CellPress

Current Biology

Review

Bacterial and archaeal symbioses with protists

Filip Husnik[1,2,*], Daria Tashyreva[3], Vittorio Boscaro[2], Emma E. George[2], Julius Lukeš[3,4], and Patrick J. Keeling[2,*]
[1]Okinawa Institute of Science and Technology, Okinawa, 904-0495, Japan
[2]Department of Botany, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[3]Institute of Parasitology, Biology Centre, Czech Academy of Sciences, 370 05 České Budějovice, Czech Republic
[4]Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic
*Correspondence: filip.husnik@oist.jp (F.H.), pkeeling@mail.ubc.ca (P.J.K.)
https://doi.org/10.1016/j.cub.2021.05.049

SUMMARY

Most of the genetic, cellular, and biochemical diversity of life rests within single-celled organisms — the prokaryotes (bacteria and archaea) and microbial eukaryotes (protists). Very close interactions, or symbioses, between protists and prokaryotes are ubiquitous, ecologically significant, and date back at least two billion years ago to the origin of mitochondria. However, most of our knowledge about the evolution and functions of eukaryotic symbioses comes from the study of animal hosts, which represent only a small subset of eukaryotic diversity. Here, we take a broad view of bacterial and archaeal symbioses with protist hosts, focusing on their evolution, ecology, and cell biology, and also explore what functions (if any) the symbionts provide to their hosts. With the immense diversity of protist symbioses starting to come into focus, we can now begin to see how these systems will impact symbiosis theory more broadly.

Introduction

The crucial role of endosymbiosis in the origin of eukaryotic cells and organelles is now accepted beyond any serious doubt, and that debate has turned to focus on how deep was its impact, and how ancient were the associations that gave rise to mitochondria and plastids[1–4]. However, it would be a mistake to codify the effects of endosymbiosis based on a few events of extreme antiquity, because bacterial and archaeal symbionts continue to play major roles today in eukaryotic cell biology, molecular biology, ecology, and evolution. The body of evidence for the importance of these ongoing relationships nearly all comes from studies of multicellular hosts, where both apparently parasitic and beneficial relationships have yielded fascinating insights into the mechanisms and outcomes of long-term cohabitation[5–9]. From these studies, new basic principles are taking shape; however, the strong focus on multicellular hosts contrasts with the fact that most of the genetic, cellular, and biochemical diversity of eukaryotes rests within single-celled organisms, called protists[10,11] (Figure 1; also see Glossary for a list of key terms used).

We know from over a century of microscopy and more recent molecular and genomic evidence that protists also form a multitude of symbiotic associations with bacteria and archaea. Yet we know considerably less about the evolution or function of these associations compared with symbioses involving animal hosts. This is important because much of our theoretical foundation for understanding symbiosis comes from well-studied animal systems that are functionally based on mitigating simple, stable nutritional deficiencies such as a lack of essential amino acid biosynthesis[5,7]. This is unlikely to be a particularly important driver of symbiosis in protists, as most do not have nutrient-limiting diets; rather, they are grazers, predators, or mixotrophs, and consequently symbioses based on nutritional supplementation are relatively rare.

The breadth of protist biology can be intimidating to synthesize or digest in general[12], and this also applies to their symbiotic interactions. Protist–prokaryote symbioses span functional spectra from facultative to obligate, and from mutualistic to parasitic. Examples of nearly every taxonomic variety of protists have formed associations with an even broader diversity of prokaryotes (Figure 2), which can completely cover their host in a layer of ectosymbionts, or be housed within the host cytoplasm, nucleus, mitochondria, or plastids. Functionally, these associations can be driven by metabolism, nutritional supplementation, defense, taxis, and probably a multitude of subtle impacts we are yet to grasp (see Table S1 in Supplemental information). The evolutionary outcomes of these associations are equally variable, ranging from stable, long-term cellular integration, to short and swiftly deleterious exploitation, each with different and at least partially predictable genomic impacts. Coming to grips with the biological diversity inherent in protist systems is indeed a challenge. But the characteristics of these systems, including how they contrast with better-studied animal systems, give us perhaps our most promising window through which to distinguish context-dependent trends from more basic evolutionary and cell-biological principles common to symbiotic associations.

Phylogenetic diversity of protist hosts and symbionts

Looking across the tree of eukaryotes, symbioses have been characterized, at least at some level, in virtually all of the well-studied supergroups (Figure 1). This distribution, however, is not even: symbiosis is abundantly documented in some lineages and rare in others, and a major outstanding question is how much of this reflects lineage-specific biological bias to hosting symbionts, and how much is simply sampling (and cultivation) bias. Distinguishing between the two is complicated: large cell size might favor symbiosis, for example, but large protists are also better studied and more often cultivated.

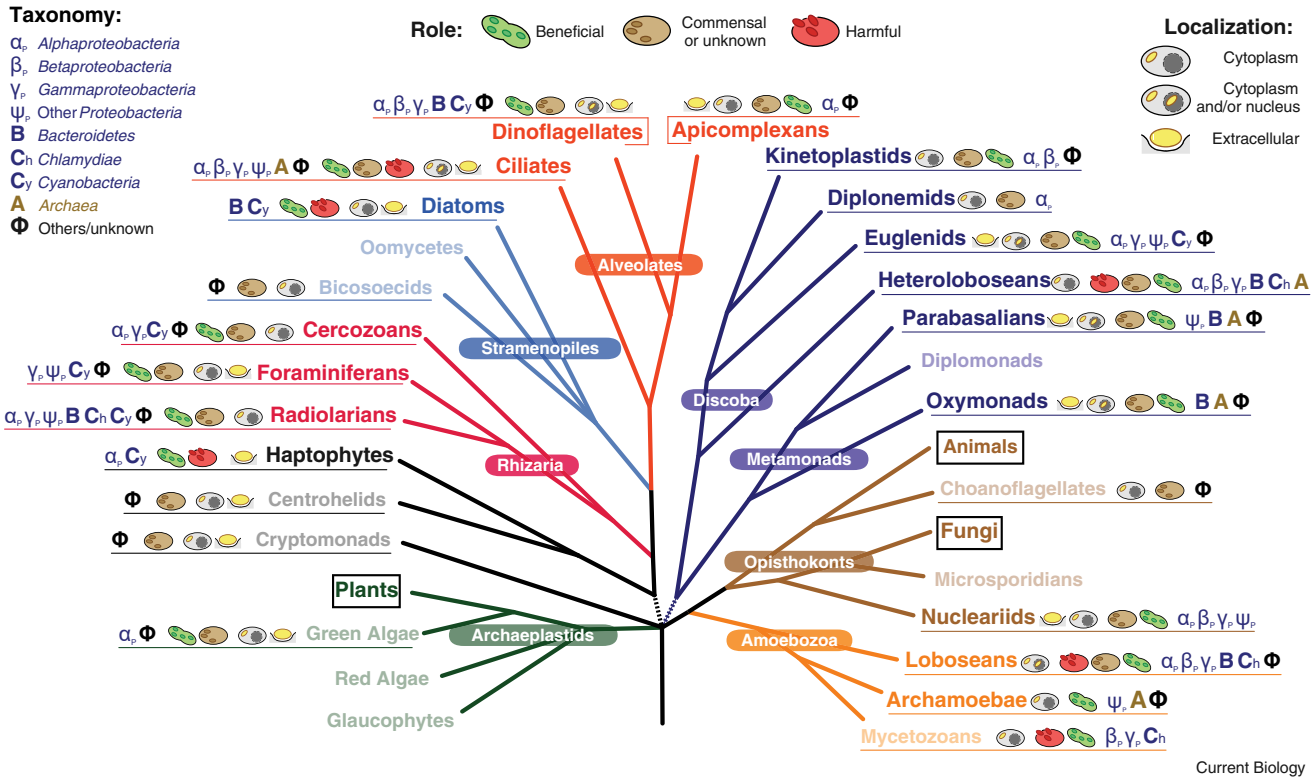


Figure 1. A phylogenetic tree of eukaryotes showing the variety of bacterial and archaeal symbioses in protists.
The main lineages of eukaryotes are depicted according to the current consensus phylogeny, and for each branch the diversity of known symbionts is summarized in terms of symbiont taxonomy, impact on the host (beneficial, harmful, or neutral), and host cell localization (cytoplasm, nucleus, extracellular). These summaries are cumulative and do not represent the frequency of symbionts present in a given lineage: to highlight this variability, lineages with few reported symbionts in the literature are labeled in faded color. Multicellular lineages, not discussed in this review, are labeled with black rectangles.

The hosts that have most frequently been documented to engage in symbiotic associations (Figure 1 and Table S1) are ciliates[13], amoebozoans[14,15], and termite-associated parabasalians and oxymonads. These are followed at some distance by certain photosynthetic lineages, such as dinoflagellates and diatoms[16], and protists that are themselves symbiotic, like the parasitic trypanosomatids[17]. Data are scarce for most free-living heterotrophs, which tend to be smaller, less-studied, and more difficult to cultivate, but nevertheless represent a major fraction of eukaryotic diversity (for exceptions, see[18–21]).

From the perspective of symbiont diversity, a similar picture emerges, but likely for different reasons. There are examples of symbionts from many bacterial and archaeal lineages, but some are far more represented. The most common symbionts are *Proteobacteria*, especially the *Alphaproteobacteria* subgroup[22–25], but *Bacteroidetes*[26,27], *Chlamydiae*[28], and *Cyanobacteria*[16] are also very common. All known symbiotic archaea are methanogens, though they are scattered through various families[29–32]. Here, sampling bias might play some role — for example, many molecular tools to detect symbionts were validated for *Proteobacteria* — but it is less likely to be a major factor because sampling is typically host-focused, and because many of these lineages have specifically adapted to a symbiotic lifestyle and therefore are more frequent symbionts. This is most evident in *Rickettsiales*, *Holosporales* (both *Alphaproteobacteria*), and *Chlamydiae*[33]. These lineages encompass specialized intracellular bacteria, with very few exceptions[34]. They have colonized most groups of eukaryotes in a pattern that emphatically does not mirror host phylogeny. Our understanding of *Rickettsiales* in particular has been re-written by investigations in aquatic protists: whereas the *Rickettsiales* were formerly known exclusively as agents of arthropod-transmitted diseases like spotted fever and typhus, we now know that all of their adaptations for a symbiotic lifestyle evolved earlier, in protist hosts. That such lineages of 'professional symbionts' should lead to a taxonomic bias is obvious, but this also has important implications for how we interpret the 'function' of symbioses that are only beginning to come into focus (as discussed in detail in the next section).

Although many famous symbiotic systems are described as partnerships between a single host and symbiont, data are emerging to challenge this assumption. Despite their unicellular nature, protists are increasingly found to harbor communities of several coexisting symbiotic species[30,35,36]. The richness and functional complexity of these communities are not very different from some model animal 'microbiomes'. Taxonomically distinct symbionts in protist cells can still be spatially compartmentalized, with organelles playing the role of tissues in animals. Most bacterial and archaeal endosymbionts inhabit the cytoplasm (Figure 2), but some appear to be freely distributed, or even swimming about[37], whereas others are surrounded by host-derived membranes[38]. Other endosymbionts are closely

Glossary.

Symbiont: Any organism in a spatially close and temporally prolonged relationship with an organism of a different species, regardless of its effect on the symbiotic partner.
Endosymbiont: A symbiont living inside its larger symbiotic partner, usually called 'host'. Since this review focuses on single-celled hosts, we use the term only for intracellular symbionts. However, the term is sometimes used in the literature also for extracellular symbionts living inside animals.
Ectosymbiont (or epibiont): A symbiont living outside of, usually attached to, its larger symbiotic partner.
Autotrophy: The ability, possessed by certain organisms, to synthesize organic compounds using CO_2 as the sole carbon source.
Heterotrophy: The inability to synthesize organic compounds from CO_2, resulting in the reliance on already reduced carbon source(s).
Mixotrophy: A nutritional strategy, fairly common amongst unicellular eukaryotes, in which an organism can pursue both autotrophy and heterotrophy, using CO_2 and/or organic compounds as carbon source(s).
Syntrophy: A symbiotic relationship in which one or both symbionts require metabolites produced by the partner as essential food source(s) (that is, they perform different steps of a shared carbon/energy metabolic pathway) or cooperate for toxic waste removal.
Plastid: A cellular organelle derived from endosymbiosis of a cyanobacteria. Chloroplast refers to green plastids specifically, but the term plastid includes a variety of other homologous organelles.
Primary plastid: A plastid derived from the ancestral symbiosis between a eukaryote and a photosynthetic cyanobacterium, typical of the supergroup Archaeplastida.
Secondary plastid: Any plastid derived from the symbiosis between a eukaryote and a different eukaryote belonging to Archaeplastida (for example, common in several ecologically important groups of unicellular algae like diatoms, haptophytes, and dinoflagellates). Many secondary plastids are photosynthetic but some have also lost photosynthesis.
Primary/secondary symbiosis: In some contexts, such as for animal symbioses, the term 'primary symbiont' is used as a synonym for a main, essential symbiont, whereas 'secondary symbiont' is used to mean an accessory, non-essential symbiont.
Methanogen: An organism producing methane as a byproduct of its energy metabolism. All known methanogens belong to Archaea and live in hypoxic environments.
Reductive acetogenesis: The ability, possessed by certain organisms, to produce acetate by reducing CO_2 or organic compounds.
Hydrogenosome: A mitochondrion-derived organelle performing an anaerobic energy-production metabolism that produces hydrogen as a byproduct.
Epixenosome: Informal name for extrusive ectosymbionts found in ciliates and euglenozoans. Epixenosomes are bacteria belonging to the phylum *Verrucomicrobia* with a complex intracellular ultrastructure.

associated with specific organelles, especially mitochondria and mitochondria-derived organelles[29,39–41]. Some bacterial symbionts colonize the outer surface of the host, often in orderly arrangements or confined to specific areas[42–44]. Finally, some of the most puzzling symbionts have invaded the host nuclear apparatus, even burrowing into the chromatin[37,45,46].

