The cryptomonad nucleomorph

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Abstract The cryptomonad nucleomorph is a vestigial nucleus of a eukaryotic red alga engulfed by a phagotrophic protist and retained as a photosynthetic endosymbiont. This review recounts the initial discovery and subsequent characterization of the cryptomonad nucleomorph focusing on the key role of Peter Sitte and his protégés in our understanding of secondary endosymbiosis to create complex plastids, one of the major transition events in the evolution of life on Earth.

Keywords Nucleomorph, cryptomonad, plastid, endosymbiosis

The first person to see a nucleomorph was apparently British phycologist Dennis Greenwood. Greenwood described structures—which he then termed 'crypto-nuclei'—with double membranes, pores, a nucleolar-like region, plus some 'distinctive particles' (Greenwood 1974). Greenwood's article is a one page abstract with just four accompanying micrograph (Greenwood 1974). Greenwood speculated that 'crypto-nuclei' might possibly contain a genophore (genome), and—somewhat remarkably for a one page article—went on to sketch out an hypothesis for the origin of the Chromophyta from a cryptomonad type ancestor via secondary endosymbiosis and loss of the nucleomorph, symbiont starch storage, invention of chlorophyll *c,* and invention of the chloroplast endoplasmic reticulum (cER) (Greenwood 1974). In a subsequent publication, again just an abstract (no micrographs) for a meeting of the British Phycological Society, Greenwood, HB Griffiths and Uwe Santore coined the term 'nucleomorph' to replace 'crypto-nuclei' and again proposed that further reduction of a eukaryotic symbiont contained within cryptomonads (such that the ribosomes, protein synthesis and starch storage in this compartment disappeared) could have given rise to four membrane bound plastids of the Chromophyta (Greenwood et al. 1977).

Two groups confirmed Greenwood's speculation that nucleomorphs contain DNA: one in Canada, and one in Germany. Martha Ludwig—then a PhD student of Sally Gibbs—stained the DNA of *Cryptomonas* with 4'-6-diamino-2-phenylindole (DAPI) (Ludwig and Gibbs 1985). DAPI accumulates in DNA structure and renders it fluorescent under UV light. Ludwig and Gibbs observed a small genome remote from the main nucleus and estimated that the nucleomorph contained between 10^8 and 10^9 Daltons of DNA on the basis that nucleomorph fluorescent intensity was somewhere between the cryptomonad chloroplast nucleoids and the nucleoids of unidentified bacteria contaminating the culture (Ludwig and Gibbs 1985). Ludwig and Gibbs also made comparisons of 5S rRNA sequences from the databases and concluded that the cryptomonads are not related to other chromophyte algae. So began a now long-running and still unresolved dispute about the origins of chromophytes: a debate placing cryptomonads at the centre of a very big puzzle in the evolution of life on Earth.

A German group, led by Peter Sitte from the Cell Biology Department at University of Freiburg, also utilised DAPI to confirm the presence of DNA in nucleomorphs, but they used acrylic sections of cryptomonad cells, providing superior resolution to the Canadian study using whole cells (Hansmann et al. 1985). The two DAPI studies thus confirmed that nucleomorphs contain DNA, corroborating preliminary evidence from regressive DNA staining of a nucleomorph by Uwe Santore (Santore 1982). Sitte's group subsequently produced very elegant images of nucleomorph DNA using immunogold labelling for electron microscopy with an anti-DNA antibody yielding the first ultrastructural localization and showed that the small osmiophylic particles, first observed in nucleomorphs by Greenwood (Greenwood 1974), contain no DNA (Hansmann et al. 1987a). To complement their localization of DNA in the nucleomorph, Paul Hansmann went on to use enzyme gold labelling to demonstrate that the ribosome-like particles observed by Greenwood in the periplasmic space contained RNA (Hansmann 1988). Furthermore, the ribonuclease gold markers also indicated that the enzyme's substrate (RNA) was concentrated in the nucleolus-like (fibrillogranular) body of the nucleomorph and absent from the dense particles (Hansmann 1988). I was then able to show that the RNA in the nucleolus like structure of the cryptomonad nucleomorph and periplastid compartment was eukaryotic type rRNA and not similar in sequence to the plastid rRNAs by using *in situ* hybridization electron microscopy with Domain specific rRNA probes (McFadden 1990b, a), thereby lending further credence to the notion that the nucleomorph was indeed a miniature nucleus. Immunogold labelling for histones by the Sitte lab was unable to detect these ubiquitous nuclear DNA binding proteins in the nucleomorph, although they were readily detectable in the host nucleus (Hansmann et al. 1987b).

