	0.24	1.17	M68929	[40]
Triticum aestivum	398	0.42	0.30	1.12	X02352	[41]	524	0.42	0.32	1.13	Y00417	[42]
Platymonas subcordiformis	343	0.33	0.14	1.54	Z47795	[43]	523	0.35	0.15	1.12	Z47795	[43]
Prototheca wickerhamii	384	0.31	0.10	1.58	U02970	[44]	515	0.35	0.13	1.11	U02970	[44]
Chondrus cripsus	381	0.30	0.13	1.88	Z47547	[45]	532	0.32	0.10	1.36	Z47547	[45]
Cyanidium caldarium	384	0.29	0.13	2.03	Z48930	unpubl.	526	0.33	0.12	1.39	Z48930	unpubl.
Reclinomonas americana	390	0.29	0.12	1.83	AF007261	[25]	531	0.34	0.15	1.12	AF007261	[25]
Rickettsia prowazekii	398	0.34	0.20	1.73	Y13854	–	534	0.36	0.20	1.39	Y13855	–
Bradyrhizobium japonicum	408	0.63	0.91	1.13	J03176	[46]	541	0.64	0.92	0.89	X54318	[47]
Paracoccus denitrificans	440	0.62	0.89	1.08	M17522	[48]	554	0.61	0.86	1.02	X05829	[49]

[a]Length in amino acid residues.
[b]Gene G+C content.
[c]G+C content at silent third codon position.
[d]Ratio of amino acids coded for by A+T-rich codons over amino acids coded for by G+C-rich codons.
[e]Accession number.

fragments of the mitochondrial *cox1* gene were isolated and one of these, BB71, was selected for sequence analysis.

A potential complication in evolutionary comparisons over long distances arises because directional mutation pressures may induce biases in amino acid composition patterns. To address this issue we have studied the base composition patterns and quantified the amino acid biases for the species included in our phylogenetic analysis (Table 1). Within the α-proteobacteria there is a wide range of variation in genomic G+C contents, from 0.29 in *Rickettsia* [20,21] to 0.60–0.70 in *Agrobacterium*, *Rhizobium*, *Bradyrhizobium* and *Rhodopseudomonas* [22,23]. The nucleotide sequences of the *cob* and *cox1* genes reflect this variation in base composition, particularly at synonymous third codon positions ($GC3_S$), which ranges from 0.20 for *cob* in *R. prowazekii* to 0.92 for *cox1* in *Bradyrhizobium japonicum*. In *R. prowazekii*, the mean $GC3_S$ value is 0.17 [24]; the value observed for *cob* and *cox1* (0.20) is thus within the range expected. All of the mitochondrial sequences are biased towards A+T, as reflected in $GC3_S$ values ranging from 0.10 for *cox1* in *Chondrus crispus* to 0.32 for *cox1* in *Triticum aestivum*. These values are also within the range expected from the base frequencies of their respective genomes.

A simple index of amino acid bias is the so-called AT/GC ratio [24], which corresponds to the number of amino acids coded for by A+T-rich codons (Asn, Ile, Lys, Phe, Tyr) divided by the number of amino acids coded for by G+C-rich codons (Ala, Gly, Pro). For the sequences analyzed in this study, the AT/GC ratio varies from 0.89 for *cox1* in *B. japonicum* (associated with a high $GC3_S$ value) to 2.03 for *cob* in *Cyanidum caldarum* (associated with a low $GC3_S$ value). The corresponding values for *cox1* and *cob* in *R. prowazekii* are 1.39 and 1.73, respectively. In this species, AT/GC ratios have been found to range from 1.2 for the most highly conserved genes to more than 7.5 for the less well conserved genes [24]. Thus, for cytochrome *b* and cytochrome *c* oxidase 1 we observe a small shift in amino acid composition patterns that correlates with the direction of the mutation pressure within each species; however, for all pairwise comparisons this effect is within a factor of two and therefore unlikely to influence the results of the phylogenetic analyses.

Alignments and trees. The deduced amino acid sequences of the *R. prowazekii cob* and *cox1* genes were aligned with the α-proteobacterial and mitochondrial protein sequences. Substitution rates in mitochondrial genomes are characteristically different among various eukaryotic lineages [9], leading to long branches that confound analyses and violate models underlying phylogenetic algorithms. In order to avoid these problems, we have selected gene sequences from the most slowly evolving mitochondrial lineages, i.e. from the plants and the green and red algae (Table 1). We have also included the *cob* and *cox1* gene sequences from *R. americana*, a freshwater protozoan and one of the most early diverging mitochondria-containing eukaryotes [25].

