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LETTER TO THE EDITOR

Plastid Isoprenoid Metabolism in the Oyster Parasite *Perkinsus marinus* Connects Dinoflagellates and Malaria Pathogens—New Impetus for Studying Alveolates

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The capacity for isopentenyl pyrophosphate (IPP) synthesis, the common precursor of isoprenoids, is universally distributed among photosynthetic and heterotrophic eukaryotes (Lange et al. 2000; this study). Land plants harbor two unrelated metabolic routes with specific substrates, intermediates, and sets of enzymes (Grauvogel and Petersen 2007). The cytosolic mevalonate-dependent MVA pathway, which is also present in metazoa and fungi, has been known since the 1960s (Katsuki and Bloch 1967; Lynen 1967), whereas the plastidial MEP (2-C-methyl-D-erythritol 4-phosphate) pathway was discovered just 10 years ago (Rohmer et al. 1993; Lichtenthaler et al. 1997). Plastid IPP generation was inherited from the cyanobacterial endosymbiont and subsequently spread to complex algae and Apicomplexa (e.g., *Plasmodium falciparum*) via eukaryote-to-eukaryote endosymbioses (Delwiche 1999). The "raison d'être" for plastids in heterotrophic parasites is their indispensable metabolic capacity, and the respective pathways are promising drug targets. A prime example is 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), an essential enzyme of plastid isoprenoid biosynthesis (MEP) that is inhibited by the natural antibiotic fosmidomycin (Jomaa et al. 1999; Wiesner et al. 2003), a promising weapon for malaria treatment (Borrmann et al. 2005).

Mass mortalities of oysters along the East Coast of the United States are caused by the eukaryotic parasite *Perkinsus marinus* (formerly *Dermocystidium marinum*), while several other species in this genus infect marine mollusks worldwide (Villalba et al. 2004). The agent of the so-called "Dermo" disease was initially described as a fungus and is now eponymous for the class Perkinsea. As part of the superensemble Alveolata, which was originally defined based on molecular data such as 18S rDNA (van de Peer and De Wachter 1997), Perkinsea are closely associated with dinoflagellates, to the exclusion of Apicomplexa (e.g., the malaria parasite) and the basal branching ciliates (Fig. 1b) (Saldarriaga et al. 2003; Grauvogel et al. 2007). The genome of *Perkinsus marinus*, whose size is estimated to be 28 MB, is currently being sequenced by The Institute of Genomic Research due to its evolutionarily key position and ecologic and economic relevance.

We analyzed the genetic distribution of the plastid isoprenoid pathway (MEP) after primary and secondary endosymbioses and identified nuclear-encoded genes for plastid *DXR* from land plants, green algae, rhodophytes, all photosynthetic orders with complex red plastids (haptophytes, cryptophytes, diatoms, dinoflagellates), and, surprisingly, also *Perkinsus* (Fig. 1a). Ten novel *DXR* clones were identified by experimental work (library screening, PCR-approach; see Supplementary Material S1) and the remaining sequences were extracted from public