The above mentioned histochemical studies from the Sitte, Gibbs, and McFadden laboratories confirmed Greenwood's surmise that nucleomorphs contained a genome and were essentially miniature nuclei, replete with a nucleolus that encoded rRNAs incorporated into ribosomes in surrounding periplasmic cytoplasm (Greenwood 1974). Meanwhile, morphological studies extended our understanding of nucleomorph size, structure and division. Sitte and Baltes did morphometric calculations to show that whilst the nucleomorph and its surrounding cytoplasm represented only 3% of the whole cell's translation capacity, the nucleomorph (which

has a relatively large nucleolus) apparently devotes three times as much of its capacity to ribosome production compared with the host nucleus (Sitte and Baltes 1990). Division of nucleomorphs was examined by Gillot and Gibbs, Morrall and Greenwood, and McKerracher and Gibbs, with the consensus being that there is one nucleomorph per plastid, the nucleomorph divides before the accompanying plastid, the dividing nucleomorph lacks a mitotic spindle, and the nucleomorph envelope persists throughout division (Gillot and Gibbs 1980; Morrall and Grenwood 1982; McKerracher and Gibbs 1983).

At this point we knew that nucleomorphs contained DNA, and that this DNA encoded eukaryotic rRNAs that were apparently present in ribosomes in the periplasmic cytoplasmic compartment surrounding the nucleomorph. The Holy Grail was now to get our hands on some nucleomorph DNA. Isolating nucleomorph DNA was a challenging quest because 1/ the amount of DNA is miniscule (Ludwig and Gibbs 1985), and 2/ volume wise the nucleomorph is a mere 0.3% of the total volume of the cell (Sitte and Baltes 1990)—a classic *needle-in-a-haystack* problem. Attempts in Freiburg to separate nucleomorph DNA from the copious amounts of nuclear, plastid and mitochondrial DNA using caesium chloride density gradients were unsuccessful (Hansmann et al. 1987b). But the Freiburg lab ultimately solved this dilemma using a very clever cell fractionation approach to isolate nucleomorphs (Hansmann and Eschbach 1990). By cunningly employing a cryptomonad (*Pyrenomonas*) in which the nucleomorph is enclosed within the pyrenoid, Hansmann and Stefan Eschbach took advantage of the robustness and high density of pyrenoids to disintegrate *Pyrenomonas* cells and then centrifugally sediment the nucleomorph-containing pyrenoids on density gradients (Hansmann and Eschbach 1990). This approach yielded the first fraction of nucleomorphs, and DAPI staining estimates provided an astonishingly small (yet remarkably accurate) nucleomorph genome size estimate of just 700kb (Hansmann and Eschbach 1990). The next step was to karyotype isolated nucleomorphs by pulse field gel electrophoresis (able to separate relatively large, chromosome-sized DNA molecules), which gave us the first insight into nucleomorph genome architecture (Eschbach et al. 1991a). The Freiburg team resolved three small linear chromosomes from the *Pyrenomonas* nucleomorphs, showing that they were indeed reduced nuclei with remarkably small genomes, weighing in at a mere 660kb—drastically smaller than the host nucleus and indeed any nucleus then known.

Sitte's Freiburg team had suddenly thrown open nucleomorph research (Eschbach et al. 1991a), and hence the secrets of secondary endosymbiosis, like never before. My lab quickly adopted the pyrenoid/nucleomorph isolation protocol and we too had three eukaryotic endosymbiont chromosomes staring at us from our agarose gels (McFadden 1993). What did they encode?

