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Review

The chastity of amoebae: re-evaluating evidence for sex in amoeboid organisms

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Amoebae are generally assumed to be asexual. We argue that this view is a relict of early classification schemes that lumped all amoebae together inside the 'lower' protozoa, separated from the 'higher' plants, animals and fungi. This artificial classification allowed microbial eukaryotes, including amoebae, to be dismissed as primitive, and implied that the biological rules and theories developed for macro-organisms need not apply to microbes. Eukaryotic diversity is made up of 70+ lineages, most of which are microbial. Plants, animals and fungi are nested among these microbial lineages. Thus, theories on the prevalence and maintenance of sex developed for macro-organisms should in fact apply to microbial eukaryotes, though the theories may need to be refined and generalized (e.g. to account for the variation in sexual strategies and prevalence of facultative sex in natural populations of many microbial eukaryotes). We use a revised phylogenetic framework to assess evidence for sex in several amoeboid lineages that are traditionally considered asexual, and we interpret this evidence in light of theories on the evolution of sex developed for macro-organisms. We emphasize that the limited data available for many lineages coupled with natural variation in microbial life cycles overestimate the extent of asexuality. Mapping sexuality onto the eukaryotic tree of life demonstrates that the majority of amoeboid lineages are, contrary to popular belief, anciently sexual, and that most asexual groups have probably arisen recently and independently. Additionally, several unusual genomic traits are prevalent in amoeboid lineages, including cyclic polyploidy, which may serve as alternative mechanisms to minimize the deleterious effects of asexuality.

Keywords: Amoebozoa; Rhizaria; asexual; evolution of sex; meiosis; karyogamy

Let us consider for a moment, a single *Ameba* ... not as a cause of disease, but as a unit mass of protoplasm which ... performs all of the fundamental vital activities common to living things ... there is no reason to doubt that [the chemical composition of these unit masses] agrees with that of other living substances, since the accompanying properties of protoplasm—metabolism, growth and reproduction—are obviously performed in the same way.

(p. 259 in [1])

1. INTRODUCTION

Microbial eukaryotes were historically classified as primitive plants and animals [2] or separated into their own kingdom [3–5]. This latter view received wide support with Whittaker's five-kingdom classification system [4] and continues to be popular in many circles. One consequence of lumping microbial eukaryotes into an artificial taxonomic unit (variously called Protista, Protoctista or Protozoa) is the implicit view that microbes are fundamentally different entities than plants, animals and fungi. As a result, microbial eukaryotes have been either dismissed as primitive or ignored in much of the theoretical work on eukaryotes, such as speciation theory [6] and theories on the evolution of sex [7], with the notable exception of Bell [8]. However, given the current classification of eukaryotes, this dismissal is no longer acceptable. In recent analyses, the eukaryotic tree of life is divided into a number of high-level lineages in which macro-organisms nest within predominantly microbial clades (figure 1), demonstrating that the evolution of multicellularity has arisen multiple times [9–14]. Hence, there is no evidence to suggest that unicellularity should be equated with 'primitive'.

The realization that there is no fundamental distinction between macrobial and microbial eukaryotes calls for reassessment of the applicability of theories on the evolution of sex in macro-organisms to be extended to their microbial relatives. Differences between macro-organisms and microbial eukaryotes must be understood, as suggested by Calkins [1], in terms of cell characteristics, habit and life cycle rather than an artificial and outdated taxonomic split. Current evidence suggests that sex has a single evolutionary origin and was present

