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The search for the missing link: A relic plastid in *Perkinsus?*

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Abstract

Perkinsus marinus (Phylum Perkinsozoa) is a protozoan parasite that has devastated natural and farmed oyster populations in the USA, significantly affecting the shellfish industry and the estuarine environment. The other two genera in the phylum, *Parvilucifera* and *Rastrimonas*, are parasites of microeukaryotes. The Perkinsozoa occupies a key position at the base of the dinoflagellate branch, close to its divergence from the Apicomplexa, a clade that includes parasitic protista, many harbouring a relic plastid. Thus, as a taxon that has also evolved toward parasitism, the Perkinsozoa has attracted the attention of biologists interested in the evolution of this organelle, both in its ultrastructure and the conservation, loss or transfer of its genes. A review of the recent literature reveals mounting evidence in support of the presence of a relic plastid in *P. marinus*, including the presence of multimembrane structures, characteristic metabolic pathways and proteins with a bipartite N-terminal extension. Further, these findings raise intriguing questions regarding the potential functions and unique adaptation of the putative plastid and/or plastid genes in the Perkinsozoa. In this review we analyse the above-mentioned evidence and evaluate the potential future directions and expected benefits of addressing such questions. Given the rapidly expanding molecular/genetic resources and methodological toolbox for *Perkinsus* spp., these organisms should complement the currently established models for investigating plastid evolution within the Chromalveolata.

Keywords

Apicoplast; Chromalveolata hypothesis; Genome; Perkinsus; Plastid; Protozoan; Targeting

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1. Introduction

1.1. Life cycle

Perkinsus spp. have a direct life cycle (Fig. 1): trophozoites proliferate intra- or extracellularly by palintomy (merogony or schizogony) giving rise to 4–32 (often 8–16) trophozoites, which are released upon rupture of the schizont cell wall (Perkins, 1996; Sunila et al., 2001). At the four-cell schizont stage, a functional diversification of the daughter cells has been proposed (Sunila et al., 2001). Whilst less frequent, binary fission has also been observed: trophozoite budding yields a mother cell surrounded by a thick wall and the daughter cell separated by a plasma membrane (Sunila et al., 2001). Sexual stages have been suggested (Perkins, 1996) and recent microsatellite analyses suggest that *Perkinsus marinus* utilises both sexual and asexual reproduction, and that over the short term selection acts upon independent parasite lineages rather than upon individual loci in a cohesive, inter-breeding population (Thompson et al., 2011). Although the overall propagation process has been described in substantial detail, various aspects of cell division and maturation at the subcellular level remain poorly understood, especially regarding the segregation and function of some organelles and macrostructures (e.g. the large vacuole containing the vacuoplast and its precursors during trophozoite proliferation and maturation) and the zoosporulation process.

1.2. Natural history and phylogenetic position

In the early 1950s, the causative agent of widespread mass mortalities of eastern oysters (*Crassostrea virginica*) on the shores of Texas (USA) was identified and named *Dermocystidium marinum*. This microorganism was later renamed *Perkinsus marinus* and closely related *Perkinsus* spp. that affect various mollusc species worldwide were described during later years (Villalba et al., 2004). The gradual expansion of the geographic distribution of P. *marinus* infections along the Atlantic coast of the USA has been associated with global warming and shellfish trade (Ford, 1996; Ford and Chintala, 2006; Ford and Smolowitz, 2007; Pecher et al., 2008), and it is currently under surveillance by the World Organization for Animal Health (http://www.oie.int/). In addition to constituting a valuable shellfisheries resource, filter-feeding bi-valves are important components of marine and estuarine environments because they play a critical role in maintaining water quality and ecosystem integrity. Since its discovery, *P. marinus* has been placed in various taxa including fungi, saprolegniales, apicomplexans and dinozoans (Mackin et al., 1950; Mackin and Ray, 1966; Levine, 1978; Goggin and Barker, 1993; Siddall et al., 1997; Kuvardina et al., 2002). More recently, the new phylum Perkinsozoa was established to include *Perkinsus* and two other genera, *Parvilucifera and Rastrimonas*, which are parasites of microeukaryotes. Parvilucifera is a parasite of the toxic dinoflagellate *Dinophysis* (Norén et al., 1999), and *Rastrimonas* infects the free-living cryptophyte *Chilomonas paramaecium* (Brugerolle, 2002). The Perkinsozoa is considered to be the earliest group diverging from the lineage leading to dinoflagellates, branching close to the node shared by dinoflagellates and apicomplexans (Saldarriaga et al., 2003; Gile et al., 2006; Moore et al., 2008; Bachvaroff et al., 2011). Members of both dinoflagellates and apicomplexans possess plastids and recent analysis of newly identified photosynthetic members of the apicomplexan lineage have shown that these plastids evolved from a single common secondary endosymbioisis with a

red alga (Moore et al., 2008; Janouskovec et al., 2010). This suggests that non-photosynthetic relatives of both lineages, including *Perkinsus*, evolved from photosynthetic ancestors, raising the possibility that these lineages retain cryptic organelles (Keeling, 2010). Similarly, expressed sequence tag (EST) evidence suggests that the non-photo-synthetic early-diverging dinoflagellate, *Crypthecodinium cohnii*, may contain a reduced plastid (Sánchez-Puerta et al., 2007). Here, we analyse the current evidence in support of the presence of a relic plastid in *Perkinsus*, as well as the remaining open questions. We also evaluate the potential future directions and expected benefits of rigorously addressing such questions in a functional and evolutionary context within the Cromalveolata. The Chromalveolata is a eukaryote supergroup established to include those taxa that comprise organisms resulting from a single secondary endosymbiosis between a line descending from a bikont and a red alga (Cavalier-Smith, 1999; Keeling, 2010). It includes the Alveolata, a group of unicellular eukaryotes characterised by the presence of cortical alveoli (Cavalier-Smith, 1998).

2. The Chromalveolata hypothesis: the apicoplast, a relic plastid in apicomplexans

2.1. Origin and architecture of the apicoplast

The apicoplast was described over two decades ago in several api-complexan parasites as a multimembrane vesicle (Kilejian, 1974; Gardner et al., 1991; Howe, 1992; Williamson et al., 1994; McFadden et al., 1996; Wilson et al., 1996; Köhler et al., 1997). Its ultrastructural hallmark is the presence of four intimately associated membranes, although three membranes have been reported in some instances (e.g. *Plasmodium* spp.), whilst in others the organelle is completely absent (e.g. *Cryptosporidium* spp.) (Hopkins et al., 1999; Zhu et al., 2000; Abrahamsen et al., 2004; Xu et al., 2004). The Chromalveolata hypothesis (Cavalier-Smith, 1999) (Fig. 2A,B) proposed that apicomplexan plastids resulted from a single secondary endo-symbiosis of a red alga that also gave rise to plastids in dinoflagellates, heterokonts, haptophytes and cryptomonads (reviewed in Keeling, 2010). Whilst this hypothesis remains contentious for several of these lineages, there is now strong evidence that at least the apicomplexan and dinoflagellate plastids did arise from a common ancestor. Both of these organelles are unusual and have proved difficult to compare but the recent discovery of photosynthetic relatives of apicomplexans (Moore et al., 2008) and the characterisation of their plastid genomes and associated genes (Janouskovec et al., 2010) have provided multiple lines of evidence for their common ancestry. Both primary and secondary endosymbiotic events are followed by drastic reductions in the genome of the engulfed endosymbiont. For example, because a chloroplast encodes for only 5–10% of the genes present in the free-living cyanobacteria, it is estimated that between 800 to 2,000 genes from the original endosymbiont were transferred to the nucleus of the primary host (Martin and Herrmann, 1998; Martin et al., 2002), and in extant species, most of the proteins targeted in a plastid are nuclear encoded. The apicomplexan plastid retained a 35–40-kb circular extrachromosomal genetic element considered as a remnant of a secondary endosymbiotic event (Fig. 2C,D), and houses 33 tRNA (e.g. *Toxo-plasma pl*DNA), two head-to-head copies of rRNA genes, RNA polymerase genes, numerous housekeeping genes (mostly related to gene expression) and several open reading frames (ORFs) (Feagin and

Parsons, 2007; Kissinger and Kuo, 2007). In dinoflagellates this reduction has been even more extreme: with only 13 genes, mostly relating to photosystems, retained on single-gene mini-circle chromosomes, they have the most reduced plastid genomes documented to date (Green, 2011) and at least some of the many missing genes are known to be located in the nucleus (Bhattacharya et al., 2004).