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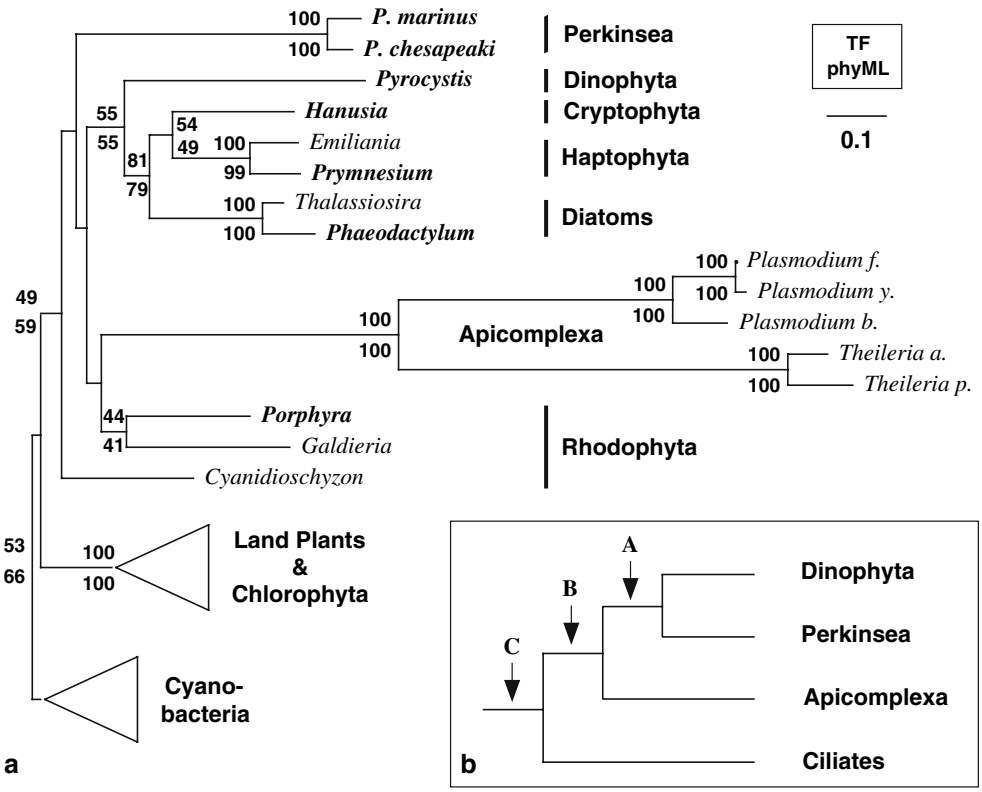

Fig. 1 **a** Phylogenetic maximum likelihood (PhyML) tree (WAG + F + Γ4 model) based on 44 *DXR* sequences and 335 amino acid positions. Sequences identified in this study are shown in boldface. The statistical support values for the internal nodes were determined by Treefinder (TF) and PhyML bootstrap analyses and values ≥40% are shown. The horizontal length of the triangles is equivalent to the average branch length. **b** Evolutionary relationships among members of the Alveolata. A, B, and C indicate possible common origins of plastids

databases (Supplementary Table S1). We searched for the presence of 12 isoprenoid biosynthesis genes in the unfinished *P. marinus* genome (TIGR) and detected 6 of the 7 genes for the plastid MEP pathway, whereas representatives of the cytosolic MVA pathway appear to be absent (Supplementary Table S2). The presence of spliceosomal introns in all preliminary sequences confirms the eukaryotic origin of these genes and excludes putative bacterial contaminants. We verified our findings by identifying and determining several MEP-related sequences from five *Perkinsus* species (*P. marinus, P. chesapeaki, P. olseni, P. honshuensis, P. mediterraneus*) (Fig. 2; Supplementary Material S1; EF140866–EF140873) and could even identify the missing seventh gene (MCT) from *P. olseni* (EF140871). The plastid localization of the MEP pathway has been previously documented for land plants, chlorophytes, and Apicomplexa (Lichtenthaler et al. 1997; Schwender et al. 2001; Gardner et al. 2002), and it is also evident for rhodophytes, diatoms, and haptophytes since these algae contain both pathways for IPP biosynthesis (this study; data not shown). In contrast, aplastidial eukaryotes exclusively harbor the cytosolic MVA pathway and there is no hint that it was ever replaced by the cyanobacterial counterpart. Thus, we propose that the presence of the complete MEP pathway in *Perkinsus* is a reliable indicator for a so far hidden plastid ("perkinsuplast"). The distribution of the isoprenoid metabolism in *Perkinsus* species is conspicuously similar to that of the evolutionarily closely related malaria parasite *Plasmodium falciparum*, where isoprenoids are exclusively synthesized within the apicoplast (Gardner et al. 2002). Our data and interpretations are consistent with novel ultrastructural data from *Perkinsus atlanticus* (Teles-Grilo et al. 2007) and the very recent findings by Stelter and colleagues (2007) of plant-type ferredoxin (*ptFd*) and ferredoxin NADP+ reductase (*ptFNR*) genes in *Perkinsus marinus*. The latter also performed pharmacological inhibitor studies, which support the presence of a plastid. However, the mature ptFd is a very small protein, about 95 amino acids long, and the ptFNR phylogeny is difficult to interpret (Stelter et al. 2007) since it exhibits a complex distribution including gene duplications and horizontal gene transfers (HGT). In contrast to that, the nuclear-encoded genes for the MEP-dependent isoprenoid biosynthesis represent promising markers for phylogenetic analyses since the corresponding metabolic pathway is exclusively located within plastids.