Untangling the biases and biological factors that underpin the distribution and diversity of symbiosis across eukaryotes is a difficult but important problem because such factors will undoubtedly reflect basic principles behind their function and evolution. As we will see below, too many of these remain unclear.

Parasites, commensals, mutualists: where to draw the line?

There is a vague but widespread perception that symbiosis is typically mutualistic. Unfortunately, there is a lot of fuzzy terminology surrounding symbiosis in general, but conflating 'symbiosis' and 'mutualism' has particularly broad potential to mislead. Terms that confer a benefit or cost to symbiotic partners are defined by fitness gains or losses that are incredibly difficult to quantify, and almost never measured[47–49]. Moreover, fitness effects are not only dependent on environmental conditions (including temperature, resources, host availability, etc.), but also time-scale dependent: an association that ecologists might see as clearly mutualistic may be closer to a death-row prison sentence for one partner on evolutionary timescales.

Overall, there is a growing body of evidence suggesting that discrete categories of fitness-defined symbioses, like parasitism and mutualism, may only really be informative for the most extreme ends of the spectrum, and that symbioses should be rather viewed as ongoing and context-dependent power struggles[50–52]. In this view, which partner is driving the association affects how it evolves, and protist–prokaryote symbioses (especially endosymbioses) provide numerous examples that blur the line between mutual benefit and exploitation. On one hand are host-driven associations with symbionts that diverged recently from free-living ancestors but are now being exploited by their hosts, and whose likely fate is to spiral down the evolutionary rabbit hole to extinction[52–55]. On the other hand are associations driven by the 'professional symbionts' belonging to lineages ancestrally adapted to an opportunistic intracellular lifestyle, and who frequently switch hosts over evolutionary time[28,33]. The frequency of host switches is likely underestimated since the natural host range of protist symbionts is rarely known and almost never tested. True mutualism in the microbial world may be extremely rare over long evolutionary timeframes, if it exists at all[52,53].

A fascinating special subtype of nutritional symbiosis (that illustrates how fuzzy our categories are) is microbial 'farming' or 'gardening'. Here, bacteria such as chemolithotrophic sulfide

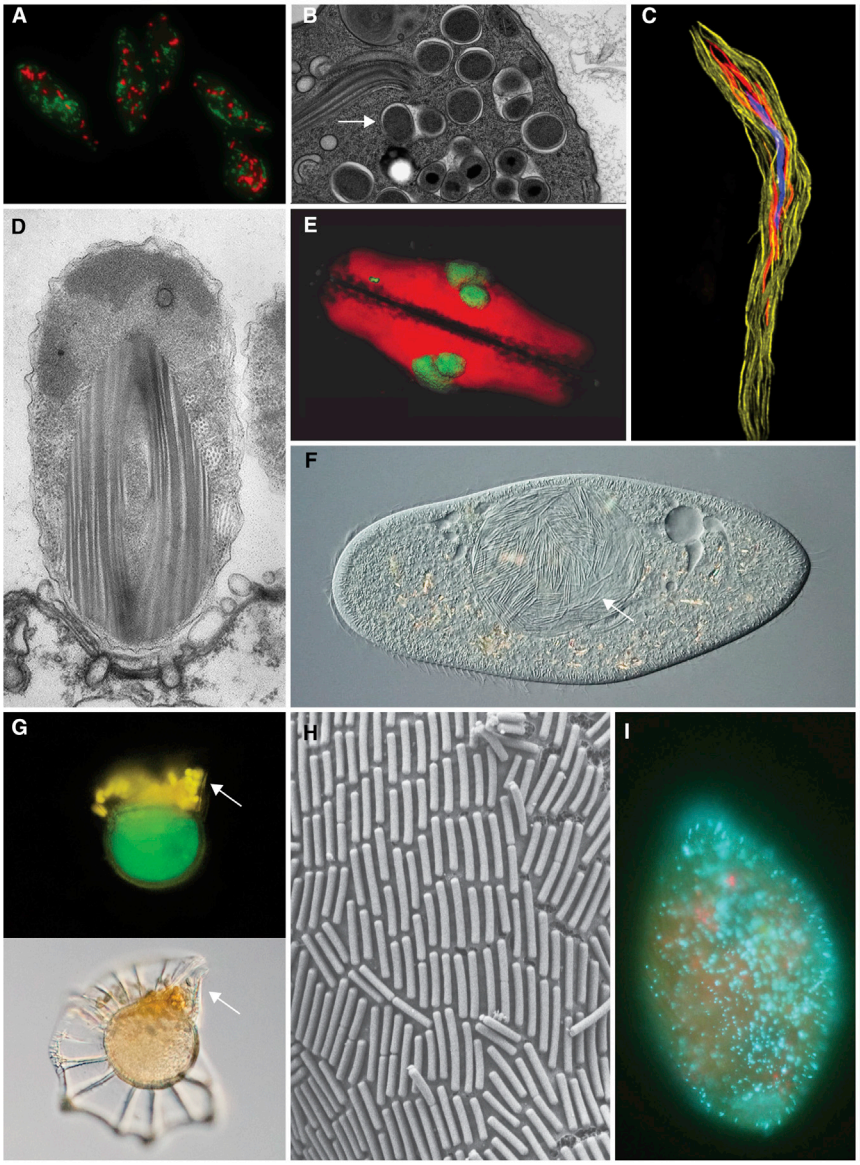


Figure 2. Light and electron micrographs of selected protists with symbionts.
(A) The diplonemid *Diplonema japonicum* with two different *Holosporaceae* endosymbionts (labeled in red and green fluorescence). (B) The diplonemid *Rhynchopus euleeides* with *Syngnamydia* endosymbionts. (C) The oxymonad *Streblomastix strix* with *Bacteroidetes* ectosymbionts (provided by Sebastian Treitli). (D) Epixenosomes of the ciliate *Euplotidium itoi* (provided by Giovanna Rosati). (E) Spheroid bodies of the diatom *Rhopalodia gibba* (provided by Stefan Zauner). (F) The ciliate *Paramecium caudatum* with *Holospora obtusa* endosymbionts inside its nucleus (provided by Hideo Dohra). (G) The dinoflagellate *Ornithocercus thumii* with cyanobacterial symbionts inside its 'green house' (provided by Takuro Nakayama). The bottom image shows a micrograph of the dinoflagellate cell, while the upper image is the same cell with fluorescently labeled yellow bacteria and the host cytoplasm in green. (H) The parabasalid *Barbulanympha* sp. with *Bacteroidetes* ectosymbionts. (I) The ciliate *Parablepharisma* sp. with methanogenic archaea (provided by Roxanne Beinart).

oxidizers or cyanobacteria are cultured — in the best known cases extracellularly, such as in the heterotrophic dinoflagellate *Ornithocercus*[56] or the ciliates *Trichodinopsis* and *Kentrophoros*[57,58] — and harvested as food. These cases are often described as mutualism, but the symbionts are grown like vegetables in tiny green houses or garden plots, so although the interaction is stable in ecological time, the host eventually eats the bacteria: this is no more a case of long-term mutualism than is cattle farming by humans. In a twist on this theme, the social amoeba *Dictyostelium* sometimes associates with *Burkholderia* symbionts that exert both pathogenic and mutualistic effects on the host in a context-dependent manner (food availability). The symbionts turn *Dictyostelium* into 'farmers' of diverse food bacteria. These bacteria are expelled when the host disperses its spores, seeding a garden of preferred food species for germinating spores to feed upon, and even secrete chemicals to inhibit non-symbiotic *Dictyostelium* hosts[59–61].

Farming is an eye-catching form of symbiosis, and we will discuss other functions for relatively well-studied cases below, but we must stress that for the vast majority of known protist–prokaryotic symbioses, any potential cost or benefit to either partner is more difficult to assess. In animal systems, symbiont genomes often (but not always[62]) reveal simple nutritional supplementation arrangements[6,7]. But the situation in protist symbionts will not likely be so simple. The genomes of protist symbionts encode large numbers of genes of unknown functions (see references in Table 1), and it is likely that many symbionts serve no specific 'function' to their hosts (for example, in the case of 'professional' symbionts, as described in the next section), or at least one not easily discerned from genome annotation (for example, defensive symbionts). Directly measuring fitness effects under laboratory conditions used for protist cultures (usually a nutrient-rich medium containing no other organisms) has been undertaken only for a few systems, and even when it is measured, the presence of symbionts is often found to lack pronounced negative or positive effects on host growth. This might change under the more stressful, heterogenous, and ever-changing conditions found in nature, especially considering complex host interactions with other organisms[63].

Genome evolution in protist symbionts

Symbionts, and endosymbionts in particular, are known for their extreme genome structure and content, and protist symbionts are no exception. In terms of genome size, protist endosymbionts cover a broad range of reduction — from ~3.6 Mbp in the

Table 1. Selected protist–prokaryotic symbioses.

Host(s)	Symbiont	Symbiont localization	Symbiont benefit and function	Symbiont genome size (# of sequenced genomes)	Select references
Paulinella spp. (Rhizaria)	'Chromatophore' (*Cyanobacteria*)	Cytoplasm	Beneficial Photosynthesis	1 Mbp (5)	94
Rhopalodia, Epithemia (Stramenopila)	'Spheroid body' (*Cyanobacteria*)	Cytoplasm	Beneficial Nitrogen fixation	2.8–3.0 Mbp (2)	85
Rhizosolenia, *Hemiaulus*, spp. (Stramenopila)	*Richelia intracellularis* (*Cyanobacteria*)	Cytoplasm	Beneficial Nitrogen fixation	3.24–3.29 Mbp (6)	155
Prymnesiophytes (Haptophyta)	*Atelocyanobacterium thalassa* / UCYN-A (*Cyanobacteria*)	Ectosymbiont	Beneficial Nitrogen fixation	1.44–1.49 Mbp (3)	92
Various hosts	*Rickettsiales* and *Holosporales* (*Alphaproteobacteria*)	Cytoplasm and/or nucleus	Unknown (parasites, commensals, mutualists)	0.62–3.18 Mbp (>30)	24,25,70
Euplotes clade B (Alveolata)	*Polynucleobacter* (*Betaproteobacteria*)	Cytoplasm	Beneficial Unknown	1.55–1.93 Mbp (9)	54
Angomonas, *Strigomonas*, *Kentomonas* (Excavata)	*Kinetoplastibacterium* (*Betaproteobacteria*)	Cytoplasm	Beneficial Provision of amino acids and cofactors	0.74–0.83 Mbp (7)	17
Calkinsia aureus-like (Excavata)	'MEB' (*Desulfarcum epimagneticum*; *Deltaproteobacteria*)	Ectosymbiont	Beneficial Magnetoreception	~3.2 Mbp (1)	102
Lenisia limosa (Opisthokonta)	*Arcobacter* sp. (*Epsilonproteobacteria*)	Ectosymbiont	Beneficial Hydrogen scavenging	~3.0 Mbp (1)	101
Trichonympha spp. (Excavata)	*Endomicrobium trichonymphae* (*Elusimicrobia*)	Cytoplasm	Beneficial Provision of amino acids and cofactors	1.13–1.15 Mbp (2)	96,156
Euplotes vanleeuwenhoeki (Alveolata)	*Pinguicoccus supinus* (*Verrumicrobia*)	Cytoplasm	Beneficial Unknown	0.16 Mbp (1)	65
Streblomastix strix (Excavata)	*Ordinivivax streblomastigis* and unnamed (*Bacteroidetes*)	Ectosymbionts	Beneficial Lignocellulose degradation, nitrogen fixation, amino acids and co-factors	~4.9 Mbp (>4 MAGs)	26
Nyctotherus ovalis (Alveolata)	*Methanobrevibacter* (Archaea)	Cytoplasm, associated with hydrogenosomes	Unclear, hydrogen scavenging	~2.0 Mbp (1)	29
Metopus contortus (Alveolata)	*Methanocorpusculum* (Archaea)	Cytoplasm, associated with hydrogenosomes	Unclear, hydrogen scavenging	~1.7 Mbp (1)	29
Heterometopus sp. (Alveolata)	*Methanobacterium* (Archaea)	Cytoplasm, associated with hydrogenosomes	Unclear, hydrogen scavenging	~2.0 Mbp (1)	31

A select group of known protist symbionts with their localization, function, and genome size. A comprehensive table with expanded information (for example, transmission mode when known) on all symbioses reviewed here is included as Table S1. MAG: metagenome-assembled genome.