2.2. Metabolic relevance of the apicoplast

Key metabolic functions associated with the apicoplast include complete pathways for isoprenoid synthesis using the non-mevalonate pathway (MEP pathway), type II fatty acid synthesis (FAS), lipoic acid, abscisic acid (ABA), iron–Sulphur (Fe–S) cluster and haeme biosynthesis; in addition the apicoplast harbours certain steps of glycolysis and phospholipid synthesis (reviewed in Seeber and Soldati-Favre, 2010). The MEP and type II FAS pathways are absent in mammals and other enzymes (e.g. Porphobilinogen synthase (PBGS) in haeme biosynthesis) differ from the human counterpart; hence, the enzymes involved in these pathways have been recognised as promising drug targets (Fichera and Roos, 1997; McFadden and Roos, 1999; Soldati, 1999; Wiesner et al., 2008; Lim et al., 2009; Jaffe et al., 2011). Currently, on a quest for new drugs to treat diseases caused by apicomplexan parasites, additional metabolic pathways associated with the apicoplast as well as other aspects of basic plastid biology are being characterised as potential targets for intervention (Muller and Hemphill, 2011).

3. *Perkinsus marinus* is a key organism for understanding plastid evolution

The Perkinsozoa (*Perkinsus, Parvilucifera and Rastrimonas*) is positioned at the base of the dinoflagellate branch close to the divergence from the Apicomplexa (Goggin and Barker, 1993; Reece et al., 1997; Siddall et al., 1997; Ellis et al., 1998; Norén et al., 1999; Leander and Keeling, 2003) and represents a key taxon for under-standing adaptations and organelle evolution within the Chromalveolata. Indeed, structures resembling a plastid have been identified in *Rastrimonas subtilis*: ‘‘Two enigmatic bodies limited by three membranes surrounding a matrix denser at the periphery than in the center’’ (Brugerolle, 2002), and in *Parvilucifera infectans*: ‘‘an unidentified organelle surrounded by two membranes. . . with the occasional indication of a third. They appear to represent small mitochondria . . .’’ (Norén et al., 1999). Interestingly, when *Parvilucifera prorocentri* was recently described, no bona fide apicoplast homologue was identified (Leander and Hoppenrath, 2008). Similarly, no reference was made to any plastid or enigmatic bodies in the description of *Parvilucifera sinerae* (Figueroa et al., 2008). Unfortunately, the lack of sequenced genomes for these species has precluded their mining for evidence of a plastid. Nevertheless, increasing ultrastructural and molecular evidence discussed below from *Perkinsus* points towards the presence of a relic plastid, which together with the currently available resources and methodological tools (two fully defined culture media formulations (Gauthier et al., 1995; La Peyre and Faisal, 1997), a large number of *Perkinsus* isolates and species (http://www.atcc.org), a *P. marinus* sequenced genome, a transcriptome (Joseph et al., 2010) and a transfection system (Fernández-Robledo et al., 2008a)) make *Perkinsus* spp. a potentially

unique model system to gain further insight into plastid evolution in this non-photosynthetic sister lineage to dinoflagellates.

3.1. A Perkinsus plastid genome?

The identification of three plastid-associated major biosynthetic pathways (MEP (Matsuzaki et al., 2006, 2008; Grauvogel et al., 2007; Joseph et al., 2010), type II FAS and Fe–S cluster (Stelter et al., 2007)) in *Perkinsus* suggests the presence of a relic plastid. However, identification of non-nuclear DNAs (*pl*DNA and *mt*DNA) in the available *P. marinus* genome have proven elusive, perhaps due to the yet incomplete genome assembly and annotation. This process has been extremely challenging due to various factors, including the repetitiveness of the genome sequences and the uncertainty of this species' ploidy. In this regard, others and we have shown genetic variation in *Perkinsus* trophozoites that suggests their diploid status (Robledo et al., 1999; Reece et al., 2001; Thompson et al., 2011). The initial assembly of the *P. marinus* genome is considerably fragmented, composed by thousand of scaffolds (more than 17,000, some composed of a single contig) and many apparent genome duplications containing up to 90 genes. This is indicative of unresolved assemblies due to either multiple haplotypes or artifacts in the automated scaffolding. Although the genome annotation also contains a large proportion of ''broken'' gene structures lacking 5' or 3' ends, mining the genome with nuclear encoded apicoplast resident genes from *Plasmodium* as queries showed hits to numerous genes (Supplementary Table S1). The fact that we have not yet been able to identify a plastid genome or even a single gene encoding a plastid-targeted protein that is related to gene expression (e.g. ribosomal proteins) seems to suggest that *Perkinsus* lacks a plastid genome. This is also consistent with what we know about dinoflagellate plastid genomes, where virtually all genes not related to photosystems have been moved to the nucleus: it is easy to see how such an organelle could loose its genome once photosynthesis was lost. Other possibilities have yet to be formally ruled out, including the plastid DNA having been lost during the isolation of DNA (by spooling, to avoid shearing (Green, 1997)) for sequencing the genome (J.A. Fernández-Robledo, G.R. Vasta, unpublished results). The plastid DNA might also be represented by a low copy number and missed bythe current sequencing coverage. Deeper sequencing is needed to resolve this possibility. Although *Perkinsus* possesses a mitochondrial genome (Masuda et al., 2010), it is also absent in the current *Perkinsus* genome draft, opening the possibility that the plastid genome might have been excluded by the same bias.

3.2. Multimembraned structures in Perkinsus

Since its initial identification (Mackin et al., 1950), seven *Perkinsus* spp. have been described to date based on their geographic location, host species, morphology by light and transmission electron microscopy (TEM) and molecular analysis (Lester and Davis, 1981; Azevedo, 1989; Blackbourn et al., 1998; McLaughlin et al., 2000; Coss et al., 2001a; Murrell et al., 2002; Casas et al., 2004; Burreson et al., 2005; Dungan and Reece, 2006; Moss et al., 2008). However, even in those *Perkinsus* spp. described after the existence of the apicoplast was reported (Kilejian, 1974; Gardner et al., 1991; Howe, 1992; Williamson et al., 1994; McFadden et al., 1996; Wilson et al., 1996; Köhler et al., 1997), no plastid-like multimembrane organelles were noticed (McLaughlin et al., 2000; Coss et al., 2001a, b; Casas et al., 2004; Dungan and Reece, 2006; Moss et al., 2008). Recently, a plastid-like

structure with four clearly defined membranes was observed in a single *Perkinsus atlanticus* (hereafter *Perkinsus olseni* (Murrell et al., 2002)) zoospore (Teles-Grilo et al., 2007b). However, the rigorous attribution of this structure to a bona fide plastid is doubtful because: (i) the putative plastid shows membranes that are further apart and appear to enclose an empty compartment; in general plastid membranes appear to be bound together by protein mass (Vaishnava and Striepen, 2006); (ii) the putative plastid is large (375–800 nm) and it is perplexing that it was not observed in the intact trophozoite; and (iii) the observed structure could be alternatively interpreted as a product of autophagy, a process that recycles macromolecules resulting from cellular remodelling or aberrant organelles (Reggiori and Klionsky, 2005; Yorimitsu and Klionsky, 2005). Similar structures with up to three membranes can be identified in both trophozoites and zoospores from previously published work (e.g. Fig. 17 (Perkins, 1976), Fig. 19 (Azevedo, 1989), Fig. 20 (Coss et al., 2001b), Fig. 12 (Sunila et al., 2001)). Nevertheless, in our micrographs of *Perkinsus chesapeaki* (syn. *Perkinsus andrewsi*) zoospores, unique multimembrane structures that resemble the apicoplast in size can also be observed (Fig. 3). Although suggestive of the presence of a secondary plastid in *P. olseni* and *P. chesapeaki*, the morphological evidence is clearly insufficient to rigorously establish the presence of this organelle. To demonstrate this, the subcellular location of putative plastid-targeted proteins will need to be analysed by in situ immunofluorescence or immuno-electron microscopy, as was used to identify the plastid in apicomplexans (Waller et al., 1998).

3.3. Protein targeting to plastids

When the genes for plastid-derived proteins moved to the nucleus of the host during integration of a secondary plastid, they acquired targeting sequences to ensure their protein products are trafficked to the correct location within the cell. These sequences generally consist of a bipartite N-terminal extension composed of a canonical signal peptide (SP) followed by a transit peptide (TP) (Foth et al., 2003; Tonkin et al., 2008a, b). This mechanism is conserved to the point that has enabled the development of effective bioinformatic tools for prediction of apicoplast-targeted proteins based solely on primary sequence (e.g. a programme developed for predicting apicoplast resident genes in *Plasmodium* (http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php) (Zuegge et al., 2001)). In addition, net charge and chaperone-binding sites are essential for robust targeting (Foth et al., 2003). It is noteworthy, however, that some proteins located in (or adjacent to) the outer apicoplast membrane lack any obvious common targeting motif (non-leader proteins) (Cavalier-Smith, 2003; Lim et al., 2009).