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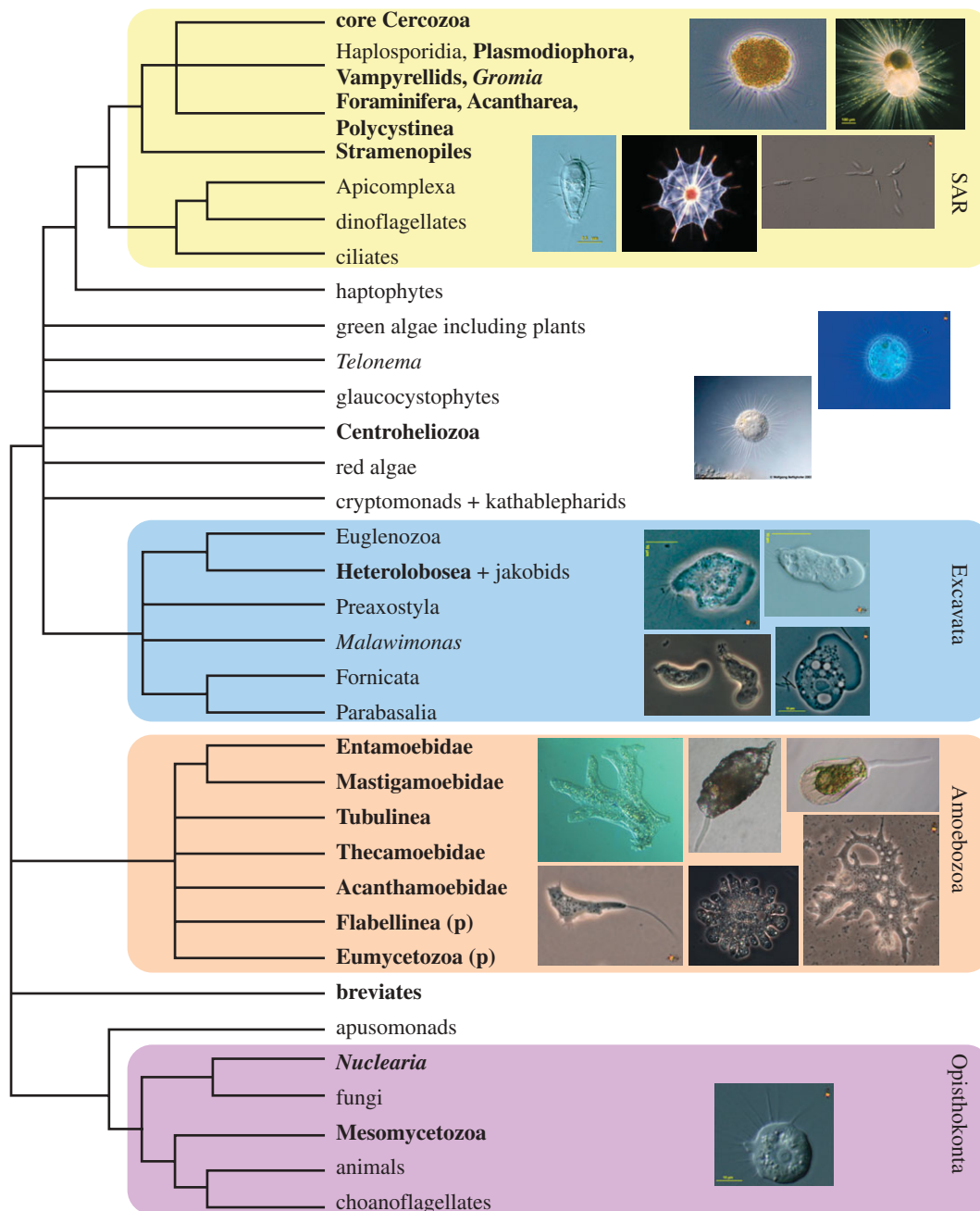
Figure 1. Distribution of amoeboid lineages in the eukaryotic tree of life. This phylogenetic hypothesis of eukaryotic evolution is adapted from Parfrey *et al.* [13], and depicts the well-supported higher-level groupings of eukaryotes. The lineages that have members with amoeboid morphology are in bold. Images depict exemplary amoeboid organisms and were retrieved from Micro*scope (http://starcentral.mbl.edu/microscope/portal.php). Paraphyletic lineages are indicated by (p). SAR indicates the group composed of Stramenopila, Alveolata and Rhizaria.

in the last common ancestor of eukaryotes [15]. Hence, sex is a synapomorphy for extant eukaryotes and, where sex is absent, it must have been secondarily lost. The patchy distribution of sexual and asexual amoeboid lineages in current phylogenetic reconstructions requires many independent losses of sex (figure 1), or may indicate that sex is present but not reported in many lineages. We argue here that the amoeboid lineages are ideal candidates to investigate whether asexuality has been lost many times, because amoebae have generally been assumed to be asexual and are spread across the tree of eukaryotes.