Acanthamoeba nucleus-invading *Berkiella aquae*[64], to the *Euplotes* endosymbiont, *Pinguicoccus supinus*[65], which at 163 kbp is scarcely larger than the smallest known insect endosymbiont genome (112 kbp)[66].

Ectosymbiont genomes are generally larger; the *Bacteroidetes* ectosymbiont *Ordinivivax streblomastigis* of *Streblomastix* has a genome predicted to be over 4.9 Mbp[26], whereas the genome of the surface-embedded *Desulfovibrio* of *Trichonympha agilis* is a mere 1.4 Mbp[67]. Insect 'ectosymbionts' (extracellular gut symbionts housed in specialized structures) with significantly smaller genomes (~270 kb) are known[68], and similarly sized ectosymbionts might also be found in protists when more are examined. Too few archaeal endosymbionts of protists are known to draw many conclusions, but current genomes range from approximately 1.7–2.0 Mbp[29,31].

All this shows is that protist symbiont genomes are prone to reduction: the far more interesting information comes from examining parallels between *how* genome reduction happens in protist and animal symbionts, and how it affects genome structure and content (Figure 3). Why gene function is lost is relatively easy to explain: symbiosis obviates the need for many functions, relaxing selection pressure on newly non-essential genes and allowing loss-of-function mutations to become fixed[54,69]. Loss of some functions is expected (for example,

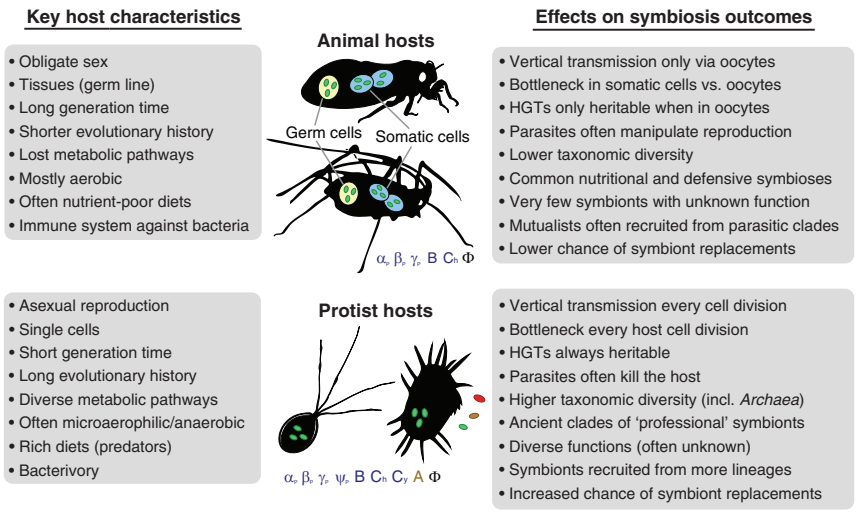


Figure 3. Host constraints and symbiosis outcomes in multicellular vs. unicellular hosts.

In this review, we have emphasized the fundamental similarities in symbiont genome evolution that stem from basic principles of population genetics. However, it is also important to consider differences in host life strategies that might affect the evolution of their symbionts. Here we list various similarities and differences between protist and animal symbioses, the mechanisms or processes responsible for these features, and how they might impact symbiont evolution.

flagella), but in others the extremity of reduction can be surprising. For example, many 'essential' metabolic pathways have been lost in endosymbionts, due to the presence of transporters — case in point, the loss of most energy metabolism due to ATP/ADP translocases that directly import ATP from the host. Genes for replication, transcription, and translation are retained the longest in all symbionts of protists and animals, but even in these processes some genes are lost[25,65,66,70].

Although the loss of many genes may simply be explained by their unnecessary function, genome reduction can go much further. One force that plays an outsized role in this process is Muller's ratchet, in which deleterious mutations are fixed in small symbiont populations due to genetic drift[71,72]. This is compounded by elevated mutation rates resulting from loss of recombination and error-correction systems, which can also bias in favor of deletions and high AT-composition. Determining whether drift or elevated mutation rate is the main non-adaptive factor in genome erosion remains a major challenge in most systems, because the symbionts are so distant from even the nearest free-living relatives that synonymous mutations are saturated. So far, only in the *Euplotes–Polynucleobacter* symbioses have substitution rates been distinguished, showing genetic drift to have the larger impact[54]. Other studies have shown that gene loss is correlated with mutation rates when time is factored into substitution rates[73], but data from more symbioses are needed.

Whether due to drift and/or mutation rate, the expected outcome is a small genome, with few genes, high substitution rates, and low GC content. Many obligate protist symbionts fit this description, especially *Holosporaceae* and *Rickettsiales*[21,25,70], and yet the evolutionary outcomes are not always the same. We can generalize these into three main functional categories that share genomic characteristics intimately tied to which partner controls and benefits from the association[52]: extinction, symbiont professionalism, and integration. We will review each in turn.

Extinction may not sound like a 'symbiosis outcome', but evidence is emerging to suggest that what may seem like a long-term symbiosis between co-evolving partners can really be a recurring, host-driven cycle of exploitation, replacement, and extinction. The symbiont genome is reduced in a seemingly chaotic and rapid fashion, with the loss of recombination and repair pathways and unchecked fixation of deleterious mutations leading to a runaway genomic breakdown that is inviable in the long term. Genome size reduction is variable, but loss of function and pseudogenization are abundant. In protist hosts, this is best characterized in *Polynucleobacter* endosymbionts of *Euplotes*[54] (Box 1). Detecting this process requires substantial data from numerous related strains (rare for protist systems), but there are intriguing data from other systems to suggest the same process. For example, recurrent replacements have been observed in methanogenic endosymbionts of anaerobic ciliates[32], and in one case little genome reduction has occurred[31], consistent with a recent uptake. Symbiont replacement in animals, specifically sap-feeding insects, has also occurred multiple times[74,75]. But it would be an oversimplification to say all such symbionts are on the road to extinction: some insect symbionts with extremely reduced genomes were acquired more than 270 Mya[53]. These may be exceptional cases that have reached a stable yet severely reduced state, or they may partly reflect the relative ease with which symbionts can be replaced in protists versus animals: a phagotrophic protist consuming bacteria might have long ago replaced such symbionts whereas sap-feeding animals have limited opportunities to do so (Figure 3). A population genomics approach is needed to understand symbiont replacement events, the pace of genome degradation and reduction, and whether these processes operate over different timescales in animal and protist hosts (Figure 3).

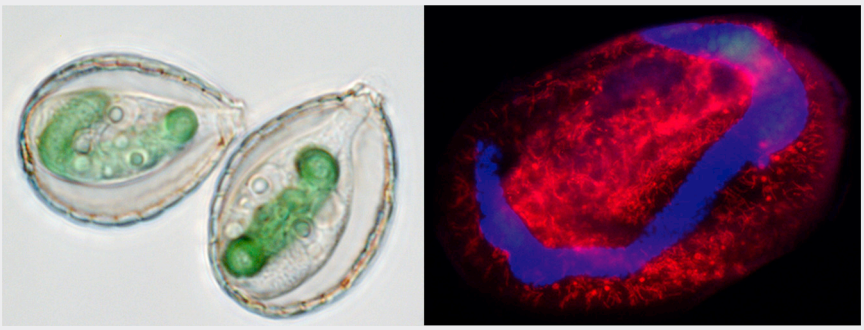
A second evolutionary outcome is the professional symbiont. These symbionts belong to lineages ancestrally adapted to symbiosis, often obligatory, and are found in a wide range of protist and animal hosts (Figure 1 and Table 1). Their genomes are reduced, but not in the haphazard or extreme way of those destined for extinction: they are compact, orderly, and streamlined, with few non-essential genes or mobile elements, but they do retain many DNA recombination and repair systems[21,24,25,70] (Table 1). These symbionts also contain a variety of systems that mediate host infection and interactions, controlling the symbiosis. This includes arsenals of secretion systems and effectors, like Type IV and Type VI secretion systems common in bacteria, which are often repurposed to secrete host-targeted effectors instead of the bacterial-targeted effectors used in interspecific competition of free-living bacteria[25,34,76]. Many

Box 1. Emerging model systems for studying protist–prokaryote symbioses.

In the long shadow of symbiosis research that has focused mainly on organelles or bacteria within animal hosts, a few protist systems have still managed to emerge as valuable models for symbiotic processes. These are important examples because the diversity and complexity of protist biology can present a daunting face to anyone hoping to acquire a deep understanding of a process using a model system-based strategy. So it is worthwhile to remind ourselves that important insights can still be gained without all the trappings of a fully developed model system.

One protist lineage that has been particularly influential in challenging our views about the origin of organelles is the rhizarian amoeba *Paulinella*[94] (shown in the left image). Most *Paulinella* species are heterotrophs that prey on bacteria, including cyanobacteria. But one small subgroup *(P. chromatophora*, *P. micropora*, and *P. longichromatophora*) has acquired cyanobacterial photosymbionts called 'chromatophores' relatively recently (90–140 Mya). Despite their young age, symbiont division is already under tight host control and it appears to be integrating with that of its host, not unlike plastids, which were acquired over 1.5 billion years ago. The *Paulinella* system represents a novel independent origin of 'primary' plastid symbiosis[94], and one that gives us glimpses into the transition of an endosymbiont into a genetically integrated organelle[94,157]. However, *P. chromatophora* lacks many of the practical characteristics that make for a good model system: it grows extremely slowly, it has a huge nuclear genome, and few genetic tools have been developed for it or any other member of the poorly studied rhizarian supergroup to which it belongs. But none of this has stopped several researchers from applying experimentally advanced methods to *Paulinella*, which shows how determined effort can make any protist a model system[157–159].

This is exemplified by recent studies into the role of horizontal gene transfer and protein import in *Paulinella*, and how these processes relate to organellogenesis more broadly. This work has pushed *Paulinella* to the frontier of our knowledge, surpassing even well-established animal–bacteria systems[160]. Although at least 229 genes of bacterial origin were found in the *Paulinella* transcriptome and draft genome, only 72 of those genes were found to be of cyanobacterial origin and thus possibly acquired from the cyanobacterial symbionts (amounting to around 30 gene transfer events followed by gene duplications[157]). In contrast, proteins from about 450 genes in the nuclear genome (including the bacterial horizontal gene transfers) were found to be imported into the cyanobacterial symbionts, and mostly these appear to functionally compensate for symbiont genome reduction[159]. These results provide strong evidence that the 'chromatophore' proteome is a mosaic, a finding that parallels recent data from mitochondrial and plastid proteomes[143,144], as well as sparser data from bacterial symbionts of animals[160]. On reflection, our thinking has been restricted more by historic barriers derived from animal symbioses that emphasized the 'special' nature of organelles[2] than by experimental barriers[52].



Paulinella illuminated ancient organelle origins because of its relatively youthful association, but another protist system, the ciliate *Euplotes* (shown in the right image), sheds light on an even more extreme timescale[22]. One subgroup of *Euplotes* species is wholly dependent on endosymbiotic bacteria (predominantly *Polynucleobacter*), which in turn cannot survive outside their host[161,162]. With related symbionts in related hosts, the system has all the hallmarks of ancient, mutualistic coevolution[162]. But a more detailed examination of symbiont genome diversity showed that this interpretation is wrong in many ways[54]. Free-living *Polynucleobacter* are abundant and common in lakes and ponds[163], and comparing their genomes with those of symbiotic strains revealed that symbionts do not share a common ancestry, as predicted by a single ancient symbiosis. Instead, symbiotic strains, even from sub-populations of the same host species, are each more closely related to different free-living strains than they are to each other, indicating many parallel origins of these endosymbionts[54]. Reconciling the ancient symbiosis with the recent symbionts shows that, over evolutionary time, the ciliate hosts are continuously replacing their symbionts with 'fresh' free-living strains from the environment. The symbionts' genomes degrade quickly, and they are likely periodically outcompeted by new symbionts and go extinct, leaving the newcomers to begin their own descent into genome erosion, dependence on the host, and eventual replacement and extinction.