3.3.1. Does P. marinus superoxide dismutase 2 (PmSOD2) localise to multiple single membrane compartments?—

Our previous work on PmSOD1 identified the presence of a leader sequence, suggesting mitochondrial targeting (Wright et al., 2002). IFA demonstrated that the protein co-localises with Mito-Tracker Red, a mitochondrial dye (Schott and Vasta, 2003). Hydropathy analysis of the deduced amino acid sequence of a second SOD (PmSOD2) identified a strong hydrophobic region of approximately 25 amino acid residues which was interpreted as a membrane-spanning domain of type II transmembrane proteins (Wright et al., 2002) and later proposed as suggestive of the presence of a relict plastid in *Perkinsus* (Saldarriaga et al., 2004). IFA

revealed, however, that PmSOD2 localises to multiple subcellular structures/organelles (Schott and Vasta, 2003). Immunogold electron microscopy (IEM) using a polyclonal antibody against recombinant PmSOD2 outlines multiple vesicles containing clustered gold grains and electrondense material of unknown nature, both of which appear to be released into a large single membrane compartment (Fernández-Robledo et al., 2008b) (Fig. 4). Transfection of *Perkinsus* trophozoites with pPmSOD2(MOE)-GFP resulted in PmSOD2-GFP localised to multiple small compartments and to what appeared as a single large compartment, supporting the hypothesis that the small vesicles visualised by the gold grains might either fuse and/or empty their contents in a larger compartment (Fernández-Robledo et al., 2008b). However, we cannot rule out that the protein was either in transit as endoplasmic reticulum (ER) vesicles to its final destination, the trophozoite was dividing or simply mislocalised due to the use of the non-native PmSOD2 flanking regions. We have observed that disruption of the flanking regions in the transfection vector may result in gene deregulation (J.A. Fernández-Robledo, G.R. Vasta, unpublished results). In either case, the localisation data are not consistent with PmSOD2 being a plastid protein.

3.3.2. ispC (1-deoxy-d-xylulose 5-phosphate reductoisomerase) also localises to multiple compartments—

Some enzymes of the MEP pathway for the synthesis of isoprenoids have been experimentally confirmed as associated with the apicoplast of apicomplexans (Jomaa et al., 1999; Ralph et al., 2004b). All six MEP enzymes and ispD, which was missed in previous genome mining and found in *Perkinsus*, have bipartite targeting peptides at the N-terminus (Matsuzaki et al., 2008) which is characteristic of proteins targeted to secondary plastids, thereby supporting the idea that *Perkinsus* has a non-photosynthetic secondary plastid. More intriguingly, immunofluorescence micros-copy of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC) reveals multiple small compartments suggesting that *Perkinsus* might have multiple plastids, even though the compartments do not appear to contain detectable amounts of DNA (Matsuzaki et al., 2008). GFP labelling of IspC under MOE gene control also highlights several structures per trophozoite (M. Matsuzaki, H. Kuroiwa, H. Nozaki, T. Nozaki and K. Kita, unpublished data).

3.4. Presence in P. marinus of other pathways/structures typically associated with plastids

3.4.1. Type II FAS enzymes—

Driven by the lack of a chemotherapeutic treatment to reduce or eliminate *P. marinus* infections in oysters and their interest in lipid metabolism (Chu et al., 2002, 2004; Lund and Chu, 2002; Lund et al., 2007), Chu's group identified Triclosan, a specific inhibitor of Fab1, which is a Type II FAS enzyme found in prokaryotes and plastids, as an inhibitor of *P. marinus* proliferation (Lund et al., 2005). The Triclosan results, however, should be interpreted with caution as revealing strictly plastid-associated FASII biosynthesis, since the apicomplexan Theileria lacks FASII genes (Gardner et al., 2005) and yet is susceptible to this drug. Furthermore, in *Plasmodium*, FabI is not the target of the antimalarial activity of Triclosan (Yu et al., 2008). Nevertheless, *Perkinsus* is sensitive to inhibitors of other typically plastid-localised enzymes involved in fatty acid biosynthesis (e.g. acetyl-CoA carboxylase) (Stelter et al., 2007). Unlike most eukaryotes, the FAS II-dependent lipoic acid synthesis has been associated with the apicoplast in both *Plasmodium*

falciparum and *Toxoplasma gondii* (reviewed in Seeber and Soldati-Favre, 2010), and we have identified a putative lipoate synthase (XP_002786331) in *Perkinsus*, although predictions for its localisation are inconclusive.

3.4.2. Fe–S cluster biosynthesis—Fe–S clusters are ubiquitous prosthetic groups required to sustain fundamental life processes including electron transfer, substrate binding/activation, Fe–S storage, regulation of gene expression and regulation of enzyme activity (Xu and Moller, 2011). In addition to the mitochondrial Fe–S cluster biosynthesis, plastid-harbouring eukaryotes usually have a second assembly for the Fe–S cluster biosynthesis in the plastid (Fleige et al., 2010; Kalanon and McFadden, 2010; Lim and McFadden, 2010), including the apicoplast (Seeber and Soldati-Favre, 2010). The identification of transcripts of the plastid-type ferredoxin and its associated reductase in in vitro-cultivated *P. marinus* has also been proposed as indicative of the presence of a plastid since this redox pair is exclusively found in cyanobacteria and plastid-harbouring organisms (Stelter et al., 2007). Other Fe–S cluster genes predicted to localise to the relic plastid include PmSufB (see Section 4.2).

3.4.3. Haeme synthesis—In most eukaryotes, haeme is an important prosthetic group on many proteins, such as cytochromes and peroxidases, which are involved in electron transfer and redox chemistry. In photo-synthetic organisms (plants/algae), the complete pathway for tetrapyrole biosynthesis is harboured within the plastid whereas in animals/fungi the pathway is distributed between the cytosol and mitochondria. In *Plasmodium*, a hybrid pathway runs cooperatively involving the cytosol, mitochondria and the apicoplast, that not only fulfil the parasite's requirements for the above processes, but also those related to the heme derived from the haemoglobin in the host erythrocytes (reviewed in Lim and McF-adden, 2010; Nagaraj et al., 2010). The PBGS (as represented by *T. gondii* and *P. falciparum* counterparts) is conserved across the phyla and is very similar to the plant enzyme in its biochemical properties (Jaffe et al., 2011). Key features in the primary sequence of the protein help to identify it as the plant type rather than the animal type of enzyme. A comparison of the PBGS enzymes from *P. marinus* and *T. gondii* and several other PBGS reveals that, unlike the apicomplexan enzyme, the *Perkinsus* enzyme (XP_002782704) is distinctly of the animal type and probably located in the cytosol rather than the plastid. There is precedence for the co-existence of animal and plant type pathways for heme biosynthesis in a single cell (e.g. *Euglena gracilis*) and it is probable that an apicomplexan ancestor contained a animal type pathway to begin with and acquired the plant type pathway via the secondary endosymbiotic event. A brief period must have existed where the apicomplexan anscestor contained both pathways, after which the different lineages developed a mosaic of both pathways. According to this hypothesis, if the common ancestor for the Apicomplexa, Chromerida, Dinophyta and Perkinsozoa contained both the animal and plant pathways, it appears that (as of now based on PBGS only), all except Perkinsozoa have retained the plant type pathway (as a consequence of the presence of the plastid in all of them). This observation alone is not sufficient to suggest that *Perkinsus* might lack a plastid as some apicomplexans have totally lost the pathway for heme biosynthesis (e.g. *Cryptosporidium* (in this case the plastid itself is lost) and *Theileria*) (Abrahamsen et al., 2004; Gardner et al., 2005; Pain et al., 2005). Therefore it is also

possible that *Perkinsus* has the plastid but harbours a complete animal type pathway for heme biosynthesis. Further characterisation is needed to characterise the plastid-like heme biosynthesis pathway.

3.4.4. ABA—ABA is a stress hormone common in plants (Nambara and Marion-Poll, 2005). More recently, it has been identified in *Toxoplasma*, where it is involved in Ca^{2+}-mediated egress from the host cell (Nagamune et al., 2008a, b). *Toxoplasma* is sensitive to fluridone, an herbicide that inhibits the indirect pathway for ABA synthesis; treatment of *Toxoplasma* cultures with fluridone resulted in both delayed egress and induction of cysts (Nagamune et al., 2008a). In plants, ABA synthesis uses an indirect pathway that involves b-carotene and resides in the plastid (Schwartz et al., 2003). In *P. falciparum* a dual localisation in the mitochondria and apicoplast has been proposed but details remain unresolved (Tonhosolo et al., 2009). Indeed, *Perkinsus* is sensitive to the herbicide fluridone (J.A. Fernández-Robledo, G.R. Vasta, unpublished results), which targets the enzyme phytoene dehydrogenase (XP_002772671); in addition, *Perkinsus* has at least three classes of genes carrying the G protein-coupled receptor 89A domain (XP_002783768, XP_002775994, XP_002775137).