The body of theory developed from macro-organismal observations holds that sexuality should be pervasive and that asexuality should be limited to recent twigs on the tree of eukaryotic life [16]. We define sex as the presence of a meiotic reduction of the genome complement followed eventually by karyogamy (nuclear fusion) in an organism's life cycle. In contrast to amphimixis [17], our definition allows autogamy to be considered sex. Sex is argued to be advantageous because it generates variability by allowing independent assortment of genetic material through recombination [18,19]. Conversely, asexual lineages are argued to be subject to the accumulation of deleterious mutations through a process described as Muller's ratchet [19,20], leading to the prediction that asexual lineages should be short-lived, and hence ancient asexuals will be rare [7,20,21]. On the other hand, sex is not beneficial for the individual in the short term, because only half of

its genetic material is transmitted to the next generation (the cost of meiosis [7]). Recent efforts in modelling the evolution of sex show that incorporating genetic drift is essential to understand the dynamics of populations with finite size: when both drift and selection are taken into account, sex and recombination bring together alleles with higher selection coefficients that tend to be found in different individuals, outcompeting asexual lineages [22]. Thus, there are two main situations where asexuality is expected: (i) in relatively young lineages (such as several species of scale insects with obligate apomictic thelytoky) [23] and (ii) in systems with very large population sizes, which rely on strategies for rapid reproduction (cell/organism replication) [21].

We posit that the purported advantages and disadvantages of sex observed in multicellular macro-organisms should also apply to microbial eukaryotes. However, some caveats must be taken into account when comparing them. Firstly, life cycles are much more varied and complex in microbial eukaryotes [24]. For instance, in most plants and animals, sex and growth are tightly linked (i.e. they cannot complete development without sex) [15]. Conversely, many microbial eukaryotes are only facultatively sexual (i.e. they turn sex on or off depending on environmental conditions).

Knowledge about the natural history of microbial eukaryotes is deeply hindered by the difficulties of observation, when compared with macro-organisms. In most cases, organisms are assumed to be asexual because no sex has been observed; the gold standard for establishing sexuality remains direct observation of sexual phases of the life cycle. Proving that sex occurs in microbial eukaryotes is further hindered as there are often no sexually dimorphic forms, and sexual life-cycle stages may not occur readily in laboratory conditions, or they may be cryptic [25]. Further, many amoebae are not culturable (e.g. polycystine radiolaria [26]). Despite these difficulties, sex has been observed in several microbial and non-microbial taxa long considered asexual when culturing conditions were modified or appropriate mating types were made available, including Darwinulid ostracods [27], arbuscular mycorrhizal fungi [28] and the filamentous mould *Aspergillus* [29], and *Dictyostelium* (see below). Thus, it may not be prudent to rely on the absence of evidence as evidence of the absence of sex [21,25].

Given the long history of study and diversity of methods used, evidence for sex in amoeboid lineages comes in a wide range of forms. We divide the continuum of evidence for sex into three categories: (i) confirmed sexual life cycle, (ii) direct evidence for sex and (iii) indirect evidence that suggests a sexual life cycle but is inconclusive. A confirmed sexual life cycle is the irrefutable combination of both meiosis and karyogamy (nuclear fusion). Direct evidence for sex is provided by microscopic observations of either meiosis or karyogamy without confirmation of the other, or the presence of meiosis-specific genes. We realize that for many biologists documenting meiosis alone is enough to confirm sexuality. However, we feel that observation of both parts of the cycle is necessary given the variation in sexual mechanisms found in microbial eukaryotes. We are defending a more logical stance: if we define a phenomenon by the union of two elements, then we must expect to see the two elements for confirmation of said phenomenon.

Conversely, the confirmation of karyogamy alone may indicate a parasexual system (one where subsequent haploidization occurs by some other means than meiosis [30]; see also the case of *Giardia* [31]) but more strongly indicates the possibility of sex. Finally, many characteristics provide indirect evidence for the hypothesis that an organism is sexual, but fall short of conclusively demonstrating sex. These include molecular evidence of recombination, cytoplasmic fusion, evidence for complex life cycles with more than one trophic stage and production of putative reproductive cells (e.g. swarmer cells that can be interpreted as gametes).