The *Euplotes* symbiont example corresponds with models and predictions made from decades of study of bacterial–animal symbioses[53,74,164], but in *Euplotes* the significantly shorter timescales allow for hypothesis testing that was impossible in animal systems[54]. Moreover, each symbiotic *Polynucleobacter* strain represents an independent transition from a free-living ancestor, and a

(Continued on next page)

Box 1. *Continued*

rare comparative window into the process at the genomic level. Lastly, the relationship between ancestrally symbiotic *Euplotes* hosts and recently evolved *Polynucleobacter* symbionts sounds unexpected, but it is actually simply the best documented example of a phenomenon that is more common in nature than the famous extremely ancient insect symbionts led us to believe[55], and might prove a model to interpret many other associations as they are better studied.

The main insights from *Paulinella* and *Euplotes* came from detailed genomics and cell biology, but neither of these systems would be recognized by mainstream cell biology as a 'model system'. Taking that same step forward for other systems across the amazing diversity of protist–bacterial symbioses will be equally profitable, and we are not short of obvious candidates; many have been simply noted by microscopy but with little information on their function or evolution[13,113,165,166]. In some cases, the host protists have barely been studied with modern methods due to challenges like lack of cultivation (for example, foraminiferans and radiolarians), unusual cellular and genomic features (such as dinoflagellates), or lifestyle (including heterotrophic predators, anaerobes, or deep-sea dwellers). Still other cases were already developed as model systems historically, but have not been revisited with many newer methods, or were lost to science because cultures died. Rejuvenating such systems, for example the classic *Amoeba–Legionella* association that transitioned from parasitic to essential in just five years[132], would certainly lead to outstanding insights.

potentially secreted proteins have known eukaryotic-interacting domains such as leucine-rich repeats and ankyrin repeats; however, most proteins with these domains are hypothetical with unknown functions[24,46,77,78]. Other professional symbionts retain the ability to actively infect their hosts, for example *Holosporaceae*[79] and *Chlamydiae*[80]. Although the professional symbionts appear to exert some control over the association, they are not immune from Muller's ratchet, and risk extinction if DNA repair and recombination pathways become compromised.

The third, and very rare, outcome for protist symbionts is integration to an organelle state. Only three prokaryotic symbiont-derived systems are currently thought to have reached this bar: mitochondria, plastids, and *Paulinella* chromatophores (Box 1). Multiple mutually exclusive definitions of 'organelle' and 'endosymbiont' exist, but the most commonly used one defines organelles based on genetic integration and protein-targeting[52,81]. In all of these cases, host-encoded proteins are targeted to the organelle (although we will discuss in the last section how even this relatively objective criterion is becoming more complex). Other endosymbionts have been proposed to be in the early stages of such integration, including the spheroid bodies of cyanobacterial origin in the diatom, *Rhopalodia gibba*[82] and *Kinetoplastibacterium* in kinetoplastid hosts[83]. No protein import has been detected in *R. gibba*[84], and the spheroid bodies have relatively large genomes with varying degrees of reduction[85]. Import has been reported in *Kinetoplastibacterium*[83], and the symbiont's cell division is synchronously tied to that of the host's[86]. Whatever the fates of *Kinetoplastibacterium* and the spheroid bodies may be, these systems will provide important insight into the processes of host–endosymbiont integration; systems such as these seem likely to further undermine any simple criterion to cleanly distinguish an 'endosymbiont' from an 'organelle'.

It will be interesting to explore how these patterns are common or distinct in protist and animal symbionts, as differences in life strategies of protist and animal hosts may influence the timescale of evolutionary processes such as genome reduction (Figure 3). Reduction of a bacterial genome from approximately 4,000 genes to 500–600 genes likely takes millions of years in animals, even though the initial phase of reduction can be rapid[66]. Faster generation times of protist hosts might speed this process substantially. Similarly, some insect symbiont populations experience bottlenecks due to the maternal transmission[87], whereas protist symbionts likely experience bottlenecks every time host cells divide.

Context-dependent plasticity of function in protist symbionts

Genomics may have explained a great deal about protist–symbiont evolution, but has shed less light on the function of these symbioses. Individual cases have been proposed to be based on a wide variety of functions, some highly specific to the partnership (for example, nucleariid ectosymbionts degrading a toxin produced by a specific prey species[36]) and others more broadly applicable across a variety of contexts (for example, sequestration of a common nutrient like nitrogen). In some cases, no 'function' exists in the simplest sense, because the symbiont drives the association: it is freeloading. In other cases, the function may be difficult to infer from genomic data alone (unlike the common nutritional supplementation in animal hosts). Some functions (such as defensive symbiosis) change with conditions, selection pressure, or over evolutionary time. Below we outline a few broad categories and how their impact varies across the tree of eukaryotes.

Metabolic symbioses

Simple nutritional supplementation is unlikely to be as dominant a function in protists as it is in animal hosts, but the relative ease by which it is identified and documented means some of the best-understood protist symbioses are based on acquiring nutrients. This appears to be especially common among non-phagotrophic protists. Many marine algae are auxotrophic for vitamin B12, for example, and rely on symbiotic bacteria for its provision[16,88,89]. Soluble iron and nitrogen are also common limiting factors in the open ocean: *Marinobacter* ectosymbionts commonly provision iron[90], whereas nitrogen is supplied to various algal lineages by endo- or ectosymbiotic *Alpha-*, *Beta-*, and *Gammaproteobacteria*[91], and cyanobacteria[82,92] that often have reduced photosynthesis[92].

Photosynthesis is itself also a common basis for cyanobacterial endosymbioses, found for example in dinoflagellates, radiolarians, and cercozoans[16,93,94]. A particularly well-studied instance is the highly integrated 'chromatophore' of the cercozoan amoeba *Paulinella* (Box 1). Anaerobic ciliates have also

Box 2. Termite symbiosis.

In addition to the specific model systems noted in Box 1, there is another symbiotic system that merits individual attention, as it has over a century of study and involves a huge variety of symbiosis in a common context: the hindgut of lower termites[100,167].

Termites eat wood but like most animals cannot digest lignocellulose or derive nitrogen from it, and instead they rely on microbial symbionts for both. Symbionts of 'higher' termites are virtually all bacteria, but 'lower' termites harbor a complex community of bacteria, archaea, and protists in symbiotic associations. These communities of hierarchical symbioses have been shaped by a mix of co-evolution with the occasional introduction of new symbionts (for example, by transfer from another termite), but within the population of a given host species, the community is remarkably stable from one individual to the next. This makes termites a fascinating model for microbial community assembly and structure, as well as the evolution and function of protist–bacteria symbioses[27,45,96,103,168]. This system is not without challenges since virtually none of the symbionts have been routinely maintained in culture outside the termite host, but if one regards the host animal as a mini-fermenter, then symbionts can nevertheless be harvested and studied repeatedly over time, presenting many unique opportunities to examine and even manipulate symbiotic communities.

The hindgut is a low-oxygen environment, and protists living in it are mostly members of two lineages, parabasalia and oxymonads, with strange mitochondrial adaptations and microaerophilic metabolism (see, for example, Figure 2C,H). Both groups host a variety of bacterial symbionts, in particular *Bacteroidetes*[108], *Spirochaetes*[97], *Elusimicrobia* (also known as *Endomicrobia* or Termite Group 1)[96,156], *Actinobacteria*[98], and *Deltaproteobacteria*[103]. Depending on the particular 'lower' termite species, representatives of these bacterial phyla are found as both ectosymbionts and endosymbionts of hindgut protists. Together with their protist hosts, they form a multifunctional consortium fixing nitrogen, hydrolyzing lignocellulose from wood particles into monosaccharides, and synthesizing amino acids and co-factors[100,167].

Interestingly, some bacterial lineages that are most commonly found in symbiotic associations with protists more generally (for example, particular subgroups of *Alpha-*, *Beta-*, and *Gammaproteobacteria*) are less abundant in the termite gut[169]. Conversely, other lineages like *Verrucomicrobia* or certain other *Alphaproteobacteria* subgroups are less abundant in the termite gut but are commonly found within termite-dwelling oxymonad and parabasalian protists, especially inside their nuclei[45]. Both parabasalids and oxymonads can also harbor methanogenic archaeal symbionts[104,105] hypothesized to scavenge molecular hydrogen, but this function still remains poorly understood.

been shown to house photoheterotrophic bacteria, but in this case use bacteriochlorophyll-harboring *Proteobacteria*[95].

Nutritional symbioses are not uncommon in protists that reside within the digestive tracts of animals. The best studied are the abundant and complex symbionts of parabasalids and oxymonads in termite hindguts (Box 2), which are often involved in nitrogen provisioning[96–98]. Some insect-associated trypanosomatids (*Angomonas*, *Strigomonas*, *Kentomonas*, and *Novymonas*) also depend on two unrelated betaproteobacterial endosymbionts for purines, heme, amino acids, and vitamins[17].

Syntrophic associations are based on metabolism, but also include detoxification and removal of metabolic byproducts. Syntrophy is typified by the associations between various bacteria or archaea and hydrogenosomes, which are anaerobic derivatives of mitochondria that produce molecular hydrogen. It has been proposed that hydrogenosome metabolism is more efficient if the hydrogen is removed by symbionts that use it for methanogenesis[29,32], reductive acetogenesis[97,99], or as a donor of reducing equivalents coupled with anaerobic respiration[67,100–103]. Methanogenic archaea are hydrogen scavengers in ciliates[29,31], amoebae[30], and termite-associated oxymonads and parabasalids[39,104,105], often physically associated with hydrogenosomes[29,41]. In ciliates, up to 95% of intracellular hydrogen is taken up by symbiotic methanogens[106], shifting hydrogenosome metabolism to produce acetate instead of butyrate, and consequently improving host growth[107].

In addition to methanogenic archaea, a variety of other symbionts also have the capacity for hydrogen scavenging, including the sulfate-reducing *Deltaproteobacteria*[67,103], *Spirochaetes*[97,100], *Bacteroidetes*[108,109] of parabasalids, and the epsilonproteobacterial ectosymbionts of breviateans[101]. The free-living anaerobic amoeba *Pelomyxa* contains an entire consortium of prokaryotes: aerobic *Rhodococcus* to eliminate trace oxygen, anaerobic *Methanosaeta* to optimize hydrogen gas levels, and *Syntrophorhabdus* to provide substrate for *Methanosaeta*[30].

Whereas some *in vitro* studies showed that elimination of hydrogen scavengers resulted in significant deceleration of the protist growth[101,110], others only reported the host survival[111]. Moreover, many anaerobic protists live without hydrogen-consuming symbionts, and the necessity for keeping them likely depends on many additional factors, such as nutrient availability, the type of prey organism, diffusion rate of hydrogen determined by the volume of host cells, partial pressure of hydrogen gas in the immediate environment, and the presence of free-living hydrogen scavengers[32,67,107,112]. In certain cases, such as in parabasalids, methanogens likely benefit their hosts primarily by supplying essential nutrients rather than by eliminating hydrogen[32,110].

Protist–prokaryote syntrophic associations are also believed to play a role in anoxic sea sediments, sometimes referred to as a 'symbiosis oasis'[113,114]. Here, ciliates and symbiontid euglenozoans withstand high concentrations of toxic sulfides thanks to sulfate-oxidizing epsilonproteobacteria that colonize their surface[58,115], an arrangement visually reminiscent of termite gut ectosymbionts (Box 2). Aerobic protists engage in similar associations, exemplified by *Bacteroidetes* and proteobacterial ectosymbionts that significantly improve growth in the diatom *Amphiprora* by removing hydrogen peroxide, a toxic product of photosynthesis[116]. Anoxic environments were also

the stage for a different type of nutritional symbiosis that evolved at least twice: both in marine benthic foraminifera[117] and in stratified lake-dwelling ciliates[118] intracellular gammaproteobacteria seem to provide energy to their hosts directly as ATP. These bacteria use nitrate instead of oxygen as a terminal electron acceptor, making their eukaryotic hosts potentially important denitrifiers, a role which is otherwise metabolically restricted to prokaryotes or eukaryotes relying on horizontally transferred genes from bacteria[119].

Defense and competition

Some protist symbionts provide the host with defense against bacterial infections or predation. Most chlamydias, for example, are parasitic, but some provide their *Acanthamoeba* host with immunity against lytic *Legionella* infection[120]. A different kind of defense is found in the ciliate *Euplotidium*, which is equipped with extrusive *Verrucomicrobia* ectosymbionts that defend against predation[42,114]. In laboratory conditions without predators, these so-called 'epixenosomes' are non-essential and often lost, but the association appears to be indispensable in the natural environment[121].

Probably the best-known example of symbiont-mediated competition is found in *Paramecium* and its *Caedibacter* 'killer' symbionts[122,123]. Harboring *Caedibacter* leads to a slightly reduced fitness, but this is offset because symbionts can infect and kill nearby uninfected hosts, eliminating competitors[124]. These functions are not immediately obvious from genomic data alone, and might prove to be much more common than currently appreciated.