3.4.5. Shikimate pathway—Shikimate and chorismate are precursors in the biosynthesis of aromatic amino acids and other aromatic secondary metabolites in microorganisms and plants. Recently, the presence of a shikimate pathway in *P. olseni* was demonstrated by inhibition studies with glyphosate, a potent inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme in the shikimate pathway. Glyphosate inhibited the in vitro proliferation of *P. olseni* in a dose-dependent manner, and this effect was reversed by addition of chorismate (Elandalloussi et al., 2005, 2008). Although in Apicomplexa this pathway is cytosolic, in dinoflagellates it localises to the plastid (Waller et al., 2006). In the *Perkinsus* genome, we have identified several genes of the shikimate pathway, including chorismate synthase (XP_002773541) and a gene (XP_002773648) with a match to the pentafunctional arom protein, which catalyses five consecutive enzymatic reactions in prechorismate polyaromatic amino acid biosynthesis,

3.4.6. ER-associated protein degradation (ERAD) system—The ERAD pathway is aimed at tagging misfolded proteins of the ER for ubiquitination and subsequent degradation, and participates in the import of apicoplast proteins (Sheiner and Soldati-Favre, 2008; Lim et al., 2009; Spork et al., 2009). Searching for the core components of the ERAD in the *Perkinsus* genome, we have identified Cdc48, Ufd1 and Npl4. The *Perkinsus* genome also has numerous Derin homologues (intramembrane serine proteases that cleave substrates in or near transmembrane domains) necessary for processing proteins into organelles. Similar to apicomplexan parasites (Agrawal et al., 2009), the presence of multiple copies of some of these genes in Perkinsus opens the possibility that some of them might reside in the putative plastid, although we must note that these proteins are also expected to exist in the absence of a plastid and consequently the identification of specific copies related to plastid-targeting will require direct experimental evidence.

4. Working hypothesis and outstanding questions

Under the Chromalveolata hypothesis, some extant non-photo-synthetic lineages, including *Perkinsus*, must have derived from photosynthetic ancestors in which the plastid membranes have evolved into diverse structures (Keeling, 2010). In this regard, membranes may arise by growth and division from pre-existing membranes (designated as ‘‘genetic membranes’’) or by differentiation from another type of membrane (‘‘non-genetic membranes’’, e.g. lysosome membranes) (Cavalier-Smith, 1995, 2003). Thus, membranes budded from either the host Golgi or the ER might have been added to those from the original endosymbiont (Cavalier-Smith, 2003). As a result, the protein-targeting and translocation machinery has been modelled by the natural history of any particular secondary endo-symbiosis within a lineage and lead to increased complexity in the host cell. Based on the fragmentary evidence described above, two working hypotheses can be posed for the presence of a relic plastid in *Perkinsus* (Fig. 5). In hypothesis 1 *Perkinsus* shares with other Alveolata a relic plastid with multiple membranes and a plastid genome. During cell propagation the organelle is segregated, unless sexual stages are confirmed, in which case it may occur that only one gamete inherits it. In hypothesis 2 *Perkinsus* has lost the plastid genome but the plastid organelle has been retained and is nevertheless segregated in zoospores and trophozoites. These hypotheses are discussed in the following sections.

4.1. Is there physical evidence for a multimembrane structure in Perkinsus trophozoites or zoospores?

Serial TEM images and three-dimensional (3D) reconstruction have proved useful to measure organellar volumes, geometries and subcellular features, and have enabled the development of 3D cellular models in protozoan parasites of sizes comparable to Perkinsus life stages (Hopkins et al., 1999; Elliott, 2007; Elliott et al., 2008). The application of similar serial TEM images and 3D reconstruction to *Perkinsus* spp. would provide a precise blueprint of its subcellular organisation and based on the reported *Perkinsus* cell size and that of the reported relic plastid (Teles-Grilo et al., 2007b), 5–10 sections should reveal the putative organelle (Elliott, 2007). The cryo tomographic X-ray techniques that, in protozoans, have enabled the detailed survey of nucleus, digestive vacuoles and membrane networks in an intact cellular context (Hanssen et al., 2010, 2011), have also been useful to show integration of the apicoplast with other organelles (Kudryashev et al., 2010; Miranda et al., 2010). Application of this approach to *P. marinus* represents a promising avenue for the high resolution of yet unidentified sub-cellular structures. Further, a putative plastid genome has not yet been identified in any member of the Perkinsozoa and DNA dyes fail to stain any subcellular structure in the trophozoite other than the nucleus and mitochondrion (Matsuzaki et al., 2008). There is no indication from the genome project for a plastid genome but neither is there data from the mitochondrion despite the fact that a partial *Perkinsus* mitochondrial genome has been reported by other means (Masuda et al., 2010). Thus, in addition to further mining of the genome, alternative approaches for identifying the plastid genome should include DNA staining by 5-bromodeoxyuridine (BrdU) incorporation followed by IEM or fluorescently-labelled anti-BrdU antibodies. This technique combined with IEM staining of alternate sections for genes predicted as targeted to the relic plastid (e.g. PmSOD2 or IspC) should reveal whether any other compartments contain DNA and

potentially represent the relic plastid. Putative tRNAs, rRNAs and ORFs typically found on plastid genomes can also be used in high resolution in situ hybridisation to localise the putative plastidic transcripts (McFadden et al., 1996) or in pulse field gel electrophoresis of *Perkinsus* cells (Teles-Grilo et al., 2007a) optimised for the size range of *pl*DNA. Usefulness of the latter approach might be hard to predict if, similar to dinoflagellates, the *Perkinsus* plastid genome is in 2–3 Kb circles (Williams and Keeling, 2003; van der Giezen et al., 2005; Howe et al., 2008; Slamovits and Keeling, 2008; Hjort et al., 2010; Keeling, 2010).

4.2. What is the subcellular destination for the Perkinsus proteins containing bipartite signal sequences?

If no plastid genome exists, the organelle can still be identified by localising nucleus-encoded plastid-targeted proteins. To date, 10 genes have been identified in *Perkinsus* that either have a bipartite signal or encode products that are expected to target the putative plastid. These include ferredoxin (PmFD) and its associated reductase (PmFNR), acetyl-CoA carboxylase and the six genes from the MEP pathway (dxs, ispC, ispE, ispF, ispG, ispH) (Matsuzaki et al., 2006, 2008; Grauvogel et al., 2007; Stelter et al., 2007; Joseph et al., 2010). In addition, the use of Prediction of Apicoplast Targeting Sequences (PATS) (Zuegge et al., 2001) resulted in numerous putative *Perkinsus* plastid genes, including most of the genes already indentified in the literature (Supplementary Table S2), some of them carrying the predicted bipartite signal (e.g. PmSufB, Fig. 6). The bipartite extension of apicomplexan proteins is well established as diagnostic for targeting to secondary plastids (Foth et al., 2003; Ralph et al., 2004a; Tonkin et al., 2006a, b, 2008a, b), however the rigorous assignment of a specific subcellular localization for any *Perkinsus* putative plastid gene will require experimental validation.

5. Technical considerations

5.1. Species and strains

Although six out of the seven *Perkinsus* spp. are available at public repositories such as the American Type Culture Collection (ATCC), USA, http://www.atcc.org/), structural and genetic evidence for the presence of a plastid have only been obtained in three species/strains. These include *P. marinus* CB5D4 (ATCC PRA-240), the strain used for the sequence of the genome and transcriptome (Fernández-Robledo et al., 2008a; Joseph et al., 2010), *P. olseni* ALG1 (Robledo et al., 2002), the species where the relic plastid was described (Teles-Grilo et al., 2007b), and *P. chesapeaki* A8–4a (ATCC #50807) (= P. andrewsi), which spontaneously sporulate in culture (Coss et al., 2001a, b; Burreson et al., 2005) and for which we have identified plastid-like structures in the zoospores (Fig. 3).

5.2. Comparative genomics and proteomics

The parallel implementation of state-of-the art deep DNA sequencing methodology to *Perkinsus* spp. such as *P. marinus* and *P. chesapeaki* will contribute enormously to a more rigorous search, annotation and curation of putative plastid genes and the search for the plastid genome (possibly with a codon bias or signature) in *Perkinsus* by yielding less partitioned and redundant assemblages that also include clearly identified data from the mitochondrial genome. Further, the identification of *Perkinsus* homologues of proteins

targeted to the apicoplast should provide bases for identifying the organelle where proteins with bipartite-N-terminal signal ultimately function. Whole-genome analysis of unicellular eukaryotes can also reveal those gene loss and gain events often associated with the lifestyle adopted by each particular group of organisms (Martens et al., 2008). Furthermore, deep sequencing should provide enough resolution to determine whether *P. marinus* and *P. chesapeaki* are distinct bona fide *Perkinsus* spp. or genetically distinct *P. marinus* assemblages. The direct comparison of putative plastid genes in the *P. marinus* and *P. chesapeaki* genomes might also shed light on the genetic/molecular basis of why the latter spontaneously sporulates in culture whilst the former does not. Similar to apicomplexans (Lim et al., 2009), a well-curated *Perkinsus* genome will further enable the application of proteomic approaches for the identification of putative genes that may reside in the relic plastid whilst lacking the canonical bipartite signal sequence.