2. AMOEBOID LINEAGES

The broad distribution of amoeboid organisms across the eukaryotic tree of life makes them an ideal system for assessing the applicability of theories on sex to microbial lineages. Amoeboid organisms are defined by the ability to produce pseudopodia for locomotion or feeding. They were historically lumped into a single group, named Sarcodina or Rhizopoda, depending on the classification system [32]. However, recent work demonstrates that amoebae are found in at least 30 distinct lineages (i.e. close to half of all described eukaryotic lineages) that are scattered throughout the tree of eukaryotes [32,33] (figure 1). The majority of these lineages are clustered in the Amoebozoa and Rhizaria [32], with the remaining lineages scattered across the tree (figure 1). The term 'amoeba' is used here descriptively as a morphological category and has no phylogenetic meaning. Here, we re-examine the sexuality of amoebae in the context of the current phylogenetic framework of eukaryotes. We review evidence for sex in lineages traditionally considered asexual, and discuss reports of sexual life cycles that were originally considered exceptions or misinterpretations.

(a) *Amoebozoa*

The Amoebozoa are a higher-level grouping encompassing over 5000 species, currently divided into approximately 14 lineages (figure 2a). These lineages include familiar amoebae, such as the star of high school biology classes *Amoeba proteus* and the human enteric parasite *Entamoeba histolytica*. The majority of organisms shown to belong within Amoebozoa have amoeboid characteristics [32], although these encompass a wide range of morphologies, such as slime moulds, lobose testate amoebae (Arcellinida) and amoeboflagellates. Asexuality in this group is thought to be a defining characteristic [34] or sexuality is assumed to be unknown [35]. However, deep inspection of the literature reveals evidence for sex in several Amoebozoa lineages: the dictyostelid sorocarpic slime moulds and myxogastrid plasmodial slime moulds, Thecamoebida, Arcellinida, Leptomyxida, the genera *Entamoeba*, *Pelomyxa*, *Mastigamoeba*, *Trichosphaerium*, the sorocarpic slime mould *Copromyxa*, and a number of protosteloid amoebae (figure 2a). We will briefly review the evidence for each of these groups.