Phylogenetic relationships were first inferred by neighbor-joining, maximum parsimony and maximum likelihood methods based on the cytochrome *c* oxidase subunit 1 sequences. The trees presented in Fig. 1 show that mitochondrial sequences from the range of eukaryotes here analyzed are monophyletic (bootstrap support 96–100%) and closely related to α-proteobacterial species such as *R. prowazekii*, *Rhizobium leguminosarum*, *B. japonicum* and *Paracoccus denitrificans*. Within the α-proteobacteria, *R. prowazekii* represents the most closely related bacterial sister group of the mitochondria with bootstrap support values of 74% and 89% in the neighbor-joining and maximum likelihood trees, respectively. However, the branching order of *R. prowazekii* in relation to the cluster *R. leguminosarum* and *B. japonicum* could not be resolved in the maximum parsimony analysis and the relative branching order among the mitochondrial groups was not strongly supported in either of the three subtrees.

In order to obtain a better resolution of the branching patterns within these lineages phylogenetic reconstructions were also derived from a concatenated alignment of cytochrome *b* and cytochrome *c* oxidase subunit 1 (Fig. 2). Since there are no bacterial representatives outside of the α-proteobacteria for which both of these genes have been sequenced, the analysis is restricted to sequences derived from the α-proteobacteria and mitochondria. This analysis shows that the mitochondrial sequences from the plants and the chlorophytes (green algae) cluster together with 100% bootstrap support to the exclusion of sequences that are representative of the rhodo-

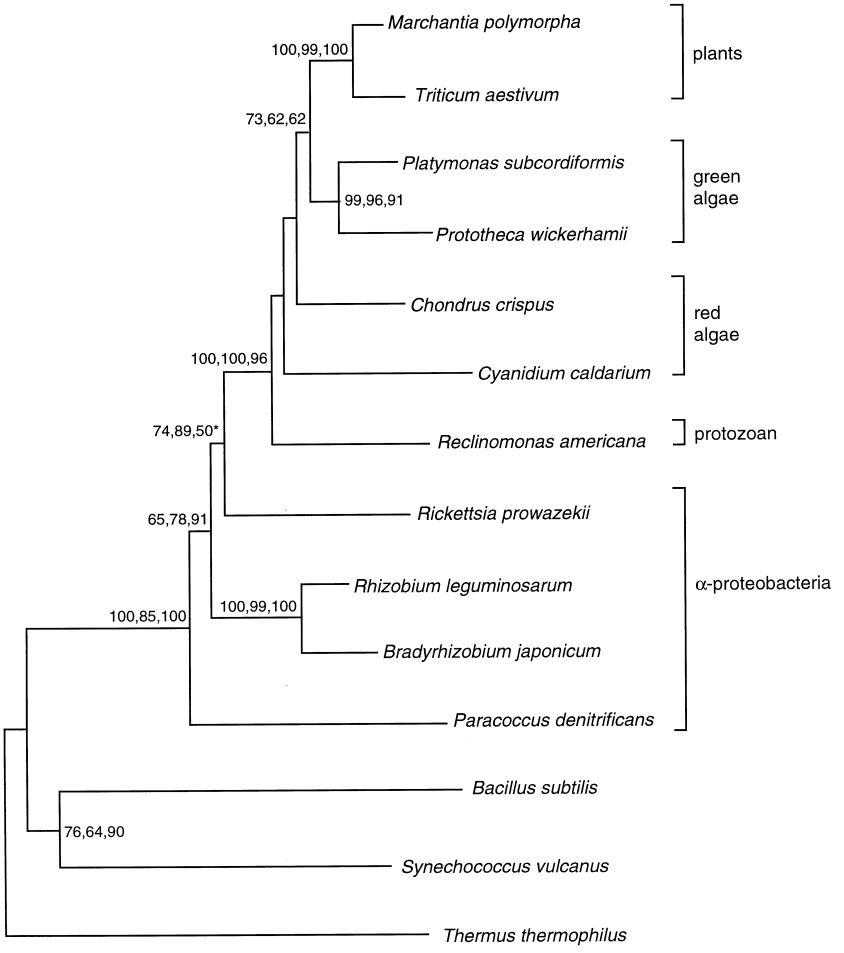

Fig. 1. Phylogenetic relationships of mitochondria and bacteria, derived from cytochrome *c* oxidase subunit 1 amino acid sequences. Branch lengths are proportional to those reconstructed under the neighbor-joining method. Values at nodes are bootstrap values indicating the degree of support for individual clusters under each method (neighbor-joining, maximum likelihood and maximum parsimony). Accession numbers are presented in Table 1. The following additional sequences were included in the analysis: *R. leguminosarum* Q08855 [50], *Synechococcus vulcanus* P50676 [51], *Thermus thermophilus* P98005 [52] and *Bacillus subtilis* P24010 [53]. *Maximum parsimony analysis yielded two topologies with equal probabilities which differed only in the relative branching order of *R. prowazekii* and the cluster *R. leguminosarum* and *B. japonicum*.