Movement and taxis

Symbioses that facilitate movement of the host are not common, but stand out as perhaps unique to protists. The classic example is the parabasalid *Mixotricha paradoxa*, which is propelled by the synchronous movement of up to 200,000 ectosymbiotic spirochaetes covering its entire surface: its four anterior flagella merely steer[125]. In another case, *Bacteroidetes* are embedded in parallel rows into the cell membrane of another parabasalid, *Caduceia*, and provide motility through the coordinated action of their connected bundles of flagella, creating helical waves that power gliding movement when the host is in contact with a substrate[126,127]. An entirely different example is the *Protochlamydia* endosymbiont of *Acanthamoeba*, which improves host ameboid locomotion by modifying host actin-remodelling systems[128].

The ectosymbiotic *Deltaproteobacteria* of symbiontid euglenozoans[129] are non-motile, and thus not involved in the protist's propulsion. However, owing to incorporation of ferrimagnetic nanoparticles, they act as magnetoreceptors guiding their hosts along the magnetic field[102]. It has been speculated that magnetoreception coupled with the host's chemical sensing may navigate the protist as a compass towards the near surface of marine anoxic sediments, where redox conditions and nutrition are optimal[130]. Chemotaxis has also been evoked to explain host and symbiont morphological adaptations in the oxymonad *Streblomastix*[131], but whether this is consistent with the system's function has not been tested[26,44].

Parasites and pathogens

Parasitic or pathogenic relationships (which for simplicity we will not attempt to distinguish here) impart a negative impact on host fitness. As we have noted, however, such measurements are rarely made for symbiotic relationships, for which the effects on host fitness can be context-dependent and changeable anyway. Nowhere is this complexity more evident than in the pathogens of protists. The co-evolutionary transition from pathogen to essential symbiont in the classic laboratory observations of the *Legionella–Amoeba* system took months[132] (Box 1), but other systems are plastic and respond to environmental conditions over periods of days or less. *Preeria* (formerly *Holospora*) *caryophila* reduces the fitness of *Paramecium biaurelia* during stationary phase, but in some strains fitness is increased during exponential growth[47]. Similarly, the 'Jekyll-and-Hyde' extracellular pathogen of *Emiliania huxleyi*, *Phaeobacter inhibens*, first promotes host growth by producing antibiotics, but subsequently produces a different toxin that kills the host[133]. Both of these systems could appear mutualistic or pathogenic, depending on what stage of the relationship was observed.

Currently the most straightforward examples of pathogenesis are the extreme cases, exemplified by the candidate bacterial phylum *Dependentiae* (or TM6), a large group of bacteria mostly known from metagenomics[78,134,135]. But two cultured examples have similar life strategies, leaving little room to doubt their pathogenic status. *Chromulinavorax* was cultured as an obligate intracellular pathogen of the heterotrophic stramenopile *Spumella*. It is reduced at the genomic, metabolic, and cellular levels, and characterized by a lethal, lytic life cycle superficially similar to giant viruses[78]. Another member of the same group, *Babela massiliensis*, also lyses its *Acanthamoeba* host, leading to the possibility that this is a common life strategy of the *Dependentiae*[134]. Although this may seem exotic, other metagenome-assembled *Dependentiae* genomes are similarly reduced (ca. 1.2 Mbp), which is consistent with this being a common life strategy, though perhaps not universal for the group since one other example is known where no pathogenic effects have yet been observed[135].

Another bacterial group potentially rich in protist pathogens is the *Chlamydiae*, with a wide variety of species having been documented to lyse several species of amoebozoans[136,137]. These examples raise the exciting possibility that entire clades have evolved to be specialist pathogens adapted to protist-killing before they became animal pathogens. However, most of this diversity remains uncharacterized, with little or no data on life cycles or hosts. This is a potential trove of interesting data on the evolution of such associations, but will require substantial effort to get more host–pathogen pairs in culture.

Genetic integration and organellogenesis

Historically, host–symbiont integration has often been portrayed as an ultimate outcome of endosymbiosis — the destination to which other cases of endosymbiosis are 'going'. But as our appreciation for the context-dependent nature of symbiosis and the prevalence of conflict over mutualism grows, this view recedes. At the same time, however, the nuances of organelle integration grow more complex as well, and parallels between 'endosymbionts' and 'organelles' not only muddy the distinction, but also provide clues supporting a completely new way to view organelle origins.

The recognition that mitochondria and plastids arose by endosymbiosis created a problem: organelle genomes did not encode sufficient genes to fulfil their functions. This problem was brought into focus by the important hypothesis that the

endosymbionts must have transferred genes to the host, and the corollary that the protein products of these genes must also be targeted back to the organelles[138], an idea that has played a major role in conceptually distinguishing organelles from other endosymbionts[3,139]. Recent findings that other systems also evolved protein-targeting (Box 1) adds an interesting twist to this character, but the really significant change in thinking comes from digging deeper into the origin of targeted proteins in well-studied organelles. The longstanding assumption is that genes for targeted proteins originate from the endosymbiont that became the organelle[138], and with this the more poorly articulated assumptions that gene transfers preceded the evolution of targeting, and that organelle origins trace back to a singular endosymbiotic event.

An emerging alternative view of the process based on phylogenetic patterns from genomic data is quite different. As opposed to a single endosymbiosis, this model is iterative, with periods of recurring 'trial-and-error' endosymbioses. And rather than organelle fixation followed by gene transfer and targeting, the order of events is the opposite; protein-targeting evolves early, before gene transfer and even fixation of the organelle. A few such models have been proposed, emphasizing different aspects of these issues. For plastids, the 'shopping bag' model focused on the iterative nature of endosymbiosis and the assembly of a chimeric proteome[140], and the 'targeting-ratchet' model focused on the iterative nature and order of events in integration[141]. In these models (which are overlapping and not mutually exclusive) the key phase is a period where endosymbionts are taken up and retained for longer and longer periods of time, but not permanently[74]. Instead, the host incrementally develops ways to use the endosymbiont resources without digesting it, selecting for longer retention times (overall more like farming than cooperation). For mitochondria, a farming model has also emerged suggesting that the endosymbiont was originally farmed as a source of nutrients and energy in stressful times[142].

These models make several predictions about phylogenetic patterns that distinguish them from traditional schemes for the genetic integration of endosymbiotic organelles, and that appear to be borne out by the data. In particular, large-scale analyses of the phylogenetic origins of organelle-targeted proteins do not appear to support the conclusion that the genes are all derived from the same lineage as the organelle, and instead they come from a variety of sources[143–145]. This has now also been found in bacterial symbionts of animals that engage in protein targeting to the symbiont[146–148], and plastids derived from eukaryote–eukaryote 'secondary' and 'tertiary' endosymbiosis[144,149,150], altogether presaging a fundamental rethink about genetic integration of endosymbionts in favor of 'shopping bag' models. These conclusions are naturally dependent on the challenging problem of inferring remote homologies and deep phylogenies, both of which require improved phylogenetic methods and taxon sampling[151,152].

In effect, Weeden's gene-transfer hypothesis[138] appears to be at least partially false, but its protein-targeting corollary is correct. Secondary and tertiary plastid endosymbioses provide even more detailed insights. Here, evidence has been found in one system that protein targeting precedes the fixation of the organelle[144], and in several systems, related hosts harboring related plastids have been found to trace back not simply to a single common endosymbiotic event[150,153,154], but instead to many parallel endosymbioses involving closely-related symbionts. This is reminiscent of the *Euplotes–Polynucleobacter* system[22], but here the endosymbionts are genetically integrated with the host and not clearly destined for rapid extinction. The appearance of these common traits over a range of symbioses based on diverse functions and involving even more diverse partners suggests that they reflect fundamental processes underpinning a wide variety of endosymbiotic interactions.

Conclusions

Protists are not a single biologically unified clade of organisms, but rather span the whole diversity of eukaryotes. This diversity alone makes them a deep pool of potentially interesting biology; however, protists are arguably also the least-studied fraction of the entire tree of life. Altogether this affects the many ways in which we interpret the norms and expectations of eukaryotic biology and evolution, since much of what we understand, and more of how we frame it, is based strongly or entirely on one biologically rather odd subgroup, the animals. This situation extends to symbiotic associations, where we know enough to conclude that these interactions are common, functionally diverse, and of great ecological and evolutionary importance. Yet we are only beginning to glimpse how a greater understanding of them will impact our general understanding of symbiosis more broadly. Genomics provided the spark that ignited an acceleration in our understanding of protist–prokaryotic symbioses, but it bears repeating that genomics alone will not reveal all we need to know to understand the function or evolution of these systems. For that, a greater push to develop a wider range of systems is needed, with the goal of establishing as deep an understanding of the symbiotic associations from a biological broad swath of eukaryotic diversity as we have from their animal cousins.

SUPPLEMENTAL INFORMATION

Supplemental information includes one table and supplemental references, and can be found with this article online at https://doi.org/10.1016/j.cub.2021.05.049.

ACKNOWLEDGEMENTS

F.H. was supported by the OIST and the Japan Society for the Promotion of Science (KAKENHI JP20K22672 and JP21K15094). D.T., J.L. and P.J.K. were supported by the Gordon and Betty Moore Foundation (#9354). D.T. and J.L. were supported by the Czech Ministry of Education (0000759), Czech Bioimaging grant (LM2018129), Czech Grant Agency (20-07186S) and ERC CZ (LL1601). V.B., E.E.G. and P.J.K. were supported by the Tula Foundation and the Gordon and Betty Moore Foundation (https://doi.org/10.37807/GBMF9201).

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Keeling, P.J., and Koonin, E.V. (2014). The Origin and Evolution of Eukaryotes: A Subject Collection from Cold Spring Harbor Perspectives in Biology (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

2. Booth, A., and Doolittle, W.F. (2015). Eukaryogenesis, how special really? Proc. Natl. Acad. Sci. USA *112*, 10278–10285.

3. Embley, T.M., and Martin, W. (2006). Eukaryotic evolution, changes and challenges. Nature *440*, 623–630.

4. Gray, M.W., and Doolittle, W.F. (1982). Has the endosymbiont hypothesis been proven? Microbiol. Rev. *46*, 1–42.

5. Moran, N.A., McCutcheon, J.P., and Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. Annu. Rev. Genet. *42*, 165–190.

6. Dubilier, N., Bergin, C., and Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat. Rev. Microbiol. *6*, 725–740.

7. McCutcheon, J.P., and Moran, N.A. (2011). Extreme genome reduction in symbiotic bacteria. Nat. Rev. Microbiol. *10*, 13–26.

8. McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., *et al*. (2013). Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. USA *110*, 3229–3236.

9. Müller, D.B., Vogel, C., Bai, Y., and Vorholt, J.A. (2016). The plant microbiota: Systems-level insights and perspectives. Annu. Rev. Genet. *50*, 211–234.

10. Keeling, P.J., and Burki, F. (2019). Progress towards the tree of eukaryotes. Curr. Biol. *29*, R808–R817.

11. Burki, F., Roger, A.J., Brown, M.W., and Simpson, A.G.B. (2020). The new tree of eukaryotes. Trends Ecol. Evol. *35*, 43–55.

12. Keeling, P.J., and Campo, J.D. (2017). Marine protists are not just big bacteria. Curr. Biol. *27*, R541–R549.

13. Görtz, H.-D. (2006). Symbiotic associations between ciliates and prokaryotes. In The Prokaryotes, M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds. (Singapore: Springer), pp. 364–402.

14. Horn, M., and Wagner, M. (2004). Bacterial endosymbionts of free-living amoebae. J. Eukaryot. Microbiol. *51*, 509–514.

15. Tekle, Y.I., Lyttle, J.M., Blasingame, M.G., and Wang, F. (2021). Comprehensive comparative genomics reveals over 50 phyla of free-living and pathogenic bacteria are associated with diverse members of the amoebozoa. Sci. Rep. *11*, 8043.

16. Foster, R.A., and Zehr, J.P. (2019). Diversity, genomics, and distribution of phytoplankton-cyanobacterium single-cell symbiotic associations. Annu. Rev. Microbiol. *73*, 435–456.

17. Harmer, J., Yurchenko, V., Nenarokova, A., Lukeš, J., and Ginger, M.L. (2018). Farming, slaving and enslavement: histories of endosymbioses during kinetoplastid evolution. Parasitology *145*, 1311–1323.

18. Hess, S. (2017). Description of Hyalodiscus flabellus sp. nov. (Vampyrellida, Rhizaria) and identification of its bacterial endosymbiont, "Candidatus Megaira polyxenophila" (Rickettsiales, Alphaproteobacteria). Protist *168*, 109–133.

