Multiple origins of anaerobic ciliates with hydrogenosomes within the radiation of aerobic ciliates

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SUMMARY

Some ciliates live anaerobically and lack mitochondria, but possess hydrogenosomes: organelles that contain hydrogenase and produce hydrogen. The origin of hydrogenosomes has been explained by two competing hypotheses: (i) they are biochemically modified mitochondria; or (ii) they are derived from endosymbiotic association(s) of ciliates and anaerobic eubacteria that possessed the hydrogenosome biochemistry. Phylogenetic analyses of representative aerobic, and anaerobic hydrogenosomal ciliates using host nuclear SSU rDNA sequences indicate a minimum of three, but more likely four, separate origins of hydrogenosomes. Whereas this does not refute either hypothesis, the implausibility of multiple convergent endosymbioses gives further support to the view that hydrogenosomes in ciliates derive from an existing organelle, which ultrastructural evidence suggests is the mitochondrion. Our results indicate a considerable potential for physiological–biochemical plasticity among a group of predominantly aerobic eucaryotes, and provide a phylogenetic framework to further refine and test hypotheses of the origins of the hydrogenosomal enzymes.

1. INTRODUCTION

Microbial eucaryotes (protists) are common in most anoxic environments (Fenchel & Finlay 1995). Some are obligate anaerobes, lacking cytochromes and mitochondria, but containing another membrane-bounded organelle: the hydrogenosome. Hydrogenosomes were discovered in Trichomonas (Lindmark & Müller 1973), but they also occur in free-living anaerobic ciliates and in rumen ciliates and chytrid fungi (Muller 1993). All hydrogenosomes are considered functionally analogous, although detailed biochemical information is limited to Trichomonas (Muller 1993), the rumen ciliate Dasytricha ruminantium (Yarlett et al. 1981) and the chytrid Neocallimastix (Marvin-Sikkema et al. 1992). Under anaerobic conditions, hydrogenosomes produce hydrogen via the activity of hydrogenase.

The origins of hydrogenosomes and their enzymes are important for understanding the evolution and metabolic diversity of eucaryotes and their organelles. The occurrence of hydrogenosomes in phylogenetically distinct eucaryotes suggests that they have been acquired independently, and differences in ultrastructure indicate that hydrogenosomes in different taxa may have different origins (Müller 1993). For example, hydrogenosomes in Neocallimastix have a single boundary membrane and resemble peroxisomes (Marvin-Sikkema et al. 1992). In contrast, hydrogenosomes in Trichomonas and most ciliates possess a double membrane and in this feature resemble mitochondria (Finlay & Fenchel 1989; Paul et al. 1990).

Ultrastructural similarities between hydrogenosomes in anaerobic ciliates and mitochondria in aerobic ciliates, have prompted the hypothesis that these hydrogenosomes are biochemically modified mitochondria (Finlay & Fenchel 1989). The transition from aerobes with mitochondria (which is considered the ancestral state in ciliates) to anaerobes with hydrogenosomes is postulated: (i) to have occurred several times; and (ii) to be a common adaptation of ciliates that occupy anaerobic habitats (Fenchel & Finlay 1995). Evidence for numerous independent losses of functional mitochondria and gain of functional hydrogenosomes, is provided by the morphological variety of hydrogenosomal ciliates (Fenchel & Finlay 1995). Balanced against this is the view that hydrogenosome origins are likely to have been complex and to have required a large number of steps (Müller 1993), rendering convergent acquisition less plausible.

The most incisive way to investigate the distribution and origins of any feature is by reference to a phylogenetic tree constructed using information independent of the trait that is being investigated. Ciliate taxonomy has traditionally relied on morphology and ultrastructure, but these data have proved to be an unsatisfactory basis for robust inferences of ciliate phylogeny, mainly because different morphological
Figure 1. (a) Maximum likelihood analysis of 1471 bases of SSU rDNA sequence from aerobic and hydrogenosomal ciliates. Details of the maximum likelihood model are given in §2. Clades X, Y and Z were consistently recovered in all unconstrained analyses. Class or subclass names of ciliates are indicated in parentheses. (b) Single maximum parsimony tree and estimates of clade reliability from analysis of informative sites of SSU rDNA sequence from the
systems often suggest radically different relationships (Corliss 1979; Lynn & Small 1989). Here we test the hypothesis of multiple separate origins of hydrogenosomes among ciliates, by phylogenetic analysis of SSU rDNA sequences from representative aerobic and hydrogenosomal ciliates.