Confirmed sexual life cycles are described for two lineages: the dictyostelid sorocarpic slime moulds and the myxogastrid plasmodial slime moulds. The dictyostelids illustrate the difficulty of observing sex in the laboratory. Known for their asexual life cycles [36,37],
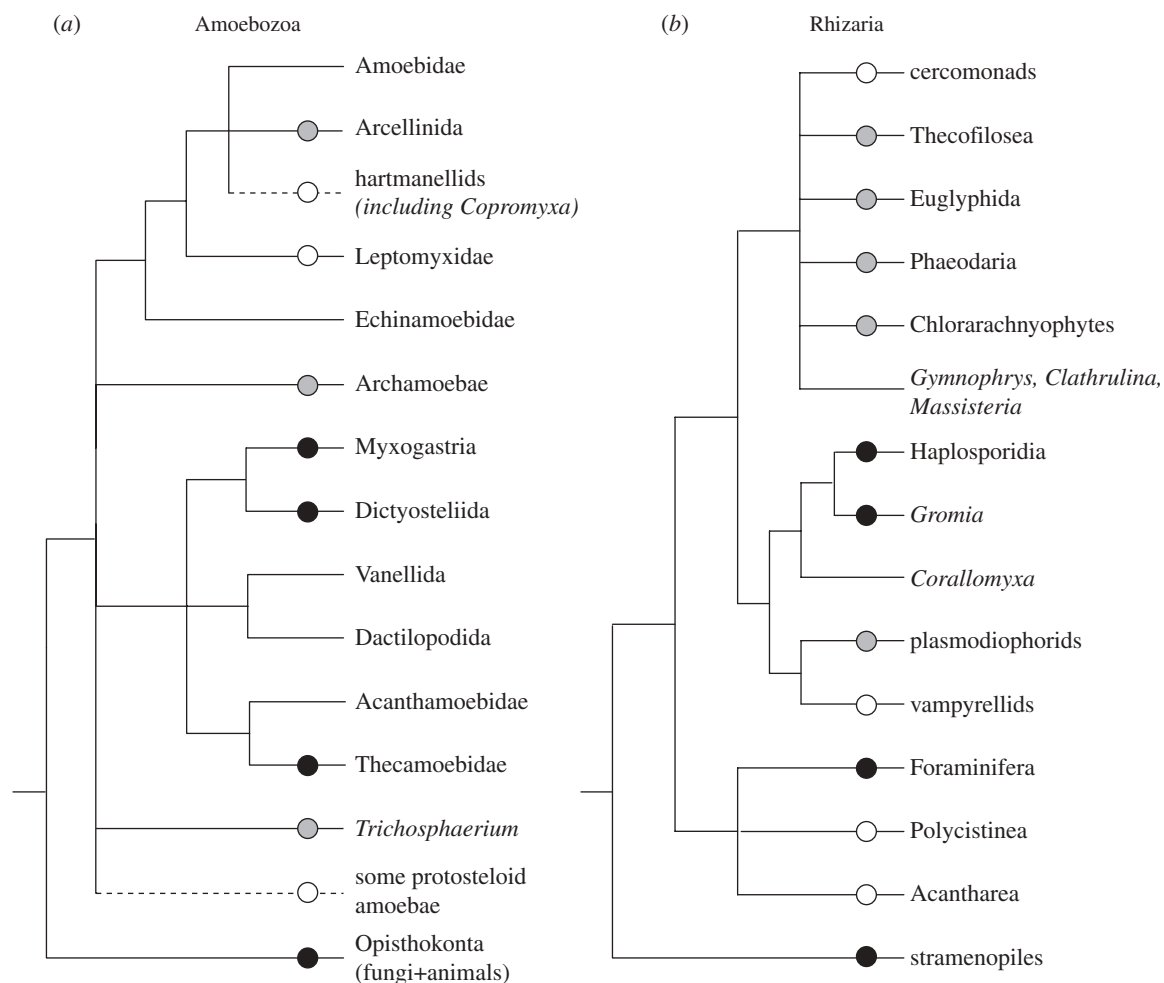
Figure 2. Distribution and types of evidence for sex in the main lineages of the two largest amoeboid groups: (*a*) Amoebozoa and (*b*) Rhizaria. The topology of these illustrative trees is a consensus of well-supported lineages derived from Tekle *et al*. [38], Pawlowski & Burki [32], Shadwick *et al*. [39] and Parfrey *et al*. [13]. Dashed lines represent non-monophyletic taxa. Black circles, confirmed sexual life cycle; grey circles, direct evidence for sex (meiosis, karyogamy or sex genes); white circles, indirect evidence for sex (cytoplasmic fusion, presence of putative gametes).

it was only in the 1970s that appropriate mating types of *Dictyostellium* were brought into culture and the sexual life cycle was fully documented [40–42]. The Myxogastria go through meiosis and fuse to form diploid plasmodia [43,44]. Myxogastria have complex mating systems, with up to 13 mating types (roughly equivalent to sexes) described [45].

Three lineages within Amoebozoa have direct evidence of sexual life cycles: the free-living thecamoebids, the sorocarpic slime mould *Copromyxa* and the testate lobose amoebae (Arcellinida). The thecamoebid *Sappinia diploidea* makes a bicellular cyst where zygote formation is thought to occur [46–48]; similar cysts have been reported in the related *Sappinia pedata* [49]. The slime mould *Copromyxa* has a life cycle that is consistent with sex, although no secondary confirmation of meiosis has been described [50]. *Copromyxa* was initially considered an acrasid sporocarpic slime mould; however, acrasids have been shown to belong to the Heterolobosea, which fall within the Excavata [9], and *Copromyxa* is its own lineage [51]. Molecular studies demonstrate that *Copromyxa* is closely related to the *Hartmanella* within the Tubulinea (figure 2a) [50]. Multiple lines of evidence indicate that the Arcellinida, also members of the Tubulinea, are sexual. *Arcella vulgaris* shows microscopic

evidence of synaptonemal complexes [52], a typical structure that forms only during meiosis [53]. Molecular data from both *Arcella hemispherica* and *A. vulgaris* also demonstrate recombination in the actin gene [54]. *Paraquadrulla* and *Heleopera* go through nuclear division and subsequent fusion [55,56]. Finally, cell fusion (which we consider indirect evidence for sex; see below) has been reported for many genera of Arcellinida, though it is unclear whether karyogamy also occurs when cells fuse, or whether gamete formation occurs at other time points (reviewed in [48]). The most complete report of karyogamy following cytoplasmic fusion is for *Difflugia lobostoma* [57], though Rhumbler [58] did not observe fusion during long-term culturing of this species. This apparent contradiction may indicate that these were different strains, a probable situation given the prevalence of cryptic species and other uncertainty in the taxonomy of Arcellinida [59,60]. Different life-cycle observations can also result from different culturing conditions.