1991 proved somewhat of a halcyon year for the nucleomorph. A new team from Canada entered the fray and used PCR to bypass nucleomorph isolation, instead taking advantage of the difference in size between the host and endosymbiont 18S rRNA genes to separate and sequence the two amplicons (Douglas et al. 1991). The two gene sequences were incorporated into the emerging rDNA tree of eukaryotes with one (tentatively assigned as originating from the host nucleus) being allied to the plants and green algae, and the other (tentatively assigned as originating from the nucleomorph) being allied to the red algae *Gracilaria* and *Gracilariopsi*s (Douglas et al. 1991). Recovering two different, evolutionarily distant genes from one cell spectacularly corroborated the hypothesis that cryptomonads harbour a reduced red algal endosymbiont (Douglas et al. 1991). But the Canadian team candidly admitted that evidence that the red algal affiliated gene from their cryptomonad came out of the nucleomorph was lacking (Douglas et al. 1991). The Freiburg team used their isolated nucleomorphs to settle this issue. Sitte's team made Southern blots of their pulsed field gel separated chromosomes from isolated nucleomorphs and showed that all three chromosomes encoded 18S and 25S rRNA genes, likely in a classic eukaryotic rRNA cistron (Eschbach et al. 1991a). Eschbach, Wolters and Sitte sequenced the 18S rRNA gene from the host nucleus of *Pyrenomnas* and modelled the secondary structure and made a phylogeny (Eschbach et al. 1991b). Another Freiburger, Uwe Maier, cut the nucleomorph chromosomes out of pulsed field gels and used PCR to amplify the nucleomorph rRNA gene for sequencing and phylogeny (Maier et al. 1991). The phylogenetic tree was satisfying in the sense that it supported the Canadian study (Douglas et al. 1991) in showing that the nucleomorph 18S rRNA gene was evolutionarily remote from the nuclear gene from the same cell, but the Freiburg analysis provided only weak indications of the ancestry of the nucleomorph positioning it close to four genes from red algae but not clustering them unequivocally (Maier et al. 1991). With hindsight, we now realise that the phylogenetic ancestry of nucleomorph gene is somewhat masked by accelerated divergence; it's a difficult gene

to infer trees with. I later rounded out these studies by using electron microscope level *in situ* hybridization with gene-specific probes to prove that transcripts of the nucleomorph rRNA gene are in the nucleolus of the nucleomorph and surrounding periplasmic cytoplasm, while transcripts of the nuclear rRNA gene are in the nucleolus of the host cell nucleus and it surrounding cytoplasm (McFadden et al. 1994). Thus, a blend of electron microscopy, histochemistry, molecular biology and phylogenetics performed across three continents confirmed that nucleomorphs are indeed vestigial nuclei of a red algal eukaryotic endosymbiont. Greenwood was right (Greenwood 1974).

Cryptomonads are inconspicuous and not particularly diverse. They come in a range of colours, but it is fair to say that if you've seen one cryptomonad, you've seen them all. However, their uniformity notwithstanding, the work described above showed that cryptomonads are among the most complicated cells on Earth, being the amalgam of two separate eukaryotes (host and endosymbiont) and two separate prokaryotes (the cyanobacterial like ancestor of their plastid and the α-proteobacterial like ancestor of their mitochondrion). Over a beer one evening in a Freiburg Biergarten, Uwe Maier laid out an audacious plan to us. Maier suggested that the previously competing teams from Germany, Canada and Australia should form a consortium and collaborate to sequence a nucleomorph genome. We announced our plan in an article called 'Bonsai Genomics', wherein we extolled the benefits of sequencing the world's smallest nucleus, pruned down by endosymbiosis to a core of eukaryotism (McFadden et al. 1997). By 2000 the team had progressed inwards from the ends of the three nucleomorph chromosomes confirming the presence of canonical (albeit novel) eukaryotic telomeres subtended by rRNA cistrons at the termini of each chromosome (Zauner et al. 2000). It was proposed that the entire repeat was kept identical by a mechanism of concerted evolution across the six ends (Zauner et al. 2000). The interior of the chromosomes carried densely packed protein and tRNA genes that were reminiscent of red algal genes in being almost completely devoid of introns (Zauner et al. 2000). The gene repertoire covered typical eukaryotic housekeeping functions, and two *raison d'être* genes (*ftsZ* and rubredoxin) encoding proteins likely essential for maintenance of the plastid, which explained why the nucleomorph persisted—it encodes crucial information for the maintenance, and biogenesis of the plastid (Zauner et al. 2000). The complete sequence (551,264 base

pairs) followed in 2001 and showed a residue of just 30 genes for chloroplast maintenance/function scattered among 464 protein coding genes with a wide range of housekeeping functions (Douglas et al. 2001) including canonical histones that Sitte's team were not able to detect histochemically (Hansmann et al. 1987b). The paucity of spliceosomal introns was further confirmed, as was the dense packing of genes consistent with severe pruning of the DNA content during the nucleomorph's tenure as an endosymbiont within the cryptomonad host (Douglas et al. 2001). John Archibald's lab in Canada sequenced several cryptomonad nucleomorphs showing that all had converged on three small chromosomes but that the mechanisms and extent of reductions and repeats varied across the genera (Lane and Archibald 2006; Lane et al. 2007; Tanifuji et al. 2011; Moore et al. 2012).