A superficial analysis of SSU rDNA sequences from the free-living hydrogenosomal ciliates Metopus palaeformis, M. contortus, Trimyema spp., T. compressum, Plagiopyla frontata, P. nasuta and Cyclidium porcatum, already suggests they are not monophyletic and that their anaerobic phenotypes may have arisen independently (Embley & Finlay 1994). Here we present a more taxonomically comprehensive and detailed analysis of the phylogenetic relationships of these species and of two hydrogenosomal rumen ciliates Dasytretia ruminantium and Entodinium simplex. The recent discovery of Cyclidium porcatum, another hydrogenosomal ciliate (Esteban et al. 1993), is important, because this genus also includes aerobic species with mitochondria. We have therefore sequenced the SSU DNA of two aerobes, Cyclidium glaucoma and C. plouneouri, for comparative purposes.

2. MATERIALS AND METHODS

(a) Culture of ciliates

Cells of Metopus contortus and Trimyema spp. were prepared for polymerase chain reaction (PCR) as described previously (Embley et al. 1992a; Finlay et al. 1993). Cyclidium porcatum cells were isolated by centrifugation from enrichments (Esteban et al. 1993) in which it was the only eucaryote. Cells (ca 100) of Plagiopyla frontata or P. nasuta were purified from enrichments by filtration and micromanipulation through a sterile (sea water for water series. Cells of Cyclidium glaucoma and C. plouneouri were a generous gift from Dr Blanca Perez-Uz (Department of Zoology, Natural History Museum). Dasytretia ruminantium and Entodinium simplex were isolated by filtration from rumen liquor taken from sheep containing defined ciliate communities (Williams & Coleman 1992).

(b) DNA extraction

Cyclidium spp., Plagiopyla spp., Metopus contortus and Trimyema spp. were lysed using 200 μl of Chelex-100 (Walsh et al. 1991). Of the centrifuged lysates, 10 μl were used for PCR. DNA was isolated from rumen ciliates using the guanidium thiocyanate-silica method (Boom et al. 1990). DNA was eluted with 50 μl TE buffer and 1 μl was used for PCR.

(c) Amplification and sequencing of ciliate SSU rDNA

PCR amplifications (50 μl or 100 μl) were done using eucaryote specific primers (Embley et al. 1992b). PCR products from Cyclidium spp., M. contortus, Plagiopyla spp. and Trimyema spp. and were sequenced directly (Embley 1991) by using published primers (Elwood et al. 1985). PCR products from D. ruminantium and E. simplex were cloned into p-GEM-T (Promega) and three clones of each species were pooled for sequencing.

(d) Analysis of SSU rDNA sequences

All sequences have been deposited in Genbank: Z29516 (Metopus contortus); Z29517 (Cyclidium porcatum); Z22879 (Cyclidium glaucoma), U27816 (Cyclidium plouneouri); Z29436 (Trimyema compressum); Z29441 (Trimyema sp.); Z29440 (Plagiopyla frontata); Z29442 (Plagiopyla nasuta); U27814 (Dasytretia ruminantium) and U27815 (Entodinium simplex). They were manually aligned against sequences from: (i) reference ciliates; (ii) two dinoflagellates and two apicomplexans as outgroup taxa; and (iii) a chrysophyte to root the tree (see figure 1), using the Genetic Data Environment (gde) software version 2.2 (Maidak et al. 1994). The aligned reference sequences were from the Ribosomal Database Project (Maidak et al. 1994), supplemented with recently published ciliate sequences (Leipe et al. 1994; Hirt et al. 1995).