19. Hess, S., Suthaus, A., and Melkonian, M. (2016). "Candidatus Finniella" (Rickettsiales, Alphaproteobacteria), novel endosymbionts of viridiraptorid amoeboflagellates (Cercozoa, Rhizaria). Appl. Environ. Microbiol. *82*, 659–670.

20. Tikhonenkov, D.V., Hehenberger, E., Esaulov, A.S., Belyakova, O.I., Mazei, Y.A., Mylnikov, A.P., and Keeling, P.J. (2020). Insights into the origin of metazoan multicellularity from predatory unicellular relatives of animals. BMC Biol. *18*, 39.

21. Muñoz-Gómez, S.A., Hess, S., Burger, G., Lang, B.F., Susko, E., Slamovits, C.H., and Roger, A.J. (2019). An updated phylogeny of the Alphaproteobacteria reveals that the parasitic Rickettsiales and Holosporales have independent origins. eLife *8*, e42535.

22. Boscaro, V., Husnik, F., Vannini, C., and Keeling, P.J. (2019). Symbionts of the ciliate Euplotes: diversity, patterns and potential as models for bacteria-eukaryote endosymbioses. Proc. Biol. Sci. *286*, 20190693.

23. Lanzoni, O., Plotnikov, A., Khlopko, Y., Munz, G., Petroni, G., and Potekhin, A. (2019). The core microbiome of sessile ciliate Stentor coeruleus is not shaped by the environment. Sci. Rep. *9*, 11356.

24. Yurchenko, T., Ševčíková, T., Přibyl, P., El Karkouri, K., Klimeš, V., Amaral, R., Zbránková, V., Kim, E., Raoult, D., Santos, L.M.A., *et al*. (2018). A gene transfer event suggests a long-term partnership between eustigmatophyte algae and a novel lineage of endosymbiotic bacteria. ISME J. *12*, 2163–2175.

25. George, E.E., Husnik, F., Tashyreva, D., Prokopchuk, G., Horák, A., Kwong, W.K., Lukeš, J., and Keeling, P.J. (2020). Highly reduced genomes of protist endosymbionts show evolutionary convergence. Curr. Biol. *30*, 925–933.

26. Treitli, S.C., Kolisko, M., Husník, F., Keeling, P.J., and Hampl, V. (2019). Revealing the metabolic capacity of Streblomastix strix and its bacterial symbionts using single-cell metagenomics. Proc. Natl. Acad. Sci. USA *116*, 19675–19684.

27. Tai, V., Carpenter, K.J., Weber, P.K., Nalepa, C.A., Perlman, S.J., and Keeling, P.J. (2016). Genome evolution and nitrogen fixation in bacterial ectosymbionts of a protist inhabiting wood-feeding cockroaches. Appl. Environ. Microbiol. *82*, 4682–4695.

28. Horn, M. (2008). Chlamydiae as symbionts in eukaryotes. Annu. Rev. Microbiol. *62*, 113–131.

29. Lind, A.E., Lewis, W.H., Spang, A., Guy, L., Embley, T.M., and Ettema, T.J.G. (2018). Genomes of two archaeal endosymbionts show convergent adaptations to an intracellular lifestyle. ISME J. *12*, 2655–2667.

30. Gutiérrez, G., Chistyakova, L.V., Villalobo, E., Kostygov, A.Y., and Frolov, A.O. (2017). Identification of Pelomyxa palustris endosymbionts. Protist *168*, 408–424.

31. Beinart, R.A., Rotterová, J., and Čepička, I. (2018). The genome of an endosymbiotic methanogen is very similar to those of its free-living relatives. Environ. Microbiol. *20*, 2538–2551.

32. Hackstein, J.H.P. (2010). (Endo)symbiotic Methanogenic Archaea (Berlin, Heidelberg: Springer).

33. Pillonel, T., Bertelli, C., and Greub, G. (2018). Environmental metagenomic assemblies reveal seven new highly divergent chlamydial lineages and hallmarks of a conserved intracellular lifestyle. Front. Microbiol. *9*, 79.

34. Castelli, M., Sabaneyeva, E., Lanzoni, O., Lebedeva, N., Floriano, A.M., Gaiarsa, S., Benken, K., Modeo, L., Bandi, C., Potekhin, A., *et al*. (2019). Deianiraea, an extracellular bacterium associated with the ciliate Paramecium, suggests an alternative scenario for the evolution of Rickettsiales. ISME J. *13*, 2280–2294.

35. Rossi, A., Bellone, A., Fokin, S.I., Boscaro, V., and Vannini, C. (2019). Detecting associations between ciliated protists and prokaryotes with culture-independent single-cell microbiomics: a proof-of-concept study. Microb. Ecol. *78*, 232–242.

36. Dirren, S., Salcher, M.M., Blom, J.F., Schweikert, M., and Posch, T. (2014). Ménage-à-trois: the amoeba Nuclearia sp. from Lake Zurich with its ecto- and endosymbiotic bacteria. Protist *165*, 745–758.

37. Vannini, C., Boscaro, V., Ferrantini, F., Benken, K.A., Mironov, T.I., Schweikert, M., Görtz, H.-D., Fokin, S.I., Sabaneyeva, E.V., and Petroni, G. (2014). Flagellar movement in two bacteria of the family Rickettsiaceae: A re-evaluation of motility in an evolutionary perspective. PLoS One *9*, e87718.

38. Kostygov, A.Y., Dobáková, E., Grybchuk-Ieremenko, A., Váhala, D., Maslov, D.A., Votýpka, J., Lukeš, J., and Yurchenko, V. (2016). Novel trypanosomatid-bacterium association: Evolution of endosymbiosis in action. mBio *7*, e01985.

39. Lee, M.J., Schreurs, P.J., Messer, A.C., and Zinder, S.H. (1987). Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr. Microbiol. *15*, 337–341.

40. Tashyreva, D., Prokopchuk, G., Votýpka, J., Yabuki, A., Horák, A., and Lukeš, J. (2018). Life cycle, ultrastructure, and phylogeny of new diplonemids and their endosymbiotic bacteria. mBio *9*, e02447-17.

41. Akhmanova, A., Voncken, F., van Alen, T., van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M., and Hackstein, J.H. (1998). A hydrogenosome with a genome. Nature *396*, 527–528.

42. Petroni, G., Spring, S., Schleifer, K.H., Verni, F., and Rosati, G. (2000). Defensive extrusive ectosymbionts of Euplotidium (Ciliophora) that contain microtubule-like structures are bacteria related to Verrucomicrobia. Proc. Natl. Acad. Sci. USA *97*, 1813–1817.

43. Bright, M., Espada-Hinojosa, S., Lagkouvardos, I., and Volland, J.-M. (2014). The giant ciliate Zoothamnium niveum and its thiotrophic epibiont Candidatus Thiobios zoothamnicoli: a model system to study interspecies cooperation. Front. Microbiol. *5*, 145.

44. Leander, B.S., and Keeling, P.J. (2004). Symbiotic innovation in the oxymonad Streblomastix strix. J. Eukaryot. Microbiol. *51*, 291–300.

45. Sato, T., Kuwahara, H., Fujita, K., Noda, S., Kihara, K., Yamada, A., Ohkuma, M., and Hongoh, Y. (2014). Intranuclear verrucomicrobial symbionts and evidence of lateral gene transfer to the host protist in the termite gut. ISME J. *8*, 1008–1019.

46. Schulz, F., and Horn, M. (2015). Intranuclear bacteria: inside the cellular control center of eukaryotes. Trends Cell Biol. *25*, 339–346.

47. Bella, C., Koehler, L., Grosser, K., Berendonk, T.U., Petroni, G., and Schrallhammer, M. (2016). Fitness impact of obligate intranuclear bacterial symbionts depends on host growth phase. Front. Microbiol. *7*, 2084.

48. Banerji, A., Duncan, A.B., Griffin, J.S., Humphries, S., Petchey, O.L., and Kaltz, O. (2015). Density- and trait-mediated effects of a parasite and a predator in a tri-trophic food web. J. Animal Ecol. *84*, 723–733.

49. Duncan, A.B., Fellous, S., and Kaltz, O. (2011). Reverse evolution: selection against costly resistance in disease-free microcosm populations of Paramecium caudatum. Evolution *65*, 3462–3474.

50. Keeling, P.J., and McCutcheon, J.P. (2017). Endosymbiosis: the feeling is not mutual. J. Theor. Biol. *434*, 75–79.

51. Garcia, J.R., and Gerardo, N.M. (2014). The symbiont side of symbiosis: do microbes really benefit? Front. Microbiol. *5*, 510.

52. Husnik, F., and Keeling, P.J. (2019). The fate of obligate endosymbionts: reduction, integration, or extinction. Curr. Opin. Genet. Dev. *58-59*, 1–8.

53. Bennett, G.M., and Moran, N.A. (2015). Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. Proc. Natl. Acad. Sci. USA *112*, 10169–10176.

54. Boscaro, V., Kolisko, M., Felletti, M., Vannini, C., Lynn, D.H., and Keeling, P.J. (2017). Parallel genome reduction in symbionts descended from closely related free-living bacteria. Nat. Ecol. Evol. *1*, 1160–1167.

55. McCutcheon, J.P., Boyd, B.M., and Dale, C. (2019). The life of an insect endosymbiont from the cradle to the grave. Curr. Biol. *29*, R485–R495.

56. Nakayama, T., Nomura, M., and Takano, Y. (2019). Single-cell genomics unveiled a cryptic cyanobacterial lineage with a worldwide distribution hidden by a dinoflagellate host. Proc. Natl. Acad. Sci. USA *116*, 15973–15978.

57. Seah, B.K.B., Antony, C.P., Huettel, B., Zarzycki, J., Schada von Borzyskowski, L., Erb, T.J., Kouris, A., Kleiner, M., Liebeke, M., Dubilier, N., *et al*. (2019). Sulfur-oxidizing symbionts without canonical genes for autotrophic CO2 fixation. mBio *10*, e01112–19.

58. Rosati, G. (2002). Ectosymbiosis in ciliated protozoa. In Symbiosis: Mechanisms and Model Systems, J. Seckbach, ed. (Netherlands: Springer), pp. 475–488.

59. DiSalvo, S., Haselkorn, T.S., Bashir, U., Jimenez, D., Brock, D.A., Queller, D.C., and Strassmann, J.E. (2015). Burkholderia bacteria infectiously induce the proto-farming symbiosis of Dictyostelium amoebae and food bacteria. Proc. Natl. Acad. Sci. USA *112*, E5029–E5037.

60. Shu, L., Brock, D.A., Geist, K.S., Miller, J.W., Queller, D.C., Strassmann, J.E., and DiSalvo, S. (2018). Symbiont location, host fitness, and possible coadaptation in a symbiosis between social amoebae and bacteria. eLife *7*, e42660.

61. Brock, D.A., Douglas, T.E., Queller, D.C., and Strassmann, J.E. (2011). Primitive agriculture in a social amoeba. Nature *469*, 393–396.

62. Oliver, K.M., Degnan, P.H., Burke, G.R., and Moran, N.A. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu. Rev. Entomol. *55*, 247–266.

63. Bjorbækmo, M.F.M., Evenstad, A., Røsæg, L.L., Krabberød, A.K., and Logares, R. (2020). The planktonic protist interactome: where do we stand after a century of research? ISME J. *14*, 544–559.

64. Mehari, Y.T., Arivett, B.A., Farone, A.L., Gunderson, J.H., and Farone, M.B. (2016). Draft genome sequences of two novel amoeba-resistant intranuclear bacteria, "Candidatus Berkiella cookevillensis" and "Candidatus Berkiella aquae. Genome Announc. *4*, e01732–15.

65. Serra, V., Gammuto, L., Nitla, V., Castelli, M., Lanzoni, O., Sassera, D., Bandi, C., Sandeep, B.V., Verni, F., Modeo, L., *et al*. (2020). Morphology, ultrastructure, genomics, and phylogeny of Euplotes vanleeuwenhoeki sp. nov. and its ultra-reduced endosymbiont "Candidatus Pinguicoccus supinus" sp. nov. Sci. Rep. *10*, 20311.

66. Moran, N.A., and Bennett, G.M. (2014). The tiniest tiny genomes. Annu. Rev. Microbiol. *68*, 195–215.

67. Kuwahara, H., Yuki, M., Izawa, K., Ohkuma, M., and Hongoh, Y. (2017). Genome of "Ca. Desulfovibrio trichonymphae", an H2-oxidizing bacterium in a tripartite symbiotic system within a protist cell in the termite gut. ISME J. *11*, 766–776.

68. Salem, H., Bauer, E., Kirsch, R., Berasategui, A., Cripps, M., Weiss, B., Koga, R., Fukumori, K., Vogel, H., Fukatsu, T., *et al*. (2017). Drastic genome reduction in an herbivore's pectinolytic symbiont. Cell *171*, 1520–1531.