5.3. Cellular and molecular tools, and surrogate models to study Perkinsus plastid targeting

Success in the search for the *Perkinsus* plastid will require availability of organelle and structural markers. Few commercial markers have been used in *Perkinsus* other than Mitotracker for mitochondria and nuclear stains (DAPI, Sybr Green). However, multiple fluorescent dyes are available (Molecular Probes, Invitrogen) that could be tested and validated in *Perkinsus*. Furthermore, our *Perkinsus* genome and transcriptome projects have enabled the identification of numerous genes predicted as targeted to most organelles and subcellular structures (Table 1, Supplementary Table S1) which, combined with the availability of numerous spectral variants of fluorescent proteins and epitope tags, should enable multicolour imaging and co-localisation studies using confocal microscopy. The development of subcellular markers for *Perkinsus* should also represent a useful tool for the scientific community interested in the Chromalveolata. The sizes of the *P. marinus* trophozoites (2.7–10 lm; 30–80 lm prior to zoosporulation) and zoospores (2–3 × 4–6 lm) are similar to those equivalent life stages from the apicomplexan parasites for which this technique has demonstrated sufficient resolution to address questions about secretion and cell division through the use of subcellular markers (Vaishnava and Striepen, 2006; Nishi et al., 2008; Sheiner and Soldati-Favre, 2008). There is mounting evidence to suggest that *Perkinsus* uses transplicing (Zhang et al., 2007; Joseph et al., 2010; Lin et al., 2011), a process where mRNA can carry a splice-leader, which is an indication of post-transcriptional regulation (Zhang et al., 2011). Since it remains unknown whether RNA interference (RNAi) (EER05359.1, EER19214.1) plays a role in these processes, coding sequence of the genes of interest should be cloned under the native flanking regions to avoid over-expression artifacts. Finally, since studies on plastid targeting in apicomplexans (Foth et al., 2003; Ralph et al., 2004a; Tonkin et al., 2006a, b, 2008a, b) have revealed that these mechanisms are very well conserved, swapping bona fide apicoplast genes with the putative *Perkinsus* homologues can be used to test whether the *Perkinsus* relic plastid and the apicoplast were similarly shaped by a similar intracellular parasitic lifestyle. For example, *T. gondii*, a well-developed genetic model with multiple tools available (Ajioka and Soldati, 2007; Weiss and Kim, 2007) may be suitable as a surrogate model to examine function and subcellular targeting of *Perkinsus* proteins.

6. Concluding remarks

The several lines of evidence described above (homologues of type II FAS, Fe–S cluster, heme, ABA, lipoic acid, shikimate and other pathway components) suggest the presence of plastid-targeted genes in *Perkinsus* spp. Although the enzymes involved in these pathways are clearly predicted to target the putative plastid, further experimental characterisation and localisation will be required. In this regard, intriguing subcellular structures noticed in earlier studies raise interesting questions about whether *Perkinsus* has a relic plastid and, if so, the number of membranes that surround it. As the first described and better characterised member of the Perkinsozoa, research on *Perkinsus* plastid localisation, targeting and biogenesis will provide fundamental insights into the biology of this phylum. Further, given its phylogenetic position within the Alveolata, the characterisation of the putative plastid genes and the potential relic organelle to which the products are targeted, should make this parasite a promising model organism for investigating various aspects of plastid biology (e.g. biogenesis, segregation, targeting, translocation, functional regulation) in an evolutionary context. Analysis of the targeting peptides will lead to the identification and functional characterisation of the plastid-targeted proteins, which may reveal *Perkinsus* adaptations to intracellular survival, proliferation and virulence. If the absence of a multimembrane compartment is established, *Perkinsus* willbe useful to gain insight into molecular innovations that compensate for plastid loss in intracellular parasites, an event that under the Chromalveolata hypothesis has taken place at multiple points during evolution. *Perkinsus marinus* is virtually ubiquitous along the Atlantic and Gulf coasts of the USA, and has devastated natural and farmed oyster populations. No effective therapeutic intervention strategies against *Perkinsus* infections are available at the present time and the impact of the disease in the environment is hardly contained by the current management practices. Soon after the hatchery-raised seed is deployed in the environment, it becomes infected and the oyster farmers harvest their product as soon as it reaches commercial size, before *Perkinsus* infections cause death. Demonstrating the presence of a relic plastid in *Perkinsus* and characterising its biology may lead to potential targets for the development of chemotherapeutic drugs for use in contained oyster farming settings or oysters genetically modified for disease resistance. Furthermore, a deep understanding of the biology of the putative plastid genes in *Perkinsus* will complement ongoing studies on apicoplast genes, thereby contributing to a better under-standing of cellular mechanisms that might result in the development of novel therapeutic targets against apicomplexan parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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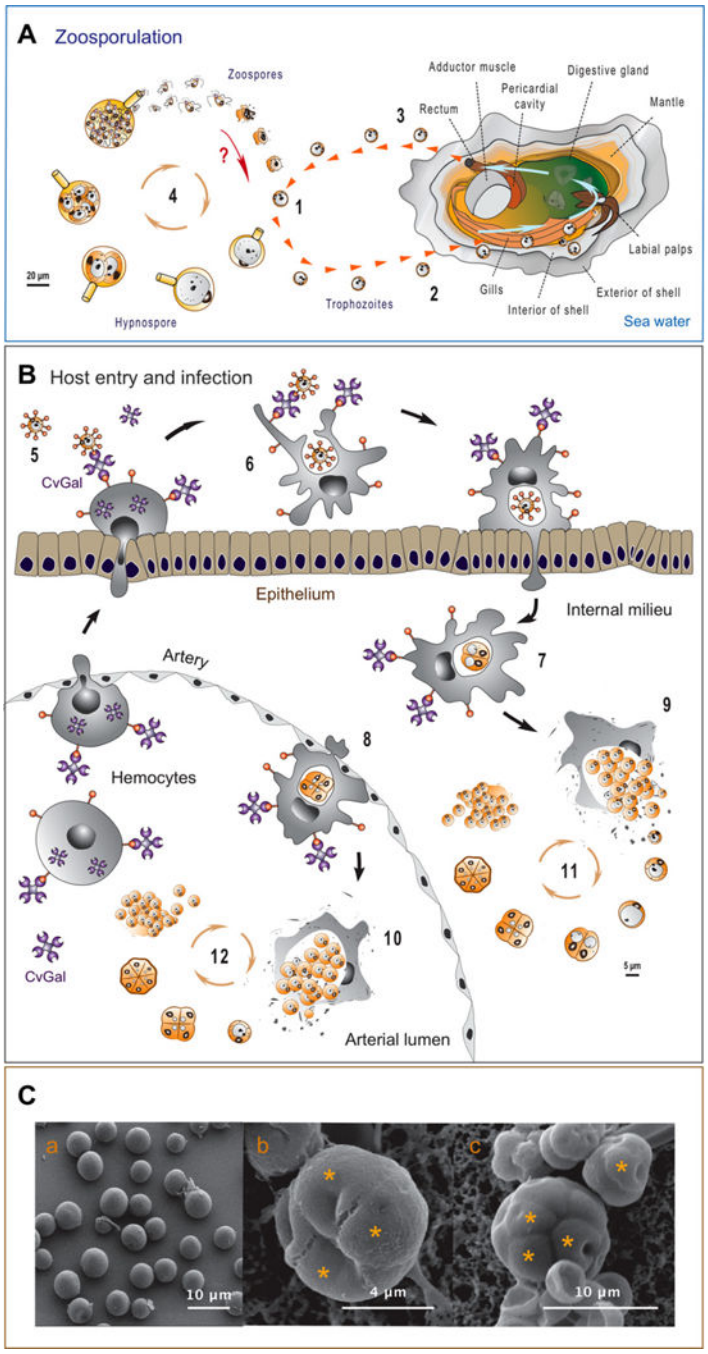


Fig. 1.