For phylogenetic analysis a mask was used to exclude positions that could not be unambiguously aligned, or positions where deletions or insertions resulted in less than half of the taxa having nucleotides at that site. The final ciliate data matrix contained 1471 positions of which 615 are informative under parsimony. The alignment is available from the first author. The program fastDNAml version 1.0.6 (from G. Olsen, see Maidak et al. 1994), was used for maximum likelihood (ML) analyses using all 1471 positions. This program does not assume constancy of evolutionary rate between lineages, and is particularly useful when, as here, rates are unknown. A transition-transversion ratio of 1 was applied because this most closely matched the pattern in the aligned data, empirical base frequencies were used to estimate substitution model parameters. The GLOBAL rearrangement option was used, with analyses repeated using different orders of sequence addition (JUMBLE in fastDNAML). The user tree—option in the program DNAML in the PHYLIP 3.5 (Felsenstein 1993) program was used to compute likelihoods for the shortest trees generated using parsimony supporting particular interpretations of hydrogenosome origins, and to compute likelihood ratios comparing these trees and the mt tree (Kishino & Hasegawa 1989). Distance matrix calculations and neighbour-joining analyses used the programs DNADIST and NEIGHBOR in PHYLIP 3.5. All maximum parsimony (MP) analyses were of informative positions only and used the heuristic search option in PAUP 3.1.1 (Swofford 1993), with one, ten or 1000 random addition sequences and TBR branch swapping. Bootstrapping (100 replicates),
was used to investigate support for groups in both distance matrix (SEQBOOT and CONSENSE in PHYLIP 3.5) and MP analyses, the latter with one random addition sequence per replicate. Topologically constrained MP analyses, with ten random addition sequences, were used to explore differences in tree length associated with specific hypotheses of ciliate relationships and determine their Bremer support (Källersjö et al. 1992).

3. RESULTS

(a) The ciliate tree

All three phylogenetic reconstruction methods consistently recovered ciliate clades, X, Y and Z as defined in figure 1. Differences between the trees, produced using different methods, are limited to the variable placements of Colpoda, relative to clades Y and Z, and of Opisthonia, Paramaecium and a well supported Plagiopyla-Trimyema clade within clade Z. Other differences are small rearrangements of closely related taxa within well supported aerobic heterotrich and litostome clades. Judged on their likelihoods, the ML tree is not significantly better than the single MP tree (table 1) and the single MP tree is just ten steps (0.38%) shorter than the ML tree. Distance and MP bootstrap proportions, and Bremer support values appear strongly correlated (see figure 1). Although many clades appear well supported, the low values for some nodes indicate that some ciliate relationships remain uncertain.

(b) Relationships of hydrogenosomal ciliates

The topologies of the distance, ML and MP trees require a minimum of four independent origins of hydrogenosomes, two within clade Y and two within clade Z, to explain, most parsimoniously, the observed distribution of these organelles among the ciliates sampled in this study. The support for each of the three lineages which comprise more than a single hydrogenosomal ciliate taxon is sufficiently high to discount more than four separate origins (see figure 1). However, the relationships of the four hydrogenosomal lineages to aerobic ciliates are less clear.

Clade Y contains a well supported litostome clade comprising the anaerobic rumen ciliates and their aerobic relatives. Other than the sister group relationship of the rumen ciliates, relationships within this litostome clade were not well supported. The anaerobic metopids are consistently recovered as the sister group of the litostomes, but judged by Bremer support and bootstrap proportions, this position is not strongly supported. Clade Z includes the Plagiopyla-Trimyema clade and the anaerobic Cyclidium porcatum. A clade containing the aerobes Cyclidium glaucoma and C. plonerei, and C. porcatum, was recovered in all unconstrained analyses (figure 1). Bootstrap proportions from the distance matrix analyses (99%) strongly support this topology, but those from the MP analysis (78%) and the Bremer support (+7) are less compelling.

(c) Testing the number of hydrogenosome origins with topological constraints

To address this question, we employed MP analyses to find the shortest trees with associations between hydrogenosomal ciliates that would require fewer origins, enforced through topological constraints. The resulting trees were compared in terms of their lengths.
Table 1. Competing hypotheses of anaerobic ciliate relationships, effects on maximum parsimony and maximum likelihood analyses

(Maximum parsimony analyses were used to find the shortest trees for associations between hydrogenosomal ciliates which would require fewer than four separate origins of hydrogenosomes. The resultant trees were compared for their lengths to the maximum parsimony tree (mp). The log likelihood of each tree was calculated using the USER tree option in Felsenstein's maximum likelihood programme DNAML. The Kishino Hasegawa (1989) test contained in DNAML was used to calculate the mean difference and its variance, between these user trees and the maximum likelihood tree (ml). Where the mean is more that 1.96 standard deviations different the hypothesis of fewer origins is considered to be worse than the ml tree which gives four origins of hydrogenosomes. Key: cp, Cyclidium porcatum; m, Metopus palaeformis; M. centrales; rc, rumen ciliates; pt, Plagiopyla and Trimyema.)