69. Belda, E., Moya, A., Bentley, S., and Silva, F.J. (2010). Mobile genetic element proliferation and gene inactivation impact over the genome structure and metabolic capabilities of Sodalis glossinidius, the secondary endosymbiont of tsetse flies. BMC Genomics *11*, 449.

70. Floriano, A.M., Castelli, M., Krenek, S., Berendonk, T.U., Bazzocchi, C., Petroni, G., and Sassera, D. (2018). The genome sequence of "Candidatus Fokinia solitaria": insights on reductive evolution in Rickettsiales. Genome Biol. Evol. *10*, 1120–1126.

71. Naito, M., and Pawlowska, T.E. (2016). Defying Muller's ratchet: ancient heritable endobacteria escape extinction through retention of recombination and genome plasticity. mBio *7*, e02057-15.

72. Moran, N.A. (1996). Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Natl. Acad. Sci. USA *93*, 2873–2878.

73. Bourguignon, T., Kinjo, Y., Villa-Martín, P., Coleman, N.V., Tang, Q., Arab, D.A., Wang, Z., Tokuda, G., Hongoh, Y., Ohkuma, M., *et al*. (2020). Increased mutation rate is linked to genome reduction in prokaryotes. Curr. Biol. *30*, 3848–3855.

74. Husnik, F., and McCutcheon, J.P. (2016). Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. Proc. Natl. Acad. Sci. USA *113*, E5416–E5424.

75. Sudakaran, S., Kost, C., and Kaltenpoth, M. (2017). Symbiont acquisition and replacement as a source of ecological innovation. Trends Microbiol. *25*, 375–390.

76. Schmitz-Esser, S., Tischler, P., Arnold, R., Montanaro, J., Wagner, M., Rattei, T., and Horn, M. (2010). The genome of the amoeba symbiont "Candidatus Amoebophilus asiaticus" reveals common mechanisms for host cell interaction among amoeba-associated bacteria. J. Bacteriol. *192*, 1045–1057.

77. Ishida, K., Sekizuka, T., Hayashida, K., Matsuo, J., Takeuchi, F., Kuroda, M., Nakamura, S., Yamazaki, T., Yoshida, M., Takahashi, K., *et al*. (2014). Amoebal endosymbiont Neochlamydia genome sequence illuminates the bacterial role in the defense of the host amoebae against Legionella pneumophila. PLoS One *9*, e95166.

78. Deeg, C.M., Zimmer, M.M., George, E.E., Husnik, F., Keeling, P.J., and Suttle, C.A. (2019). Chromulinavorax destructans, a pathogen of microzooplankton that provides a window into the enigmatic candidate phylum Dependentiae. PLoS Pathog. *15*, e1007801.

79. Duncan, A.B., Fellous, S., Accot, R., Alart, M., Chantung Sobandi, K., Cosiaux, A., and Kaltz, O. (2010). Parasite-mediated protection against osmotic stress for Paramecium caudatum infected by Holospora undulata is host genotype specific. FEMS Microbiol. Ecol. *74*, 353–360.

80. Taylor-Brown, A., Vaughan, L., Greub, G., Timms, P., and Polkinghorne, A. (2015). Twenty years of research into Chlamydia-like organisms: a revolution in our understanding of the biology and pathogenicity of members of the phylum Chlamydiae. Pathog. Dis. *73*, 1–15.

81. McCutcheon, J.P., and Keeling, P.J. (2014). Endosymbiosis: protein targeting further erodes the organelle/symbiont distinction. Curr. Biol. *24*, R654–R655.

82. Nakayama, T., Kamikawa, R., Tanifuji, G., Kashiyama, Y., Ohkouchi, N., Archibald, J.M., and Inagaki, Y. (2014). Complete genome of a nonphotosynthetic cyanobacterium in a diatom reveals recent adaptations to an intracellular lifestyle. Proc. Natl. Acad. Sci. USA *111*, 11407–11412.

83. Morales, J., Kokkori, S., Weidauer, D., Chapman, J., Goltsman, E., Rokhsar, D., Grossman, A.R., and Nowack, E.C.M. (2016). Development of a toolbox to dissect host-endosymbiont interactions and protein trafficking in the trypanosomatid Angomonas deanei. BMC Evol. Biol. *16*, 247.

84. Adler, S., Trapp, E.M., Dede, C., Maier, U.G., and Zauner, S. (2014). Rhopalodia gibba: The first steps in the birth of a novel organelle? In Endosymbiosis, W. Löffelhardt, ed. (Vienna: Springer), pp. 167–179.

85. Nakayama, T., and Inagaki, Y. (2017). Genomic divergence within nonphotosynthetic cyanobacterial endosymbionts in rhopalodiacean diatoms. Sci. Rep. *7*, 13075.

86. Catta-Preta, C.M.C., Brum, F.L., da Silva, C.C., Zuma, A.A., Elias, M.C., de Souza, W., Schenkman, S., and Motta, M.C.M. (2015). Endosymbiosis in trypanosomatid protozoa: the bacterium division is controlled during the host cell cycle. Front. Microbiol. *6*, 520.

87. Mira, A., and Moran, N.A. (2002). Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb. Ecol. *44*, 137–143.

88. Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., and Smith, A.G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature *438*, 90–93.

89. Harke, M.J., Frischkorn, K.R., Haley, S.T., Aylward, F.O., Zehr, J.P., and Dyhrman, S.T. (2019). Periodic and coordinated gene expression between a diazotroph and its diatom host. ISME J. *13*, 118–131.

90. Amin, S.A., Green, D.H., and Hart, M.C. (2009). Photolysis of iron–siderophore chelates promotes bacterial–algal mutualism. Proc. Natl. Acad. Sci. USA *106*, 17071–17076.

91. Farnelid, H., Tarangkoon, W., Hansen, G., Hansen, P.J., and Riemann, L. (2010). Putative N2-fixing heterotrophic bacteria associated with dinoflagellate–Cyanobacteria consortia in the low-nitrogen Indian Ocean. Aquat. Microb. Ecol. *61*, 105–117.

92. Tripp, H.J., Bench, S.R., Turk, K.A., Foster, R.A., Desany, B.A., Niazi, F., Affourtit, J.P., and Zehr, J.P. (2010). Metabolic streamlining in an open-ocean nitrogen-fixing cyanobacterium. Nature *464*, 90–94.

93. Escalera, L., Reguera, B., Takishita, K., Yoshimatsu, S., Koike, K., and Koike, K. (2011). Cyanobacterial endosymbionts in the benthic dinoflagellate Sinophysis canaliculata (Dinophysiales, Dinophyceae). Protist *162*, 304–314.

94. Nowack, E.C.M., and Weber, A.P.M. (2018). Genomics-informed insights into endosymbiotic organelle evolution in photosynthetic eukaryotes. Annu. Rev. Plant Biol. *69*, 51–84.

95. Fenchel, T., and Bernard, C. (1993). Endosymbiotic purple non-sulphur bacteria in an anaerobic ciliated protozoon. FEMS Microbiol. Lett. *110*, 21–25.

96. Hongoh, Y., Sharma, V.K., Prakash, T., Noda, S., Taylor, T.D., Kudo, T., Sakaki, Y., Toyoda, A., Hattori, M., and Ohkuma, M. (2008). Complete genome of the uncultured Termite Group 1 bacteria in a single host protist cell. Proc. Natl. Acad. Sci. USA *105*, 5555–5560.

97. Ohkuma, M., Noda, S., Hattori, S., Iida, T., Yuki, M., Starns, D., Inoue, J.-I., Darby, A.C., and Hongoh, Y. (2015). Acetogenesis from H2 plus CO2 and nitrogen fixation by an endosymbiotic spirochete of a termite-gut cellulolytic protist. Proc. Natl. Acad. Sci. USA *112*, 10224–10230.

98. Strassert, J.F.H., Mikaelyan, A., Woyke, T., and Brune, A. (2016). Genome analysis of "Candidatus Ancillula trichonymphae", first representative of a deep-branching clade of Bifidobacteriales, strengthens evidence for convergent evolution in flagellate endosymbionts. Environ. Microbiol. Rep. *8*, 865–873.

99. Ikeda-Ohtsubo, W., Strassert, J.F.H., Köhler, T., Mikaelyan, A., Gregor, I., McHardy, A.C., Tringe, S.G., Hugenholtz, P., Radek, R., and Brune, A. (2016). "Candidatus Adiutrix intracellularis", an endosymbiont of termite gut flagellates, is the first representative of a deep-branching clade of Deltaproteobacteria and a putative homoacetogen. Environ. Microbiol. *18*, 2548–2564.

100. Ohkuma, M. (2008). Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol. *16*, 345–352.

101. Hamann, E., Gruber-Vodicka, H., Kleiner, M., Tegetmeyer, H.E., Riedel, D., Littmann, S., Chen, J., Milucka, J., Viehweger, B., Becker, K.W., *et al*. (2016). Environmental Breviatea harbour mutualistic Arcobacter epibionts. Nature *534*, 254–258.

102. Monteil, C.L., Vallenet, D., Menguy, N., Benzerara, K., Barbe, V., Fouteau, S., Cruaud, C., Floriani, M., Viollier, E., Adryanczyk, G., *et al*. (2019). Ectosymbiotic bacteria at the origin of magnetoreception in a marine protist. Nat. Microbiol. *4*, 1088–1095.

103. Takeuchi, M., Kuwahara, H., Murakami, T., Takahashi, K., Kajitani, R., Toyoda, A., Itoh, T., Ohkuma, M., and Hongoh, Y. (2020). Parallel reductive genome evolution in Desulfovibrio ectosymbionts independently acquired by Trichonympha protists in the termite gut. ISME J. *14*, 2288–2301.

104. Tokura, M., Ohkuma, M., and Kudo, T. (2000). Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol. Ecol. *33*, 233–240.

105. Inoue, J.-I., Noda, S., Hongoh, Y., Ui, S., and Ohkuma, M. (2008). Identification of endosymbiotic methanogen and ectosymbiotic spirochetes of gut protists of the termite Coptotermes formosanus. Microbes Environ. *23*, 94–97.

106. Fenchel, T., and Finlay, B.J. (1992). Production of methane and hydrogen by anaerobic ciliates containing symbiotic methanogens. Arch. Microbiol. *157*, 475–480.

107. Yamada, K., Kamagata, Y., and Nakamura, K. (2006). The effect of endosymbiotic methanogens on the growth and metabolic profile of the anaerobic free-living ciliate Trimyema compressum. FEMS Microbiol. Lett. *149*, 129–132.

108. Hongoh, Y., Sharma, V.K., Prakash, T., Noda, S., Toh, H., Taylor, T.D., Kudo, T., Sakaki, Y., Toyoda, A., Hattori, M., *et al*. (2008). Genome of an endosymbiont coupling N2 fixation to cellulolysis within protist cells in termite gut. Science *322*, 1108–1109.

109. Inoue, J.-I., Saita, K., Kudo, T., Ui, S., and Ohkuma, M. (2007). Hydrogen production by termite gut protists: characterization of iron hydrogenases of Parabasalian symbionts of the termite Coptotermes formosanus. Eukaryot. Cell *6*, 1925–1932.

110. Odelson, D.A., and Breznak, J.A. (1985). Nutrition and growth characteristics of Trichomitopsis termopsidis, a cellulolytic protozoan from termites. Appl. Environ. Microbiol. *49*, 614–621.

111. Messer, A.C., and Lee, M.J. (1989). Effect of chemical treatments on methane emission by the hindgut microbiota in the termite Zootermopsis angusticollis. Microb. Ecol. *18*, 275–284.

112. Hongoh, Y. (2011). Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. Cell. Mol. Life Sci. *68*, 1311–1325.

113. Bernhard, J.M., Buck, K.R., Farmer, M.A., and Bowser, S.S. (2000). The Santa Barbara Basin is a symbiosis oasis. Nature *403*, 77–80.

114. Radek, R. (2010). Adhesion of bacteria to protists. In Prokaryotic Cell Wall Compounds: Structure and Biochemistry, H. König, H. Claus, and A. Varma, eds. (Berlin, Heidelberg: Springer), pp. 429–456.

115. Edgcomb, V.P., Leadbetter, E.R., Bourland, W., Beaudoin, D., and Bernhard, J.M. (2011). Structured multiple endosymbiosis of bacteria and archaea in a ciliate from marine sulfidic sediments: A survival mechanism in low oxygen, sulfidic sediments? Front. Microbiol. *2*, 55.

116. Hünken, M., Harder, J., and Kirst, G.O. (2008). Epiphytic bacteria on the Antarctic ice diatom Amphiprora kufferathii Manguin cleave hydrogen peroxide produced during algal photosynthesis. Plant Biol. *10*, 519–526.

117. Bernhard, J.M., Edgcomb, V.P., Casciotti, K.L., McIlvin, M.R., and Beaudoin, D.J. (2012). Denitrification likely catalyzed by endobionts in an allogromiid foraminifer. ISME J. *6*, 951–960.