Finally, three taxa have direct, but controversial, evidence for sex: cell fusion reports in the free-living naked amoebae Leptomyxida, a complement of meiotic gene in the human pathogen *E. histolytica* and life cycles consistent with sex in *Trichosphaerium* and others. Cell fusion is widely reported for Amoebozoa [61,62].

Among the leptomyxids, *Leptomyxa reticulata* [61], *Flabellula baltica* [63] and multiple strains of flabellulids [64] are observed to fuse. Subsequently, the cells separate or persist as multinucleate stages. It is unclear whether this fusion facilitates genetic exchange or serves another purpose [35]; hence, we consider this as only supporting evidence for sex.

Entamoeba histolytica has long been considered asexual despite numerous pieces of evidence pointing to the contrary, such as the appearance of putative heterozygote populations after mixing of homozygotic populations for certain isozyme classes [65,66]. The availability of the whole genome [67] shows that *E. histolytica* has the full complement of genes required for meiosis [68,69], which should have decayed if the species had abandoned a sexual life cycle. The enigmatic genus of marine amoebae *Trichosphaerium* is reported to have an alternation of generations with gamont (sexual, including karyogamy) and schizont (asexual) stages [70]. Since meiosis has not been properly documented [71,72], we consider there is only direct evidence for sex in *Trichosphaerium*. Complex life cycles with multiple types of trophic cells that are consistent with sex have been described from a number of lineages: the polyphyletic protosteloid amoebae *Clastostelium recurvatum*, *Protosporangium* spp., *Cavostelium apophysatum*, *Ceratiomyxa fruticulosa* and *Ceratiomyxella tahitiensis* [39], and the archamoebid *Pelomyxa palustris* [73].

(b) *Rhizaria*

The Rhizaria are a heterogeneous assemblage encompassing lineages such as Foraminifera, radiolarians and euglyphid testate amoebae, chlorarachniophytes, parasitic groups (Phytomyxea, Haplosporidia), as well as a multitude of other lesser-known flagellates (figure 2b) that emerge as having fundamental ecological roles [13,74–76]. Filamentous pseudopodia are a recurrent morphological feature among amoeboid members of Rhizaria, in contrast to the lobose or broad pseudopodia of many Amoebozoa. Complete sexual life cycles are documented for two lineages (Foraminifera and *Gromia*); karyogamy or meiosis (direct evidence) has been observed in five lineages (Euglyphida, Thecofilosea, Chlorarachniophyta, Plasmodiophorida and Phaeodarea); and indirect evidence (such as cell fusion or formation of putative gametes) has been witnessed in five lineages (Acantharea, Polycystinea, *Cercomonas*, *Helkesimastix* and *Lateromyxa*).

There are at least two lineages in the Rhizaria with confirmed sexual life cycles. Foraminifera are marine amoebae defined by a dynamic network of anastomosing pseudopodia [77], and well known for producing intricate shells. They exhibit complex sexual life cycles, with meiosis and gamete production occurring at separate stages [78]. The Gromiidae also have confirmed sexual life cycles [79]. These large protists (up to several centimetres) have been observed in shallow and deep-sea sediments [80], where they are capable of denitrification in anoxic environments [81]. *Gromia* was originally classified as a genus of Foraminifera, based on gross morphology, but lacks the distinctive anastomosing pseudopods of Foraminifera and branches separately in molecular phylogenies [76]. The life cycle of *Gromia* resembles that of Foraminifera, with meiosis and gamete fusion occurring at different stages.

