TECHNICAL COMMENT

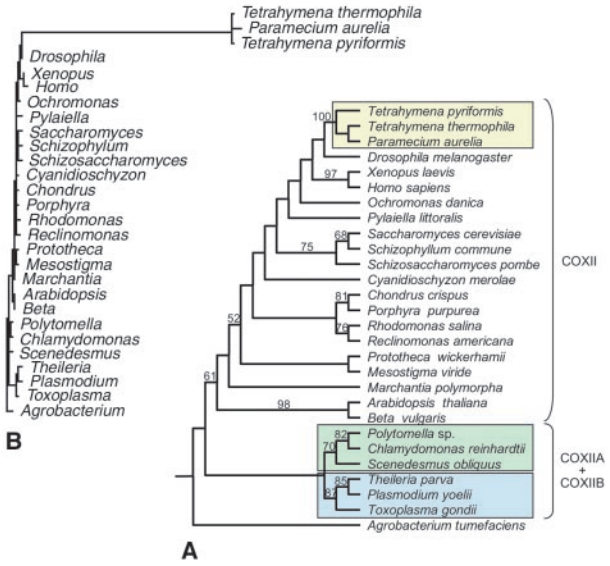
Response to Comment on "A Green Algal Apicoplast Ancestor"

We suggested lateral transfer of split *cox2a* and *cox2b* genes from a chlorophyte algal ancestor to an apicomplexan ancestor (*1*). Waller *et al.* (*2*) oppose this interpretation based on a phylogeny implying close affiliation of apicomplexan *cox2a* and *cox2b* genes with ciliate *cox2* homologues; a 900-bp insertion that separates the corresponding domains in ciliate mitochondrial *cox2* genes; and low hydrophobicity values for ciliate mitochondrial COXII and apicomplexan COXIIA and COXIIB subunits. They suggest that mitochondrial *cox2* genes were split and transferred to the nuclei of the apicomplexan and chlorophyte ancestors during independent events.

We find their phylogenetic argument unconvincing because, as they admit, statistical support for the position of the ciliate sequences is lacking. Affiliation of ciliates and apicomplexans previously noted in mitochondrial sequence phylogenies should also be interpreted with caution because both lineages are rapidly evolving and may be artifactually grouped due to long-branch attraction (*3*). Furthermore, the addition of ciliate *cox2* sequences to our previous phylogenetic analysis (*1*), does not show a relationship between ciliates and apicomplexans (Fig. 1, A and B). Nevertheless, we are pleased that in agreement with our findings in (*1*), Waller *et al.* (*2*) confirm the common branch for apicomplexan and chlorophyte COXIIA and COXIIB sequences—an otherwise unexpected affiliation.

The presence of an insertion in the mitochondrial *cox2* genes of ciliates does not necessarily imply kinship with apicomplexan fragmented *cox2a* and *cox2b* genes. The *cox2* gene is susceptible to insertions between regions that encode the hydrophobic and hydrophilic domains of COXII. Insertions of variable lengths have been described in *cox2* genes of unrelated organisms (*4*), including brown marine algae (NC_003055, NC_004024), microflagellates (NC_000946), and bacteria (NC_003888). Localization of insertions in *cox2* is most likely constrained by the difficulty of inserting in the gene without disrupting the structure of the COXII protein (*5*). Ciliates also exhibit an insertion in *cox1* and a fragmented mitochondrial *nad1* (*3*). These rearrangements most likely occurred after the divergence of ciliates from apicomplexans and dinoflagellates (*6*), since no sequence remnants of insertions are present in *cox1* in apicomplexans (*7*) or dinoflagellates (*8*). The *cox1* and *cox2* insertions were most likely absent in the alveolate ancestor and were acquired by ciliates independently. Therefore, the proposed vertical inheritance of *cox2a* and *cox2b* genes in apicomplexans is not directly supported by the presence of insertions in ciliate *cox2*.

The hydrophobicity analysis presented by Waller *et al.* (*2*) provides no evidence for a common origin of ciliate COXII and apicomplexan COXIIA and COXIIB sequences. We agree that hydrophobicity is one of the rate-limiting steps in functional gene transfer from the mitochondrion to the nucleus (*5, 9–11*). It is therefore not surprising that apicomplexan COXIIA and COXIIB subunits exhibit diminished hydrophobicity. Nevertheless, the low mean hydrophobicity values of ciliate mitochondrial COXII sequences merely indicate that, from the hydrophobicity point of view, the corresponding genes are ready to migrate—not that they migrated in the distant past. Ciliate mitochondrial DNA (mtDNA) contains more than the standard set of genes encoded by mtDNA in other eukaryotes (*3*) and therefore does not seem to show an increased rate of gene migration. The hydro-