We investigated the distribution of the *DXR* sequences and our phylogenetic analyses indicate that Plantae including green and red algae (Rodrigues-Ezpeleta et al. 2005) recruited this gene from the cyanobacterial endosymbiont and donated it in the course of secondary endosymbiosis (Fig. 1a; Supplementary Fig. S1). Haptophytes, diatoms, the cryptophyte *Hanusia*, and the peridinin-containing dinoflagellate *Pyrocystis* probably obtained their *DXR* genes via a single endosymbiotic gene transfer from a red alga, in agreement with the evolutionary origin of their complex

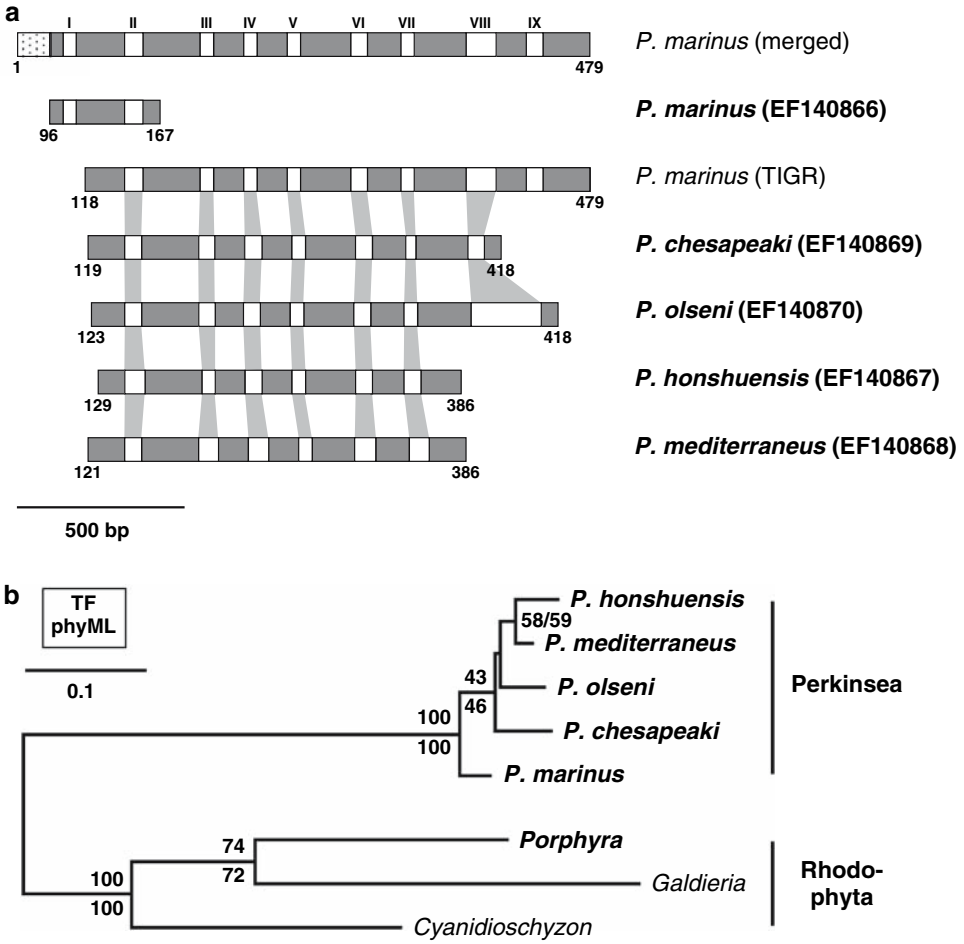

Fig. 2 **a** Structure of the *DXR* genes from various *Perkinsus* species. Exons and introns are shown with gray and white boxes, respectively. Introns are named with roman numerals. Arabic numerals indicate the deduced amino acid position of the established clones using the *DXR* of *Arabidopsis* (NP_201085) as a reference for numbering. The conservation of nine intron positions in phase -0, -1, or -2 (I, 109-0; II, 154B-2; III, 210-1; IV, 241-0; V, 268-2; VII, 317-0; VIII, 401-1; IX, 431-0) is shown in bright gray. All clones with the exception of the partial TIGR sequence (*P. marinus* Contig: 16110) were determined for this study. The stippled box of the merged *P. marinus DXR* gene assumes the presence of an N-terminal presequence equivalent to the transit peptide found in the *Arabidopsis* reference sequence. **b** Phylogenetic maximum likelihood (phyML) tree (WAG + F + I + Γ4 model) based on eight *DXR* sequences and 249 amino acid positions. The statistical support for the internal nodes was determined by bootstrap analyses (TF, PhyML). Support values >40% are shown. Clones sequenced for this study are shown in boldface

plastids (Delwiche et al. 1999; Petersen et al. 2006; Teich et al. 2007), and the same explanation may also apply to the *Perkinsus* species. The extremely divergent Apicomplexan *DXR* sequences are obviously subjected to long-branch attraction (LBA [Brinkmann et al. 2005]) (Supplementary Fig. S1), but they most likely also have a red algal affiliation (Fig. 1a) (Foth and McFadden 2003). The relationship between the *Perkinsus* and the *Pyrocystis DXR* genes is not resolved, but a common origin would be compatible with the phylogeny of the host cell (Fig. 1b) (Saldarriaga et al. 2003; Grauvogel et al. 2007).