A crucial insight from the nucleomorph genome was that it lacked many genes for functions considered essential for the maintenance and replication of the nucleomorph and its expression machinery—proteins for this apparatus clearly had to be encoded by the host nucleus and imported into the nucleomorph/periplastid cytoplasm for the cells-within-cells that comprise a cryptomonad to work (Douglas et al. 2001). Herein lay the next major challenge to understand these cellular equivalents of the Russian matryoshka dolls.

Plastid proteins encoded by the nucleomorph must be made in the periplastid space and imported across the two inner cryptomonad plastid membranes. By analogy, it was early reasoned that this would happen by the same mechanism used by the red algal ancestor of the cryptomonad endosymbiont. Indeed, the first two nucleomorph encoded plastid proteins (FtsZ and rubredoxin) bear N-terminal extensions proposed to act as transit peptides (Zauner et al. 2000), as do many of the plastid proteins identified in the whole nucleomorph genome (Douglas et al. 2001). Indeed, two of these putatively targeted proteins (Tic 110 [Iap100] and Tic22) (Douglas et al. 2001) are components of the plant chloroplast import machinery, which is apparently highly conserved across all types of plastids including those of cryptomonads (McFadden and van Dooren 2004).

More puzzling was how proteins from the cryptomonad host nucleus could cross the four membranes bounding these complex plastids. Work with diatoms, which also

have a CER and were proposed by Greenwood to derive from cryptomonad-like ancestors (Greenwood 1974), showed that host nucleus encoded plastid proteins use classic secretory signal peptides to cross the outermost, ribosome-encrusted plastid membrane (the so-called cER)(Bhaya and Grossman 1991), and similar arguments were made for cryptomonad proteins(Reith 1996). This left the traversal of only one of the four cryptomonad plastid bounding membranes (known as the periplastid membrane and second in from the outermost) as unexplained. Uwe Maier's group solved this conundrum by oing back to first principals. They reasoned that the translocon pore would be a multimembrane pass protein encoded by the nucleomorph (Sommer et al. 2007). From there they were able to demonstrate that cryptomonads employ a translocation motor originally derived from the endosymbiont's ER that has been relocated into the periplastid membrane (Sommer et al. 2007). This translocation system, known as the ER-associated protein degradation (ERAD) translocon, normally extracts misfolded proteins from the ER lumen for degradation in the cytoplasm (Bagola et al. 2010; Mehnert et al. 2010). In other words, the translocation system re-imports proteins back into the cytosol from the lumen of the ER, which is technically an external compartment. This is exactly the direction required for import across the periplastid membrane of the apicoplast, so the ERAD system was apparently ideal for the task. Moreover, the ERAD machinery recognises unfolded proteins, which is a characteristic of plastid targeting transit peptides (Ralph et al. 2004). Maier's team proposed that relocation of the ERAD translocon from the endosymbiont's ER to its plasma membrane, which are effectively continuous, was the critical invention for import of proteins into the secondary plastid (Sommer et al. 2007). Indeed, by simply relocating its ERAD translocon into its plasma membrane, the endosymbiont was apparently able to import proteins from the lumen of the host's ER—the space between the outermost secondary plastid membrane and the periplastid membrane.

Maier refers to the endosymbiont ERAD system as SELMA (symbiont-specific ERAD-like machinery (Hempel et al. 2009). Like canonical ERADs, SELMA comprises multiple components, the identity and roles of which have now been explored in other complex plastids in organisms such as diatoms and apicomplexan parasites, all of which use the SELMA system discovered in the cryptomonad nucleomorph (Gould et al. 2015; Maier et al. 2015; Fellows et al. 2017).

The SELMA system also traffics host nucleus encoded proteins into the periplastid space and nucleomorph. The routing decision for plastid versus periplastid/nucleomorph destinations after SELMA translocation is heavily influenced by the newly exposed terminal residue of the transit peptide after signal peptide cleavage (Gould et al. 2006). If this residue is a phenylalanine, the protein typically goes into the plastid via the Toc and Tic machinery. Other residues result predominantly in a periplastid space/nucleomorph localization (Gould et al. 2006).

The collaborative nature of nucleomorph research reached its zenith with the completion of the entire genome (nucleomorph, nucleus, mitochondrion and plastid) (Curtis et al. 2012) for the model cryptomonad *Guillardia theta* (named after the eminent phycologist Bob Guillard). Peter Sitte had long since retired, but the team he created collaborated with researchers from many countries to complete the work begun in Freiburg three decades ago. Sitte's gentlemanly style brought researchers together to solve big questions: his flair for microscopy and cell biology inspired them to address these questions with the very best tools of the day.

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