The Euglyphid testate amoebae and the Thecofilosa have many reports of cytoplasmic fusion, which we consider indirect evidence, and also reports of karyogamy, a form of direct evidence. Euglyphid testate amoebae have primarily been studied from a faunistic perspective, as bioindicators of past and present environmental conditions [82,83], and recently from a molecular phylogenetic perspective [84–86]. In the family Euglyphidae, *Euglypha alveolata* [87], *Euglypha scutigera* [88] and *Euglypha* sp. [89] combine their cellular contents to form a cyst, or in one case a third larger shell (*E. alveolata* [90]). Similar processes have been observed in other closely related families: Assulinidae [89], Trinematidae [88,91] and Cyphoderiidae [58,91], and in the unclassified *Tracheleuglypha dentata* [92]. The formation of a third, larger cell has been reported only in Assulinidae and Euglyphidae [93,94], and not in Trinematidae and Cyphoderiidae, where cell fusion occurs within one of the copulating cells.

In some Euglyphids, cytoplasmic fusion is followed by karyogamy, providing direct evidence for sex. In *Trinema lineare*, *Valkanovia delicatula* [95], *Assulina muscorum* and *Valkanovia elegans* [94], karyogamy was documented but the ultimate fate of the synkaryon (fused nuclei) remains unknown. In *Corythion delamarei* (family Trinematidae), the synkaryon divides into four nuclei, interpreted as the result of meiosis [96]. The cytoplasm is then distributed around the four nuclei, and four naked daughter cells leave the mother shell, which is left empty. These naked cells eventually secrete a test. If the interpretation is correct and *C. delamarei* indeed goes through meiosis after cytoplasmic and karyogamy, these organisms spend most of their life cycle in a haploid stage, being diploid only when karyogamy occurs. In contrast, *T. lineare* (Trinematidae) performs 'conventional' binary divisions in addition to a sexual life cycle similar to *C. delamarei* [97]. Binary divisions were not observed in *C. delamarei*, or its sister species *Corythion dubium* [96]. This suggests that *Corythion* is a genus of obligate sexual organisms. In sum, there is direct evidence for sex in four families out of the five that compose Euglyphida.

The other lineage of filose testate amoebae, Thecofilosea (*sensu* [98]), presents direct evidence for sex. Recent phylogenetic analyses show they are not sister to the Euglyphida [13,76]. These amoebae may have proteinaceous or agglutinated tests and are often overlooked in environmental samples owing to their small size. Cytoplasmic fusion followed by karyogamy has been observed in both *Pseudodifflugia gracilis* and *P. fascicularis*. The fate of the synkaryon is unknown [95].

Chlorarachniophytes, a group known for their ancient secondary endosymbiosis [99], go through an elaborate alternation of flagellate and amoeboid life-cycle stages, and show indirect evidence for sex. In *Chlorarachnion reptans*, flagellate cells fuse with coccoid cells; these are interpreted as 'male' and 'female' gametes [100]. In *Cryptochlora perforans*, two morphologically identical amoeboid cells fuse and produce a cyst where meiosis is thought to occur in a manner similar to euglyphids. The DNA content of the cyst is double that of the amoeboid stages, suggesting karyogamy [101]. As meiosis has not been confirmed, we consider this direct evidence of a sexual life cycle as opposed to confirmation. The Plasmodiophorida are obligate intracellular parasites of plants,

characterized by a specific type of mitotic division named cruciform nuclear division [102]. They have a complex life cycle with a plasmodial amoeboid phase, and meiosis has been confirmed in the group. However, karyogamy has not yet been observed [102].

The organisms collectively designated 'Radiolaria'—a non-monophyletic assemblage containing Phaeodarea, Acantharea and Polycystinea—are large pelagic cells ubiquitous in the oceans. These organisms are extremely difficult to maintain in laboratory conditions, and their full life cycle has never been documented, but observations reveal evidence that suggests sex. All three groups of radiolarians generally produce small biflagellated cells, whose fate remains unclear [26,103], but may be gametes that are released into the water column.