phytes (red algae). The association of chlorophytes with land plants is in agreement with the protein and ribosomal RNA phylogenies currently available [26,27]. Similarly, the rhodophytes are well recognized as a monophyletic lineage [26,27]. The protozoan *R. americana* represents the most early diverging lineage among the eukaryotes here analyzed, as was also expected [25]. In summary, our phylogenetic analysis supports an origin of mitochondria from within the α-proteobacteria and more specifically from within the Rickettsiaceae, in accordance with phylogenetic reconstructions based on rRNA and heat shock protein sequences [1–4].

Substitution rates. In order to determine the rate at which the *cob* and *cox1* genes evolve within the mitochondrial lineages, we have exploited the fossil-based divergence time between bryophytes and higher plants as our calibration point. The earliest land plant fossils have been dated to be 420 million years old [28,29], whereas the first fossils from distinct progymnosperms and bryophytes have been determined to be about 350 million years old [30–32]. These two dates are therefore considered to represent lower and upper estimates for the split between bryophytes and progymnosperms [33]. In this study, *Marchantia polymorpha* has been used as a representative of the

bryophytes and *Triticum aestivum* as a representative of the angiosperms. Using both the lower and the upper values for the divergence between *M. polymorpha* and *T. aestivum*, we obtain rate estimates ranging from 5.8 to 7.0×10^{-11} and from 14.4 to 17.2×10^{-11} non-synonymous substitutions per position and per year for the *cox1* and the *cob* genes, respectively. To obtain an estimate of the substitution rate at the most highly conserved positions in the *cob* and *cox1* genes, we have calculated the non-synonymous substitution frequency based on a concatenated, minimal alignment of these genes based exclusively on positions that are present in all species. We estimate that this combined core region evolves at a rate of $7.1–8.6\times10^{-11}$ non-synonymous substitutions per position and per year. This rate is slow enough to be expected to accurately record substitutional events over the last 1–2 billion years.

Divergence times. By comparing the number of non-synonymous substitutions separating the bacterial and mitochondrial lineages, we have attempted to roughly estimate the date at which the *cox1* and *cob* genes in *R. prowazekii* separated from their mitochondrial homologs. The date of divergence between the α-proteobacteria and the plant mitochondrial sequences was first estimated by assuming rate constancy in the lineages leading to *Rickettsia* and plant mitochondria. This assumption obtains support from the observation that the branch lengths of the neighbor-joining trees presented in Figs. 1 and 2 are