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and their likelihoods. Table 1 summarizes these analyses and figure 2 shows mp trees for three, two, and one origin(s) of hydrogenosomes. None of the alternative topologies add less than 13 steps (three separate origins) to the length of the tree (by way of comparison the ciliate clade has a Bremer support of +10), and most alternatives require substantial increases in tree length. Judged by their likelihoods, trees requiring less than three origins are significantly worse than the ml tree (see table 1). However, of the nine trees requiring three origins, the one enforcing an association between the Plagiopyla–Trimyema clade and Cyclidium porcatum (see figure 2 a) requires only 13 extra steps and is not significantly different from the ml tree.

4. DISCUSSION

(a) Ciliate molecular phylogeny

The results of our multiple analyses of ciliate relationships are in general agreement with recently published molecular-based trees that support the reality of the ciliate clade; indicating that some main ciliate subclades can be identified, but also that some relationships are not well resolved (for examples, see Leipe et al. 1994; Hirt et al. 1995). In contrast, there is only part agreement with published ciliate taxonomies or phylogenies based upon morphology or ultrastructure (Corliss 1979; Lynn & Small 1989). Here we focus on relationships of the hydrogenosomal ciliates and their significance for the interpretation of the origins of hydrogenosomes.

(b) Plagiopyla and Trimyema

Trimyema and Plagiopyla have traditionally been classified within different families of the subclass Vestibulifera by the presence of a vestibular cavity preceding the mouth (Corliss 1979). Others have argued that the morphology of Trimyema places it in the subclass Gymnostomata (Serrano et al. 1988). From a third perspective based upon analysis of basal...
while we cannot entirely discount it, we consider that
Plagiopyla
clusively. The bootstrap proportions, is completely absent. Thus, a positive support for this topology, as provided by
not significantly worse than the

hydrogenosomes (metabolism) must have originated after the origin of their hydrogenosomes. However, analyses using topological constraints show that associations of this clade with Cyclidium porcatum (and hence a common origin of their hydrogenosomes) cannot be completely discounted (see §4c).

c) Cyclidium porcatum and aerobic Cyclidium species

Cyclidium porcatum is a fascinating protist; not only does it contain a functional hydrogenosome, but this is organized into a stable intracellular symbiosis containing Bacteria and Archaea (Esteban et al. 1993; Embley & Finlay 1994). Interspecies transfer of metabolites, particularly hydrogen, is thought to underpin the symbiosis and no other similar complex is known (Fenchel & Finlay 1995). The morphology of C. porcatum supports its classification with its aerobic congeners C. glaucoma and C. ploumecouri (Esteban et al. 1993), as do our unconstrained analyses. If this association is correct, then hydrogenosomes in C. porcatum are most parsimoniously explained as being acquired independently from other anaerobic ciliates, and its complex symbiosis (which depends on hydrogenosome metabolism) must have originated after the organelle was acquired. However, our analyses do not provide compelling evidence (i.e. consistently high bootstrap support), for a monophyletic Cyclidium, and further sampling of other scuticociliates may be necessary to resolve their relationships more conclusively. The MP tree constrained to place C. porcatum with the Plagiopyla–Trimyema clade and thereby imply a single origin of hydrogenosomes within clade Z, and three separate origins overall (see figure 2a, table 1), is not significantly worse than the ML tree. In contrast, positive support for this topology, as provided by bootstrap proportions, is completely absent. Thus, while we cannot entirely discount it, we consider that a single origin of the hydrogenosomes of C. porcatum, Plagiopyla and Trimyema is improbable.

d) Metopus

Our inferred relationships among heterotrich ciliates are not congruent with classical taxonomy (Corliss 1979), but support a recent molecular analysis (Hirt et al. 1995) which revealed that heterotrichs are not monophyletic. In all unconstrained analyses the metopids comprise a distinct clade requiring a separate origin of their hydrogenosomes.

