TECHNICAL COMMENT

Comment on "A Green Algal Apicoplast Ancestor"

Discovery of a plastid in apicomplexan parasites such as *Toxoplasma* and *Plasmodium* (*1, 2*) has prompted intense debate over whether the plastid originates from a red algal or a green algal ancestor (*3–5*). Funes *et al.* (*6*) argued for a green algal ancestry based on analysis of the *cox2* gene, which encodes COXII, a subunit of the mitochondrial cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain). Apicomplexan parasites are unusual in that COXII is encoded in the nucleus (*7*). In all other organisms studied, with the notable exceptions of certain green algae and leguminous plants, COXII is encoded by the mitochondrial genome (*8, 9*). Intriguingly, the COXII protein of apicomplexan parasites comprises two polypeptides corresponding to the NH_2-terminal and COOH-terminal domains of the canonical COXII. The two domains are encoded by two nuclear genes, *cox2a* and *cox2b* (*7*). This gene separation also occurs in certain green algae, where it appears that the *cox2* gene split in the mitochondrial DNA before *cox2a* and *cox2b* transferred to the nucleus (*6*). Funes *et al.* (*6*) presented a phylogeny of COXII indicating that apicomplexan genes are most closely related to the *cox2* genes of green algae. They further suggested that apicomplexa acquired their split *cox2a* and *cox2b* genes through lateral gene transfer (presumably nucleus to nucleus) from the endosymbiotic (green) alga that gave rise to the plastid.

We reanalyzed COXII phylogeny to include the mitochondrion-encoded COXII proteins of ciliates. Ciliates are crucial to the interpretation of COXII phylogeny because they are closely related to apicomplexa (together with dinoflagellates, ciliates and apicomplexa form the protist supergroup alveolates), but were not included by Funes *et al.* (*6*). If apicomplexan *cox2* genes were inherited vertically (the null hypothesis) and not acquired laterally from a green algal endosymbiont, then they should be related to ciliate homologues. COXII phylogenies including ciliates indeed show that the apicomplexan *cox2a* and *cox2b* genes group with the ciliate *cox2* genes (Fig. 1A). However, COXII data provide poor overall phylogenetic resolution [as with the Funes *et al.* analysis (*6*), there is very little support at the phylum level], and the ciliate genes are remarkably divergent. Still, this tree is consistent with simple, vertical inheritance of *cox2* in alveolates, and therefore provides no grounds to reject the null hypothesis in favor of lateral transfer of *cox2a* and *cox2b* from a green alga. It is thus possible that the *cox2* gene underwent independent splitting and relocation from the mitochondrion to the nucleus after the ancestor of ciliates and apicomplexa diverged.

Funes *et al.* argued that parallel transfer of *cox2* to the nucleus is unlikely (*6*), but it clearly happened twice—for green algae and the legumes (*8, 9*)—and the phylogeny in Fig. 1A is consistent with a third transfer in an ancestor of apicomplexan parasites. The mitochondrial genome of apicomplexans is the smallest known and encodes a mere three proteins (*10*), a fact that suggests heavy gene loss accompanied by gene transfer to the nucleus. One factor proposed to limit relocation of genes from organelles to the nucleus is hydrophobicity of the encoded protein. If a gene product is too hydrophobic to undergo retrograde targeting to the organelle, relocation of the gene is not feasible (*11, 12*). COXII is a hydrophobic membrane protein, and organisms containing nuclear *cox2* genes appear to have conceived two mechanisms for solving the hydrophobicity problem. One is a hydrophilic shift in the sequence of the protein, with certain legumes having a single nuclear *cox2* gene that encodes a relatively hydrophilic COXII protein (*12*). The second mechanism entails splitting proteins into smaller modules that are more amenable to transport (*11–13*), which appears to be the case with *cox2a* and *cox2b*. Analysis of protein hydrophobicity shows that, in comparison with