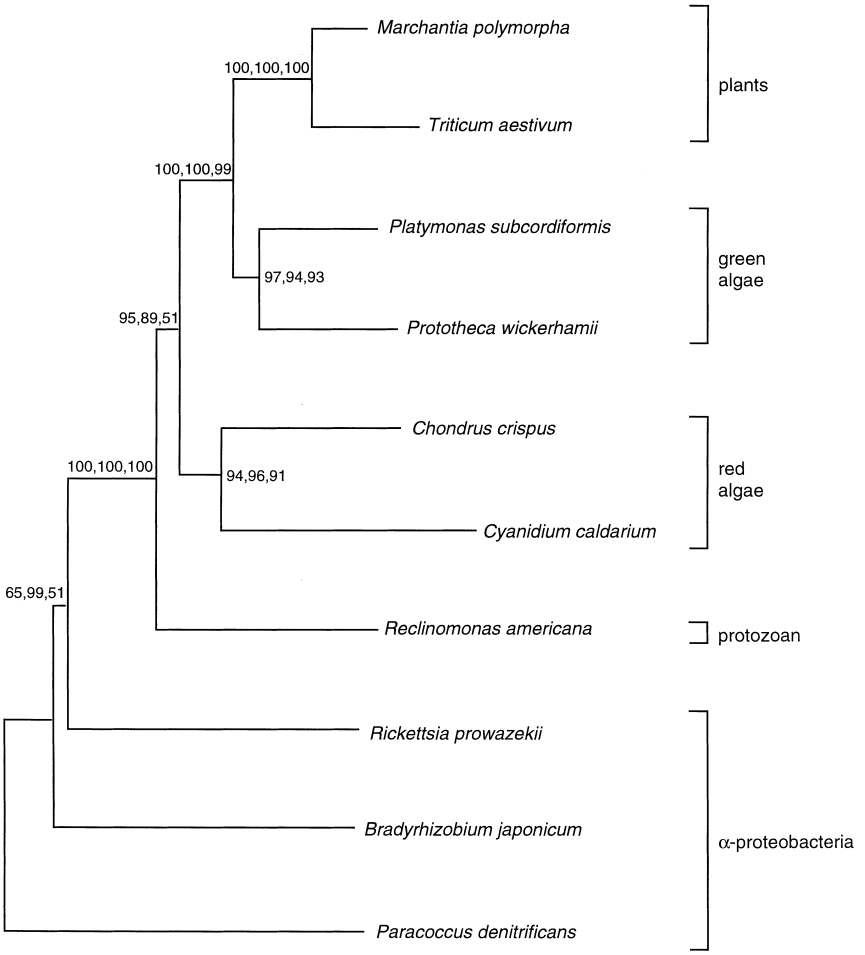

Fig. 2. Phylogenetic relationships of mitochondria and α-proteobacteria, derived from the combined amino acid sequences of cytochrome *b* and cytochrome *c* oxidase subunit 1. Neighbor-joining, maximum parsimony and maximum likelihood methods gave identical topologies. Branch lengths are proportional to those reconstructed under neighbor-joining. Values at nodes are bootstrap values indicating the degree of support for individual clusters under each method (neighbor-joining, maximum likelihood and maximum parsimony).

very similar in the α-proteobacterial and mitochondrial lineages. Using a substitution rate of 7.1–8.6×10^{-11} non-synonymous substitutions per site and per year for the core region of the *cob* and *cox1* genes, a date of 1360–1650 Myr (million years) was estimated for the split between *R. prowazekii* and mitochondria.

The divergence time has also been inferred from the branch length of the node separating *R. prowazekii* and mitochondria divided by the branch length of the calibrated node separating *M. polymorpha* and *T. aestivum*. In the distance neighbor-joining tree based on the cytochrome *c* oxidase 1 amino acid sequences, a divergence ratio of 5.0 extrapolates to a divergence time of 1750–2100 Myr. Under the assumption that the neighbor-joining tree based on the combined cytochrome *b* and cytochrome *c* oxidase 1 alignment is rooted at the node separating *P. denitrificans* from the other α-proteobacteria, the divergence ratio in this tree can be estimated to 4.0. This ratio extrapolates to a divergence time of 1400–1680 Myr, which is in excellent agreement with the estimate based on rate constancy along the branches.

Oxidative metabolism of the sort found in mitochondria could not have originated until the partial pressure of oxygen (pO_2) increased to concentrations capable of supporting aerobic respiration. Geochemical data suggest that long periods of environmental stability were followed by short periods of drastic increases in pO_2. The first such steep increase in the oxygen level corresponded to a step of 1–2% of present-day atmospheric levels that occurred around 2800–2400 Mya (million years ago) [34]. Recognizable eukaryotes, interpreted to be protistans, are relatively frequent in the fossil record at around 1700–1900 Mya [35]. Megascopic fossils resembling *Grypania spiralis*, a probable eukaryotic alga have been dated to 2100 Myr [36]. If this assignment is correct, mitochondria may have appeared more than 2000 Mya. The oldest known multicellular protist is thought to be a bangiophyte red alga, and these findings have been dated to between 1260 and 950 Mya [37]. The appearance of red algae as well as other multicellular eukaryotes at high abundance during this time period suggests that the first mitochondrial lineages originated well before this time.

The calculations presented in this paper, which are based on the non-synonymous substitution rates of the *cob* and *cox1* genes, suggest that *Rickettsia* and mitochondria diverged around 1500–2000 Mya, but possibly even earlier since the dating of the early diversification of land plants may be slightly underestimated [38]. Previous attempts to date the origin of mitochondria based on SSU rRNA sequences have resulted in a divergence time of 900 Myr for the split between bacteria and mitochondria [39]. Since this estimate as well as ours is associated with high uncertainty values and because mitochondria may have a closer bacterial relative that has either not yet been discovered or already gone extinct, these datings should only be taken as very rough approximations for the origin of mitochondria. Nevertheless, our dating based on the genes coding for cytochrome *b* and cytochrome *c* oxidase subunit 1 provides the first estimated time span that is well within the limits inferred from a combination of fossil data and geochemical reasonings.

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