118. Graf, J.S., Schorn, S., Kitzinger, K., Ahmerkamp, S., Woehle, C., Huettel, B., Schubert, C.J., Kuypers, M.M.M., and Milucka, J. (2021). Anaerobic endosymbiont generates energy for ciliate host by denitrification. Nature *591*, 445–450.

119. Woehle, C., Roy, A.-S., Glock, N., Wein, T., Weissenbach, J., Rosenstiel, P., Hiebenthal, C., Michels, J., Schönfeld, J., and Dagan, T. (2018). A novel eukaryotic denitrification pathway in foraminifera. Curr. Biol. *28*, 2536–2543.

120. König, L., Wentrup, C., Schulz, F., Wascher, F., Escola, S., Swanson, M.S., Buchrieser, C., and Horn, M. (2019). Symbiont-mediated defense against Legionella pneumophila in amoebae. mBio *10*, e00333–19.

121. Rosati, G., Petroni, G., Quochi, S., Modeo, L., and Verni, F. (1999). Epixenosomes: peculiar epibionts of the hypotrich ciliate Euplotidium itoi defend their host against predators. J. Eukaryot. Microbiol. *46*, 278–282.

122. Kusch, J., Czubatinski, L., Wegmann, S., Hubner, M., Alter, M., and Albrecht, P. (2002). Competitive advantages of Caedibacter-infected paramecia. Protist *153*, 47–58.

123. Grosser, K., Ramasamy, P., Amirabad, A.D., Schulz, M.H., Gasparoni, G., Simon, M., and Schrallhammer, M. (2018). More than the "killer trait": infection with the bacterial endosymbiont Caedibacter taeniospiralis causes transcriptomic modulation in Paramecium host. Genome Biol. Evol. *10*, 646–656.

124. Schrallhammer, M. (2010). The killer trait of Paramecium and its causative agents. Palaeodiversity *3*, 79–88.

125. Wenzel, M., Radek, R., Brugerolle, G., and König, H. (2003). Identification of the ectosymbiotic bacteria of Mixotricha paradoxa involved in movement symbiosis. Eur. J. Protistol. *39*, 11–23.

126. Hongoh, Y., Sato, T., Dolan, M.F., Noda, S., Ui, S., Kudo, T., and Ohkuma, M. (2007). The motility symbiont of the termite gut flagellate Caduceia versatilis is a member of the "Synergistes" group. Appl. Environ. Microbiol. *73*, 6270–6276.

127. Tamm, S.L. (1982). Flagellated ectosymbiotic bacteria propel a eucaryotic cell. J. Cell Biol. *94*, 697–709.

128. Okude, M., Matsuo, J., Nakamura, S., Kawaguchi, K., Hayashi, Y., Sakai, H., Yoshida, M., Takahashi, K., and Yamaguchi, H. (2012). Environmental chlamydiae alter the growth speed and motility of host acanthamoebae. Microbes Environ. *27*, 423–429.

129. Edgcomb, V.P., Breglia, S.A., Yubuki, N., Beaudoin, D., Patterson, D.J., Leander, B.S., and Bernhard, J.M. (2011). Identity of epibiotic bacteria on symbiontid euglenozoans in O2-depleted marine sediments: evidence for symbiont and host co-evolution. ISME J. *5*, 231–243.

130. Edgcomb, V. (2019). Symbiotic magnetic motility. Nat. Microbiol. *4*, 1066–1067.

131. Dyer, B.D., and Khalsa, O. (1993). Surface bacteria of Streblomastix strix are sensory symbionts. Biosystems *31*, 169–180.

132. Jeon, K.W. (1972). Development of cellular dependence on infective organisms: micrurgical studies in amoebas. Science *176*, 1122–1123.

133. Seyedsayamdost, M.R., Case, R.J., Kolter, R., and Clardy, J. (2011). The Jekyll-and-Hyde chemistry of Phaeobacter gallaeciensis. Nat. Chem. *3*, 331–335.

134. Pagnier, I., Yutin, N., Croce, O., Makarova, K.S., Wolf, Y.I., Benamar, S., Raoult, D., Koonin, E.V., and La Scola, B. (2015). Babela massiliensis, a representative of a widespread bacterial phylum with unusual adaptations to parasitism in amoebae. Biol. Direct *10*, 13.

135. Delafont, V., Samba-Louaka, A., Bouchon, D., Moulin, L., and Héchard, Y. (2015). Shedding light on microbial dark matter: a TM 6 bacterium as natural endosymbiont of a free-living amoeba. Environ. Microbiol. Rep. *7*, 970–978.

136. Thomas, V., Casson, N., and Greub, G. (2006). Criblamydia sequanensis, a new intracellular Chlamydiales isolated from Seine river water using amoebal co-culture. Environ. Microbiol. *8*, 2125–2135.

137. Bou Khalil, J.Y., Benamar, S., Di Pinto, F., Blanc-Tailleur, C., Raoult, D., and La Scola, B. (2017). Protochlamydia phocaeensis sp. nov., a new Chlamydiales species with host dependent replication cycle. Microbes Infect. *19*, 343–350.

138. Weeden, N.F. (1981). Genetic and biochemical implications of the endosymbiotic origin of the chloroplast. J. Mol. Evol. *17*, 133–139.

139. Timmis, J.N., Ayliffe, M.A., Huang, C.Y., and Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nat. Rev. Genet. *5*, 123–135.

140. Larkum, A.W.D., Lockhart, P.J., and Howe, C.J. (2007). Shopping for plastids. Trends Plant Sci. *12*, 189–195.

141. Keeling, P.J. (2013). The number, speed, and impact of plastid endosymbioses in eukaryotic evolution. Annu. Rev. Plant Biol. *64*, 583–607.

142. Zachar, I., Szilágyi, A., Számadó, S., and Szathmáry, E. (2018). Farming the mitochondrial ancestor as a model of endosymbiotic establishment by natural selection. Proc. Natl. Acad. Sci. USA *115*, E1504–E1510.

143. Gray, M.W. (2015). Mosaic nature of the mitochondrial proteome: Implications for the origin and evolution of mitochondria. Proc. Natl. Acad. Sci. USA *112*, 10133–10138.

144. Hehenberger, E., Gast, R.J., and Keeling, P.J. (2019). A kleptoplastidic dinoflagellate and the tipping point between transient and fully integrated plastid endosymbiosis. Proc. Natl. Acad. Sci. USA *116*, 17934–17942.

145. Husnik, F., and McCutcheon, J.P. (2018). Functional horizontal gene transfer from bacteria to eukaryotes. Nat. Rev. Microbiol. *16*, 67–79.

146. Nakabachi, A., Ishida, K., Hongoh, Y., Ohkuma, M., and Miyagishima, S.-Y. (2014). Aphid gene of bacterial origin encodes a protein transported to an obligate endosymbiont. Curr. Biol. *24*, R640–R641.

147. Bublitz, D.C., Chadwick, G.L., Magyar, J.S., Sandoz, K.M., Brooks, D.M., Mesnage, S., Ladinsky, M.S., Garber, A.I., Bjorkman, P.J., Orphan, V.J., *et al*. (2019). Peptidoglycan production by an insect-bacterial mosaic. Cell *179*, 703–712.

148. Mao, M., and Bennett, G.M. (2020). Symbiont replacements reset the coevolutionary relationship between insects and their heritable bacteria. ISME J. *14*, 1384–1395.

149. Hehenberger, E., Burki, F., Kolisko, M., and Keeling, P.J. (2016). Functional relationship between a dinoflagellate host and its diatom endosymbiont. Mol. Biol. Evol. *33*, 2376–2390.

150. Novák Vanclová, A.M.G., Zoltner, M., Kelly, S., Soukal, P., Záhonová, K., Füssy, Z., Ebenezer, T.E., Lacová Dobáková, E., Eliáš, M., Lukeš, J., *et al*. (2020). Metabolic quirks and the colourful history of the Euglena gracilis secondary plastid. New Phytol. *225*, 1578–1592.

151. Ku, C., Nelson-Sathi, S., Roettger, M., Garg, S., Hazkani-Covo, E., and Martin, W.F. (2015). Endosymbiotic gene transfer from prokaryotic pangenomes: Inherited chimerism in eukaryotes. Proc. Natl. Acad. Sci. USA *112*, 10139–10146.

152. Strassert, J.F.H., Irisarri, I., Williams, T.A., and Burki, F. (2021). A molecular timescale for eukaryote evolution with implications for the origin of red algal-derived plastids. Nat. Commun. *12*, 1879.

153. Takano, Y., Hansen, G., Fujita, D., and Horiguchi, T. (2008). Serial replacement of diatom endosymbionts in two freshwater dinoflagellates, Peridiniopsis spp. (Peridiniales, Dinophyceae). Phycologia *47*, 41–53.

154. Gast, R.J., Moran, D.M., Dennett, M.R., and Caron, D.A. (2007). Kleptoplasty in an Antarctic dinoflagellate: caught in evolutionary transition? Environ. Microbiol. *9*, 39–45.

155. Hilton, J.A., Foster, R.A., James Tripp, H., Carter, B.J., Zehr, J.P., and Villareal, T.A. (2013). Genomic deletions disrupt nitrogen metabolism pathways of a cyanobacterial diatom symbiont. Nat. Commun. *4*, 1767.

156. Izawa, K., Kuwahara, H., Kihara, K., Yuki, M., Lo, N., Itoh, T., Ohkuma, M., and Hongoh, Y. (2016). Comparison of intracellular "Ca. Endomicrobium trichonymphae" genomovars illuminates the requirement and decay of defense systems against foreign DNA. Genome Biol. Evol. *8*, 3099–3107.

157. Nowack, E.C.M., Price, D.C., Bhattacharya, D., Singer, A., Melkonian, M., and Grossman, A.R. (2016). Gene transfers from diverse bacteria compensate for reductive genome evolution in the chromatophore of Paulinella chromatophora. Proc. Natl. Acad. Sci. USA *113*, 12214–12219.

158. Nowack, E.C.M., and Grossman, A.R. (2012). Trafficking of protein into the recently established photosynthetic organelles of Paulinella chromatophora. Proc. Natl. Acad. Sci. USA *109*, 5340–5345.

159. Singer, A., Poschmann, G., Mühlich, C., Valadez-Cano, C., Hänsch, S., Hüren, V., Rensing, S.A., Stühler, K., and Nowack, E.C.M. (2017). Massive protein import into the early-evolutionary-stage photosynthetic organelle of the amoeba Paulinella chromatophora. Curr. Biol. *27*, 2763–2773.

160. McCutcheon, J.P. (2016). From microbiology to cell biology: when an intracellular bacterium becomes part of its host cell. Curr. Opin. Cell Biol. *41*, 132–136.

161. Vannini, C., Ferrantini, F., Ristori, A., Verni, F., and Petroni, G. (2012). Betaproteobacterial symbionts of the ciliate Euplotes: origin and tangled evolutionary path of an obligate microbial association. Environ. Microbiol. *14*, 2553–2563.

162. Heckmann, K., and Hagen, R. (1983). Freshwater Euplotes species with a 9 type 1 cirrus pattern depend upon endosymbionts. J. Eukaryot. Microbiol. *30*, 284–289.

163. Hahn, M.W., Jezberová, J., Koll, U., Saueressig-Beck, T., and Schmidt, J. (2016). Complete ecological isolation and cryptic diversity in Polynucleobacter bacteria not resolved by 16S rRNA gene sequences. ISME J. *10*, 1642–1655.

164. Buchner, P. (1965). Endosymbiosis of Animals with Plant Microorganisms (Geneva: Interscience Publishers).

165. Bernhard, J.M., Tsuchiya, M., and Nomaki, H. (2018). Ultrastructural observations on prokaryotic associates of benthic foraminifera: Food, mutualistic symbionts, or parasites? Marine Micropaleontol. *138*, 33–45.

166. Görtz, H.-D., Rosati, G., Schweikert, M., Schrallhammer, M., Omura, G., and Suzaki, T. (2009). Microbial Symbionts for Defense and Competition among Ciliate Hosts (Boca Raton: CRC Press).

167. Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. Nat. Rev. Microbiol. *12*, 168–180.

168. Carpenter, K.J., Weber, P.K., Davisson, M.L., Pett-Ridge, J., Haverty, M.I., and Keeling, P.J. (2013). Correlated SEM, FIB-SEM, TEM, and NanoSIMS imaging of microbes from the hindgut of a lower termite: methods for in situ functional and ecological studies of uncultivable microbes. Microsc. Microanal. *19*, 1490–1501.

169. Tai, V., James, E.R., Nalepa, C.A., Scheffrahn, R.H., Perlman, S.J., and Keeling, P.J. (2015). The role of host phylogeny varies in shaping microbial diversity in the hindguts of lower termites. Appl. Environ. Microbiol. *81*, 1059–1070.