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Droso  ----------------------------------------------------------NIEFDSYMIPTNELMTDGF-RLLDVDNRVVLPM
Homo   ----------------------------------------------------------GLIFNSYMLPPLFLEPGDL-RLLDVDNRVVLPI
Arabi  ----------------------------------------------------------SSDEQS-LTFDSYMIPEEDLELGQS-RLLEVDNRVVVPA
Mesos  ----------------------------------------------------------SDEQDSNILFDSYMVPEDELELGDL-RLLEVDNRVVIPA
Theil  ----------MLRGILESILGNVYAEGPLLDSDLERSRERYPIPKKYLDNPELIPKYYSFQSNLVTDEDLQPGML-RQLEVDKRLTLPT
Plasm  MDKIKSWLGFNKKIVFTENKFLLRNVNDQESLQAENVRAEDYPIPEKYLEDPDKIPKYYVFQSNMVTDEDLQPGML-RQLEVDKRLTLPT
Toxop  MDVFRSFWNRRGAAPGGAATSSGRPAGCREENSSVLLQSLKANSQQKYLDDPDKIPTYYVFQSNMVTDEDLLPGML-RNLEVDKRLTLPT
Chlam  ----------MSESKDQLKEKLKADPSFRAELKDRIKNALLSKVPASVPISYNFDSYMLT--EVQPGQL-RVLEVDERLVLPT
Polyt  ----------MSDAKDQLKEQLKASPSFRAELKDKLKAALLSKVPASQPIQYNFDSYMVT--DVQPGQL-RMLEVDERLVLPT
Tther  YLTLKQKRYNRKKVVAPQIKYYKDDNGNKTDLVKYTGKPYLSNDKLLKQSIYDQTTQYKLIKKNKKRGELIPVTLARRILRTKKTLVLPA
Tpyri  YLTLKQKRYNRKKVVAPQVKYYKDSNENKTDIARYSGKPYLSNDKILKQSIYDQTTQYKLIKKNKKRGELIPVTLARRILRTKKTLVLPA
Param  YLTLKQKRYKRKKNIPLRIRLDKNDLSANTASIKFTDKPYLVANKIIKSLEFNATSLYRCIKKNKLRSENFSVQLSRRLLRTKKTLVLPS
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Fig. 1. Phylogenetic analysis of COXII. (**A**) Maximum likelihood (ML) tree. COXIIA and COXIIB (excluding MTS and extensions) were fused in silico as a single polypeptide and aligned with orthodox mitochondrial COXII sequences (*14*). (**B**) Phylogram showing branch lengths estimated with ML implementing the data, model, and parameters used to perform the ML search. COXII sequences for *Tetrahymena pyriformis* (NC_000862), *T. thermophila* (NC_003029), and *Paramecium aurelia* (NC_001324) were obtained from GenBank. (**C**) Multiple protein sequence alignment of the N-terminal regions of COXIIB sequences. Shown are the N-terminal extensions of nuclear *cox2b* genes from chlorophytes and apicomplexans (blue), the conserved residues in these domains (yellow), the conserved residues found only in chlorophytes and ampicomplexans (black background), the corresponding region of the 300-residue insertion in ciliate mitochondrial COXII (gray), and the conserved residues between ciliates and apicomplexans (bold). *Droso* (*Drosophila melanogaster*), *Homo* (*Homo sapiens*), *Arabi* (*Arabidopsis thaliana*), *Mesos* (*Mesostigam viride*), *Theil* (*Theileria parva*), *Plasm* (*Plasmodium yoelli*), *Toxop* (*Toxoplasma gondii*), *Chlam* (*Chalmydomonas reinhardtii*), *Polyt* (*Polytomella* sp.), *Tther* (*Tetrahymena termophila*), *Tpyri* (*Tetrahymena pyriformis*), and *Param* (*Paramecium aurelia*).

phobicity of contemporary ciliate COXII is unlikely to bear on either the location of its gene or apicomplexan COXIIA and COXIIB phylogeny. In addition, the hydrophobicity analysis does not argue against the proposal that the original apicomplexan mitochondrial *cox2* gene was eliminated after functional acquisition of chlorophyte *cox2a* and *cox2b* sequences (*1*).

COXIIB has a unique N-terminal extension that most likely arose since the splitting of the original mitochondrial *cox2* gene of the chlorophyte ancestor (*1*). Protein sequencing has confirmed the presence of this extension in mature chlorophyte COXIIB (*5*). All chlorophyte and apicomplexan sequences share N-terminal extensions of COXIIB containing the conserved PxxxPxxY motif not present in the canonical COXII. These extensions seem homologous and suggest a common origin for chlamydomonad and apicomplexan COXIIA and COXIIB sequences. The proposal of Waller *et al.* (*2*) would imply that this domain conservation is due to convergent evolution, which we consider unlikely. The corresponding borders of the ciliate COXII insertions and the apicomplexan COXIIB extensions reveal no similarities (Fig. 1C).

We originally described rare characteristics that are shared solely between apicomplexans and certain chlorophyte algae (*1*), namely, the presence of nucleus-encoded split *cox2a* and *cox2b* genes and a conserved domain present in a region of COXIIB that is not derived from orthodox COXII. Functional fragmentation of a mitochondrial gene followed by functional integration in the nucleus is an extremely rare event, unlikely to happen several times. The current evidence suggests that this phenomenon was confined to the ancestor of chlorophyte algae. Our analyses support a close relationship between apicomplexan and chlorophyte *cox2a* and *cox2b* sequences specific to the mitochondrion, whereas analysis of apicoplast genome organization has suggested a red algal origin of the organelle (*12*). Clearly, there is still conflicting evidence for green versus red algal ancestry in the apicomplexans (*13*). Whatever the outcome of this debate, the presence of nucleus-encoded, fragmented *cox2a* and *cox2b* genes of green origin in apicomplexans must be considered whenever the evolutionary story of these parasites is reconstructed.

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14. Sequences were aligned using ClustalX (*15*), with subsequent manual refinements. The corresponding aligned nucleotide sequences were used in the analysis. *Agrobacterium* was specified as outgroup. The model that best fits the data (GTR+I+ Γ) and its parameters (base frequency: 0.3349, 0.1732, 0.1828, 0.3091; rate matrix: 1.9883, 3.2172, 2.1240, 2.9095, 4.8212, 1.0000; proportion of invariable sites: 0.0086; shape of gamma distribution: 0.8863) were identified with Modeltest (*16*) and implemented in heuristic searches with TBR branch swapping, to obtain a ML tree (score of –ln L = 25559.03416). Branch support was obtained from 100 ML bootstrap replicates conducted under the same model and parameters as the ML search. Analyses were performed using PAUP* 4.0b10 for Macintosh and UNIX (*17*).
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