Perkinsus life cycle. (A) Trophozoites in the water column (1) are taken by the oyster during filter-feeding (2), enter the paleal cavity and are directed through gills and palps towards the mouth. Trophozoites may be released into the water (3) from live oysters together with the pseudofeces and upon death of the oyster, from the decaying infected tissues (Bushek et al., 2002). Once released into the water column, trophozoites may sporulate (4): trophozoites enlarge, develop a discharge tube and after multiple rounds of division, release hundreds of zoospores into the water column. Whether zoospores develop into trophozoites remains an

open question. (B) Once in the paleal cavity or the digestive tract (5), trophozoites displaying surface ligands for the oyster galectin CvGal (Tasumi and Vasta, 2007) are recognised and phagocytosed by the hemocytes (6) that can transmigrate to the internal milieu (7) and eventually into the vascular system (8). Parasites remain inside phagosome-like vesicles where they remain viable and multiply. When hemocytes disintegrate (9, 10), the released trophozoites can either be phagocytosed by neighbouring hemocytes or multiply extracellularly in both the internal milieu (11) and arterial lumen (12). The infected circulating hemocytes migrate throughout the host tissues where they may lyse and release trophozoites, leading to systemic infection and eventually host death. (C) In vitro culture of *Perkinsus marinus* trophozoites. Under scanning electron microscopy the cultured *P. marinus* trophozoite surface appears smooth (a, b). In trophozoites undergoing schizogony, the shapes of the daughter cells become apparent on the exterior surface (stars, b, c).

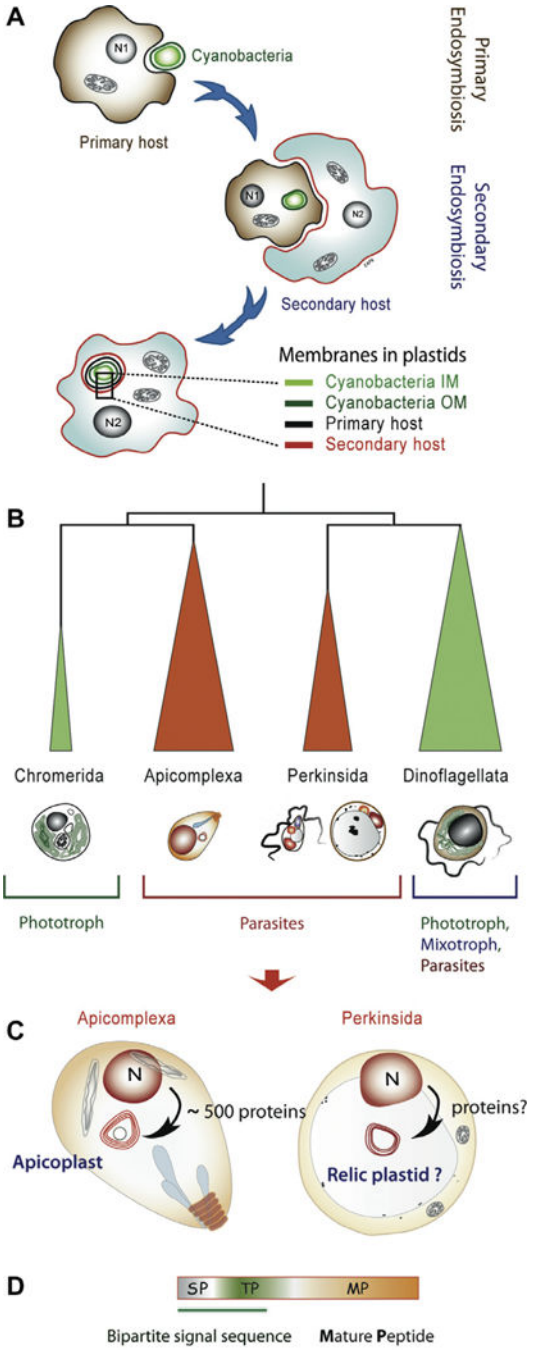


Fig. 2.

Plastid origin and evolution. (A) Under the Chromalveolata hypothesis (Cavalier-Smith, 1999; Keeling, 2010) eukaryotic plastids are DNA-containing organelles that have evolved via secondary endosymbiosis resulting from the initial engulfment of a free-living photosynthetic cyanobacterium by a heterotrophic protist, followed by its secondary engulfment by a heterotrophic eukaryotic host. The hallmark of this process is the presence of a three- to four-membrane organelle that has maintained a remnant of the original cyanobacterium genome and essential metabolic functions. (B) Both the Apicomplexa and

Perkinsida are constituted by parasitic protista that have lost photosynthetic capabilities. (C) In addition to genes encoded by the plastid genome derived from the secondary endosymbiosis in apicomplexans and perkinsids, approximately 500 nuclear-encoded apicomplexan proteins are predicted to be targeted back to the plastid through a bipartite targeting sequence (as depicted in D); similarly, nuclear genes encoding putative plastid pre-proteins containing an N-terminal signal peptide have been identified in Perkinsus. (D) In apicomplexans, the nuclear-encoded plastid pre-proteins contain an N-terminal signal peptide (SP) which targets the protein to the secretory pathway, followed by a transit peptide (TP) which targets the protein to the plastid; whether this process also takes place in perkinsids remains an open question. N, nucleus. MP, mature peptide.

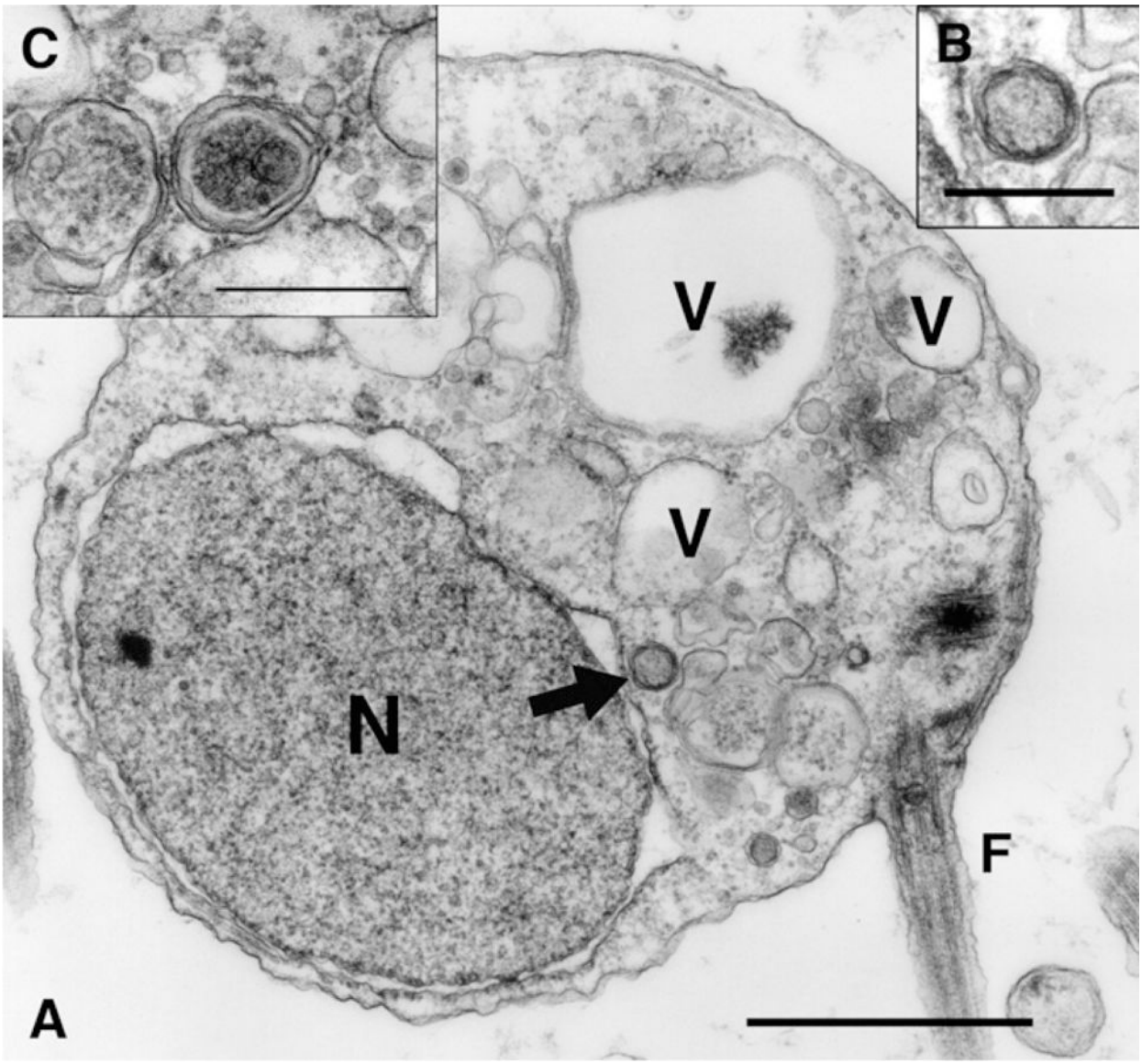


Fig. 3.