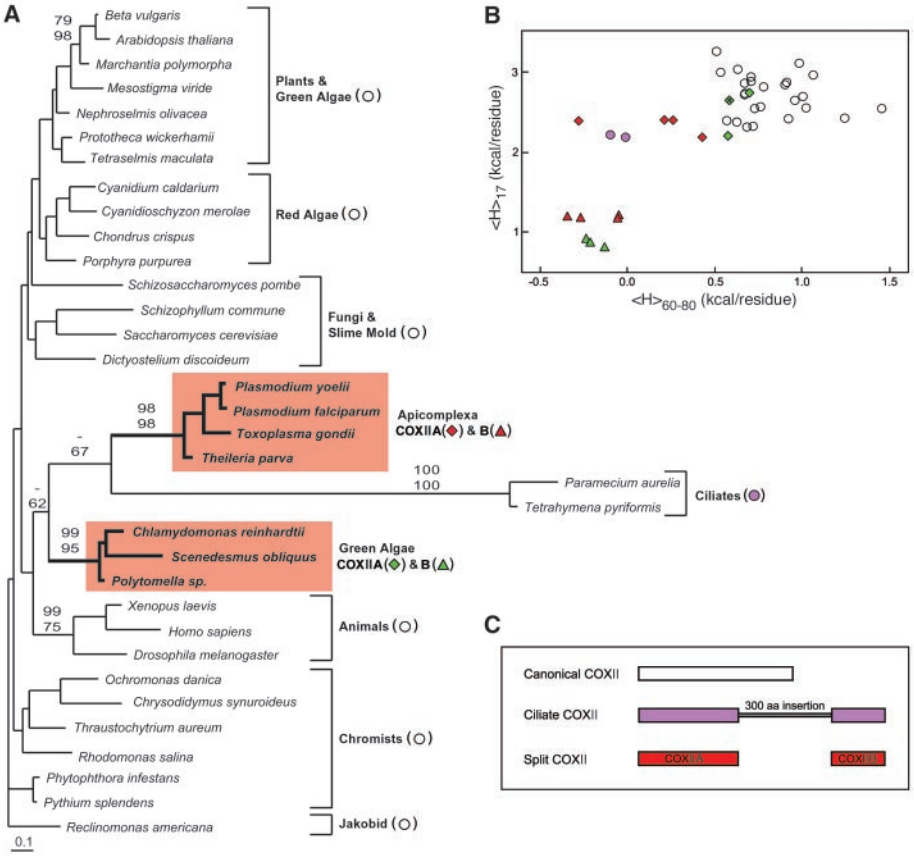

Fig 1. Analysis of COX II. (**A**) Maximum likelihood (ML) analysis (*17*) including ciliate sequences. Bootstrap support >50% for ML (above) and Fitch-Margoliash (below) analyses is indicated for major nodes. The phylogeny groups apicomplexans with ciliates, consistent with vertical inheritance (rather than lateral gene transfer) of the COXII coding sequence in apicomplexa (*19*). (**B**) Mesohydrophobicity ($<H>_{60\text{-}80}$) versus maximal local hydrophobicity ($<H>_{17}$) plot of COXII proteins from the phylogeny (*20*). Circles indicate intact COXII proteins encoded in the mitochondrion. Diamonds and triangles indicate split COXII proteins, which (except for *S. obliquus* COXIIA, indicated with a cross) are nucleus-encoded and imported into the mitochondrion. Split COXII proteins cluster away from their intact mitochondrion-encoded counterparts. (**C**) Schematic of COXII protein forms.

mitochondrion-encoded COXII proteins, the split COXII proteins of apicomplexans obey a defined trend of reduced hydrophobic characters that are necessary for mitochondrial import (Fig. 1B) (*11*). Thus, in view of the highly reduced mitochondrial genome in apicomplexans, we believe that independent splitting and relocation of *cox2* has occurred. Interestingly, ciliate COXII proteins, which are unusually hydrophilic for mitochondrion-encoded COXII (Fig. 1B), contain a 300-amino-acid insertion exactly where the apicomplexan COXII is split (Fig. 1C)—which demonstrates that this region of the protein is amenable to alterations. Protein plasticity at this site in alveolates further bolsters the likelihood of a convergent *cox2* split rather than lateral transfers of the split gene from a putative green algal endosymbiont.

The only other support for a green algal ancestry of the apicoplast is the phylogeny of plastid *tufA*, but the support for this phylogeny is weak (*3*). In contrast, several independent lines of evidence point to a red algal origin of the apicomplexan plastid based on structural characteristics of the plastid genome and on a shared gene duplication of a nucleus-encoded, plastid-targeted protein (*14*–*16*). Indeed, the apicomplexa are related to a number of other lineages with red algal plastids, so this conclusion should not come as a surprise.

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17. Protein maximum likelihood (ML) phylogeny was inferred using ProML [Felsenstein, J. 2002. PHYLIP (Phylogeny Inference Package) version 3.6a3] with site-to-site rate variation modeled with 6 variable rate categories and invariable sites. Rates and frequencies were estimated using TREE-PUZZLE 5.0 (*18*). Gamma-corrected distances were calculated using TREE-PUZZLE and Fitch-Margoliash trees inferred using FITCH.
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19. A putative common intron is used as a further phylogenetic marker by Funes *et al*. (*6*). However, this intron occurs in the poorly conserved 5′-region of *cox2a*, which cannot be reliably aligned. Moreover, the intron is present in only one of several apicomplexan taxa. It is more likely an independently acquired intron in *T. gondii,* and therefore not useful for this study.
20. Protein mesohydrophobicity (average regional hydrophobicity over a 60- to 80-residue window) and maximal local hydrophobicity (over 17 residues) were calculated with Mitoprot (*21*) using the GES hydrophobicity scale (*22*).
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