The presented findings may improve the understanding of alveolate evolution. Many dinoflagellates are heterotrophic and the phototrophic species exhibit a broad spectrum of different plastids with characteristic accessory pigments that originated via independent secondary and tertiary endosymbioses (Chesnick et al. 1997; Tengs et al. 2000; Saldarriaga et al. 2001; Hackett et al. 2003; Patron et al. 2006). The prevalent type of dinoflagellates, including *Pyrocystis lunula*, contain peridinin, but it was hitherto disputed if they represent the ancestral lineage and it was also unclear if the primordial dinoflagellates were photosynthetic (Taylor 1980, 2004; Saldarriaga et al. 2001; Yoon et al. 2002; Inagaki et al. 2004; Sanchez-Puerta et al. 2007). Under the assumption that the plastid specific MEP pathway represents a shared trait, as indicated by the *DXR* genes identified and described above, these dinoflagellates and Perkinsea have a common photosynthetic ancestry (Fig. 1b; scenario A), whereas heterotrophic representatives lost their plastids secondarily. Moreover, a common origin of plastids

in the ancestor of dinoflagellates and Apicomplexa was previously proposed (Fig. 1b; scenario B) (McFadden and Waller 1997). The *Perkinsus* genome may represent the ideal reference system for testing this hypothesis, especially since complete genome sequencing of dinoflagellates is largely prohibited due to their huge genome size (3000 to 215,000 MB) (Spector 1984). Concerning the evolution of the superensemble Alveolata as a whole, a common plastidial origin of all classes including ciliates is the most far-reaching scenario (Fig. 1b; C). It is a sine qua non for the so-called chromalveolate hypothesis (Cavalier-Smith 1999), which is subject to continuous controversial discussion (Harper and Keeling 2003; Bodyl 2006; Teich et al. 2007). However, the recently released genome of *Tetrahymena thermophila* gives no hint of a photosynthetic ancestry of ciliates (Eisen et al. 2006) and therefore argues against an endosymbiotic event at the origin of alveolates.

Taken together, the identification of all seven genes for the strictly plastid specific MEP pathway in the alveolate parasite *Perkinsus* gives strong support for the lately predicted (Stelter et al. 2007; Teles-Grilo et al. 2007) presence of a so far unknown "perkinsuplast." The phylogenetic position of *Perkinsus* within the Alveolata distinguishes it as the connecting link between dinoflagellates and apicomplexans, including the malaria pathogen *Plasmodium*, and therefore, this key species will be an ideal reference taxon for the study of alveolate biology, evolution, and infection strategies.

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