Ultrastructure of *Perkinsus* chesapeaki zoospores. A putative plastid has recently been identified in both zoospores and trophozoites of *Perkinsus olseni* (Teles-Grilo et al., 2007b). We have also identified putative plastid-like organelles in *P. chesapeaki* zoospores: A, plastid-like structure close to the nucleus (arrow); B and C, Detail of putative plastid-like organelles showing tightly associated membranes. N, nucleus; V, vacuole; F, flagellum; A, scale bar = 1 lm; B, scale bar = 500 nm; C scale bar = 300 nm.

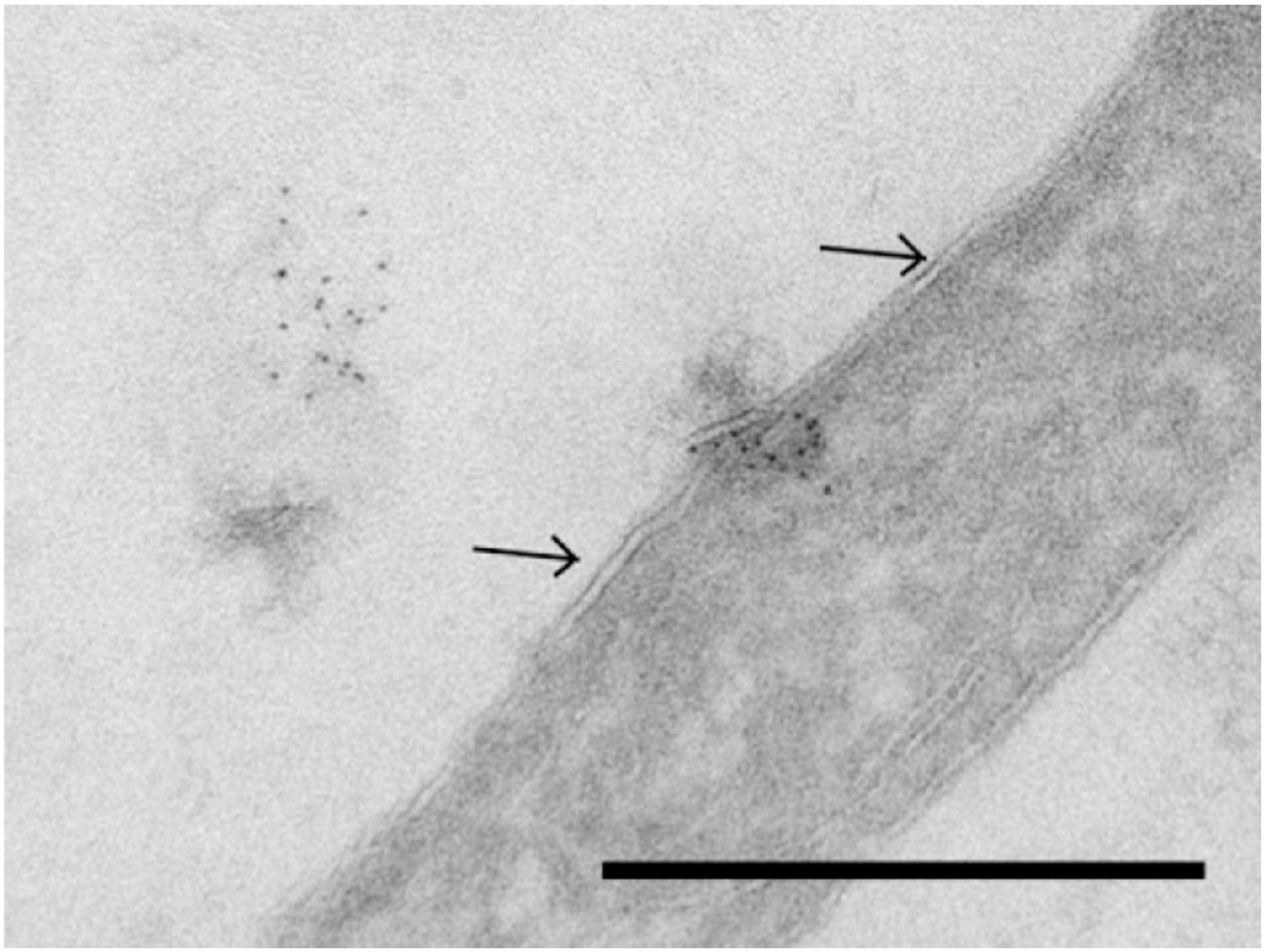


Fig. 4.

Immunoelectron microscopy of *Perkinsus marinus* superoxide dismutase 2 (PmSOD2) in *P. marinus* trophozoites. Anti-recombinant PmSOD2 (rPmSOD2)-specific antibodies localised the gold grains to compartments (vacuoles) limited by single membranes (arrow). The thickness of these membranes (14 nm) was within the range of most eukaryotic membrane lipid bilayers. The gold particles appeared to localise to vesicle-like structures on the outer face of the membrane surrounding the vacuole, and either free or associated to amorphous material in the vacuole lumen, probably derived from the content of the above mentioned vesicles. Scale bar = 500 nm.

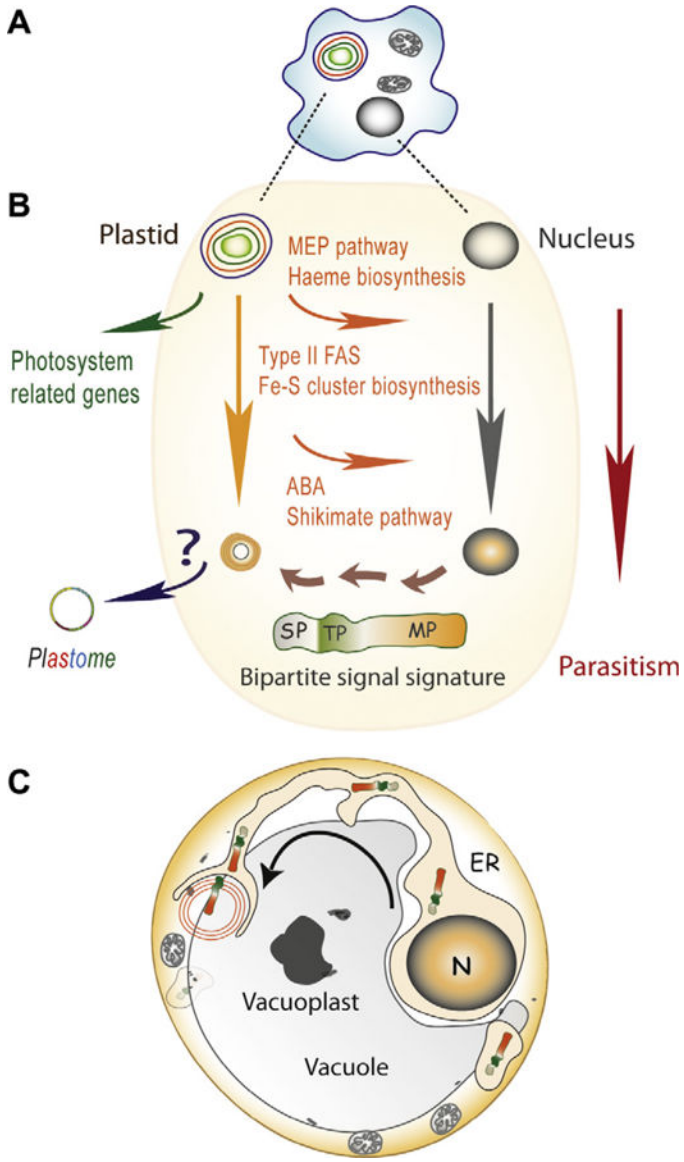
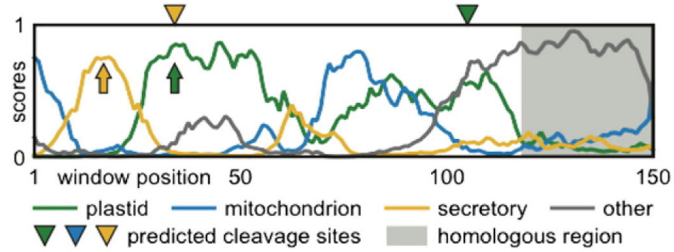


Fig. 5.