(e) Rumen ciliates

The phylogenetic position of the entodiniomorphs, represented here by Entodinium, has always been controversial. Formerly placed in the Spirotrichea (with the heterotrichs) based upon oral ciliature they were reclassified within the Vestibulifera (Corliss 1979), then in the Litostomatea (Lynn & Small 1989). The present analysis agrees with this latest placement (see figure 1): Dasytricha ruminantium is classified in the litostomes, although in a different order to Entodinium (Small & Lynn 1985). The evolutionary origins and diversification of the rumen ciliates have been the subject of much speculation (Williams & Coleman 1992). Our contribution to this debate is to demonstrate that representatives of the two most important groups; the holotrichs and entodiniomorphids, are more closely related to each other than was previously thought. That Dasytricha ruminantium and Entodinium simplex are sister taxa, and that their hydrogenosomes are therefore not independently derived, are among the most strongly supported inferences from our data. This raises the possibility, which should be tested by sampling more species, that the morphologically heterogeneous rumen ciliates are the products of a single radiation rather than multiple colonizations. Our analyses also demonstrate that Dasytricha and Entodinium, and the aerobic litostomes (represented by Spathidium, Lexophyllum and Homolazoon) comprise a well supported litostome clade, and that hydrogenosomes have evolved within this clade independently of their origin elsewhere among the ciliates.

(f) Origins of hydrogenosomes among ciliates

The problem of hydrogenosome origins can be broken into two parts: (i) where does the organelle come from; and (ii) where do the hydrogenosomal enzymes originate from. The most direct source of evidence for organelle origins is its genome (Gray 1992), but as yet there is no published evidence for an organelle genome in hydrogenosomal ciliates. In the absence of direct evidence, one must use data which speaks indirectly to the problem to help formulate working hypotheses. The evidence for hydrogenosomes in ciliates being modified mitochondria is based upon structural similarities between the two organelles. Hydrogenosomes in the free-living anaerobic ciliates sampled in this study are all double membraned (Zwart et al. 1988; Finlay & Fenchel 1989), and hydrogenosomes in Cyclidium porcatum strongly resemble mitochondria in aerobic Cyclidium spp. (Fenchel & Finlay 1995). The data for Dasytricha and Entodinium are insufficient to determine unequivocally if their hydrogenosomes have one or two membranes, although one has been cited (Muller 1993). Polyplastron is in the same family as Entodinium and it has two membranes (Paul et al. 1990). The alternative theory of hydrogenosomal origins is that they are the descendants of endosymbioses of anaerobic bacteria that contained the hydrogenosomal enzymes (Muller 1993).

Our phylogenetic analysis cannot resolve the origins of hydrogenosomes or hydrogenase in free-living ciliates. Our sampling of taxa must needs be incomplete, there are several thousand described ciliate species, most of which have not been brought into culture, but some of which have been observed to live anaerobically (Fenchel & Finlay 1995). However, our analyses do clearly demonstrate, for the best chara-
cterized hydrogenosomal ciliates, that these organelles have been acquired independently by at least four phylogenetically distinct groups. A single alternative involving three origins among the ciliates sampled cannot be completely discounted, but it is less plausible. In our view this observed frequency makes repeated convergent endosymbioses as the source of hydrogenosomes in ciliates implausible, because the steps required to convert an endosymbiont into an organelle are very complex (Cavalier-Smith 1992).

If we dismiss multiple origins of a new organelle, we need to explain the multiple occurrence of hydrogenase: an enzyme that is not found in mitochondria (Müller 1993). A single ancestral origin of hydrogenase is more parsimonious than multiple acquisitions, and it is known that some anaerobic protozoa that separated from other eucaryotes early in evolution, also contain hydrogenase (Müller 1993). An ancestral acquisition would predict that aerobic ciliates contain hydrogenase genes, this might be tested by searching for these genes in aerobic ciliates, particularly the aerobic Cyclidium spp. and aerobic Litostomes.

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