Working hypotheses for the presence of a plastid in the protozoan *Perkinsus*. A secondary endosymbiosis resulted in an ancestor plastid with multiple membranes and a plastid genome. (B) *Perkinsus* adaptation to intracellular parasitism resulted in plastid size reduction, photosystem gene loss, gene transfer to the nucleus (N) and acquisition of bipartite signal signatures for re-targeting proteins to the plastid. (C) Hypothetical targeting of *Perkinsus* nuclear-encoded proteins to the relic plastid via the endoplasmic reticulum (ER), based on the most parsimonious *Plasmodium* model (Tonkin et al., 2008b). MEP, non-mevalonate; FAS, fatty acid synthesis; Fe–S, iron–Sulphur; ABA, abscisic acid; SP, signal peptide; TP, transit peptide; MP, mature peptide.

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                                                                                                10        20        30
40        50        60        70        80        90        100
                              |         |         |         |         |         |         |         |         |         |
Thiomicrospira       ----------------------------------------------------------------------------------------------------
Vibrio               ----------------------------------------------------------------------------------------------------
Roseobacter          ----------------------------------------------------------------------------------------------------
Ketogulonicigenium   ----------------------------------------------------------------------------------------------------
Perkinsus            MRPPPPLSYLRRSAALPGLTLLFMGCYLLYSSVAFTASLFIAPLSSSSLGHMTASGVVPYASVPASSVSDGGIISFSLAQRALQLLAISGAVGAASRVMG

Prim.cons.           MRPPPPLSYLRRSAALPGLTLLFMGCYLLYSSVAFTASLFIAPLSSSSLGHMTASGVVPYASVPASSVSDGGIISFSLAQRALQLLAISGAVGAASRVMG

                              110       120       130       140       150       160       170       180       190       200
                              |         |         |         |         |         |         |         |         |         |
Thiomicrospira       -----------------MLLKVENLHAQVAEDESKQILKGLNLEVKPGEVHAIMGPNGAGKSTLSSVLAGREDYEVTEGSAIFNGEDLLELDPEDRAR-K
Vibrio               ------------------MLTIKNLKAQIDDTP---ILNGIDLEIKPGEVHAIMGPNGSGKSTLASTLAGREDYEITEGEVMLNGKDLLALDPDERAG-E
Roseobacter          ------------------MLSIKNLQVKLEEED-KEILKGVDLEIEAGKVHAIMGPNGSGKSTLSYVLSGKDGYEVTAGSAELEGEDVLDMDPEERAA-A
Ketogulonicigenium   ------------------MLTIKNLHVKLEEED-KAILKGVDLVIKPGEVHAIMGPNGSGKSTTSYVLAGRDGYEVTDGEVEFEGENLLEMEPEERAA-A
Perkinsus            RIRMRASEGSNAPAESPSGLHVSNLHTVLSEDPEMPILNGVTLDLEPGEVVALLGPNGCGKSSFARTLMGDSQYVVTHGGVEMNREDVLSLDVDERASKL
                                       * :.**:. : :      **:*: * ::.*:* *::****.***: : .* * . * :* * . :: ::* :: ::**
Prim.cons.           RIRMRASEGSNAPAESP2MLTIKNLH22LEE222K4ILKGVDLEIKPGEVHAIMGPNGSGKSTLS2VLAGR22YEVTEG2VE2NGEDLLELDPEERAAKA

                              210       220       230       240       250       260       270       280       290       300
                              |         |         |         |         |         |         |         |         |         |
Thiomicrospira       GVFLAFQYPVEIPGVSNKLFMQTAVNAIREENGLPVLDMFDFDEYAQAKIDLLNMRKDLLD---RSVNVGFSGGEKKRNDIFQMALLEPKLCILDETDSG
Vibrio               GVFLAFQYPVEIPGVSNHLFLQASVNAVRSYRGLPELERFELADLIEEKIELLNMPEDLLS---RSVNVGFSGGEKKRNDILQMAVLDPKLCILDETDSG
Roseobacter          GLFLAFQYPVEIPGVGNMTFLRTAVNAQRKARGEEEMSAAEFLKVVRARAKELKIDADMLK---RPVNVGFSGGEKKRNEILQMAMLEPKMCILDETDSG
Ketogulonicigenium   GVFLAFQYPVEIPGVGNLTFLRTALNAQRKSRGEEELSAADFLKLVRARAASLKIDAEMLK---RPVNVGFSGGEKKRNEILQMAMLEPKLCILDETDSG
Perkinsus            GLFMSWQAPVEVPGLTNFTLLHAAMNATRQAKNLPQLSPFEFYDFAKSRLMEVERGEELLEYLDRPVNTGMSGGERKRVELLHAVVLRPRLCIFDEIDSG
                     *:*:::* ***:**: *  :::::** *. ..    :.  :: .  . :   ::   ::*.   *.**.*:****:** :::: .:* *::**:** ***
Prim.cons.           GVFLAFQYPVEIPGV2N5TFL2TAVNAQRKARGLPELSAFEFL2L2RAR252L22D2DLLKYLDRPVNVGFSGGEKKRNEILQMA2LEPKLCILDETDSG

                              310       320       330       340       350       360       370       380
                              |         |         |         |         |         |         |         |
Thiomicrospira       LDIDAMKVVANGVNSLRTKDR--SFIVVTHYQRLLDYIKPDYVHVLYDGKIIKSGGFELVHELEEKGY--DDIIQAHDDTTQTNSK
Vibrio               LDIDALKEVSNGVNSLRDGTR--SFIIVTHYQRILDYIKPDFVHVLYQGKIIKSGDFSMVAELEENGY--GWLTEGE---------
Roseobacter          LDVDAMKLVAEGVNALRNEGR--GFLVITHYQRLLDHIKPDYVHIMSDGRIVKSGGPELALEVEHNGY--ADILAEVA--------
Ketogulonicigenium   LDVDAMKLVAEGVNALRSPER--SFLVITHYQRLLDHIKPDVVHIMANGRIVKSGGPELALEVENNGY--GDLLAGV---------
Perkinsus            LDVDALRQVATTLRSLREVDPSITMLIVSHYKRMLDELHPTRVLVMQDGVIVHQGGPDLINVVDRGGFKGGWLQQQEDDDDP----
                     **:**:: *:  :.:**          :::::**:*:** ::*  * :: :* *::.*. .:   ::. *:     :
Prim.cons.           LDVDAMKLVA2GVNSLR55DRSISFLVVTHYQRLLD2IKPDYVHVMYDG2IVKSGGPEL2LEVEENGYKGGDLL2G2DD222TNSK
```

Fig. 6.

Clustal W alignment of *Perkinsus marinus* putative iron–Sulphur (Fe–S) assembly ATPase SufB. The box above the alignment displays the results of Sliding Window Iteration of TargetP (SWIT) (Matsuzaki et al., 2008) that shows the distribution of targeting preferences for the N-terminal pre-sequences. The neural network score (y axis) of the TargetP prediction is plotted for the N-terminal 150 amino acids (x axis). Although the signal peptide (SP) prediction is not very strong, the chloroplast transit peptide (cTP) is predicted to be near the SP cleavage site and, therefore, as a good candidate for a class II bipartite leader. Green, plastid target peptide; light blue: mitochondrial target peptide; yellow. signal peptide; grey, other locations. *Thiomicrospira: Thiomicrospira crunogena; Vibrio: Vibrio caribbenthicus; Roseobacter: Roseobacter litoralis; Ketogulon-icigenium: Ketogulonicigenium vulgare*; *Perkinsus: P. marinus*.

Table 1

Proposed Perkinsus genes for co-localisation studies derived from published studies or from genome mining.

Organelle	Marker	Accession	Reference
Nucleus	Histone H3 Dmc1	XM_002788843 XM_002784146	*Perkinsus genome*
Centrosome	Centrin	XM_002787551	*Perkinsus genome*
Golgi	Ras Rab6 Sys1	XM_002786157 XM_002788779 XM_002781689	*Perkinsus genome*
ERAD	Cdc48 Ufd1 Npl4	XM_002782252 XM_002786555 XM_002778993	*Perkinsus genome*
Membranes/vesicles	SNARE SNAP	XP_002784625 XP_002769618	*Perkinsus genome*
ER	KDEL	Pmar PMAR017428	*Perkinsus genome*
Mitochondria	PmSOD1 Cit c oxydase	AY095212 AB513789	Schott and Vasta (2003) Masuda et al. (2010)
Putative plastid	MEP pathway Fe–S cluster pathway		Matsuzaki et al. (2008) Stelter et al. (2007)
Large vacuole	PmAPX2	XP_002767285	Schott et al. (2004)
Cell wall	PmMOE? GH18 Chitinase	EF632302 XP_002768327	Fernández-Robledo et al. (2008a) *Perkinsus genome*
Cytoskeleton	Actin Tubulin	AY436364 XM_002788606	Reece et al. (1997) *Perkinsus genome*
Cytosol	Glycolytic enzymes	XM_002788328 XM_002783157	*Perkinsus genome* Joseph et al. (2010)