The strongest evidence for sex within the 'Radiolaria' is found in Phaeodarea; specifically, in the well-studied species *Aulacantha scolymantha*, which falls in the Cercozoa [76]. Synaptonemal complexes have been documented between the numerous (1000+) composite chromosomes. Each of these composite chromosomes subsequently segregates into developing biflagellated swarmer cells [104] and divides into eight chromosomes. However, complete evidence for sex is still lacking for this group, as cellular fusion and karyogamy have not been documented. Production of small biflagellated swarmer cells has also been observed in Polycystinea and Acantharea, which are closely related to Foraminifera [13]. These have been interpreted as 'isogametes' in the case of Acantharea [105], but cell fusion has not been observed for either lineage [106].

Evidence for sex becomes scarcer as organisms get smaller and more difficult to observe. For the small amoeboflagellate forms, there are reports of cell fusions with subsequent encystment in *Helkesimastix faecicola* [107] and *Cercomonas longicauda* [108]. In *Cercomonas*, cells can aggregate and fuse in some species, thus forming plasmodia containing up to 100 nuclei [109,110]. Such plasmodia have also been documented in the vampyrellid *Lateromyxa gallica* [111,112], though the fate of these nuclei is unknown.

(c) Other amoeboid lineages: Heliozoa, Heterolobosea, Stramenopila and Opisthokonta

There are other amoeboid lineages scattered in the tree of eukaryotes, most with limited information on sex. The 'Heliozoa' have been split into four morphological lineages [33], three of which have been confirmed in molecular reconstructions [113]. One lineage—the Actinophryida nested within the Stramenopila—is reported to go through autogamy in the cyst [114]. The life cycles of all three remaining 'heliozoan' lineages (the Desmothoracida, Centrohelida and Gymnosphaerida) remain poorly documented.

The Heterolobosea are a lineage of amoeboflagellates nested within the Excavata [115]. *Heteramoeba clara* is reported to have a sexual life cycle consisting of a two-mating-type system [116], although there is a certain amount of doubt to these experiments. The genome of *Naegleria gruberi* was recently sequenced, and reveals the presence of meiosis-specific genes, supporting the presence of sex in this clade [117]. The acrasid cellular slime moulds have been shown to fall within the Heterolobosea rather than with other sorocarpic slime moulds in Amoebozoa [9]. Complete life cycles have been documented for acrasids, but these contain no evidence for meiosis or karyogamy. Hence, we consider there is no evidence pointing to sex in this group.

The Labyrinthulidae and Thraustochytriidae are amoeboid organisms currently placed within the Stramenopila (or Heterokonta), which also includes the diatoms, brown algae and water moulds, in which sex is well established. A complete sexual cycle is described for both of these amoeboid lineages, with well-documented meiosis [118,119].

A number of orphan amoeboid lineages have recently been placed amid the Opisthokonta (which also includes the Fungi and Metazoa). *Amoebidium parasiticum*, originally thought to be a fungus, has a multi-stage life cycle, but no sex has been reported [120]. Similarly, the nucleariid amoebae and *Fonticula alba* have shown no evidence of sex [50]. However, only a limited number of studies have focused on these taxa.

3. CONCLUSION

Evolutionary theory predicts that long-lived lineages should be sexual [7], and that asexual lineages derived from sexual ancestors will be short-lived owing to the negative effects of Muller's ratchet on the genome [121,122]. The two major clades that are dominated by amoebae, the Rhizaria and Amoebozoa (figure 2), are certainly very ancient. Fossil Arcellinida, a clade of testate amoebae within the Amoebozoa, has been found in 750 Myr old rocks [123]; Foraminifera and Polycystinea, two clades within Rhizaria, have fossil records that extend back at least to the Cambrian (i.e. 488–542 Myr ago) [26,124]. Sex is a complex character and it is unlikely to have evolved independently in multiple lineages, or lost and regained multiple times [25]. Thus, the presence of sexual lineages scattered across Amoebozoa and Rhizaria suggests that these clades were ancestrally sexual. As in other branches of the eukaryotic tree, sex may then have been lost independently in derived lineages.

Some amoeboid lineages may be genuinely asexual. One candidate for asexuality is *A. proteus*, which is the textbook example of binary fission in eukaryotes. A multitude of research groups have been culturing *A. proteus* and its relatives for more than a century without uncovering evidence supporting the existence of sex in this group. Yet assuming asexuality may be precarious given the uncertainties regarding culturing conditions. Although the ultimate proof for sex, as defined here, is the observation of meiosis and subsequent karyogamy, genomic data from populations of *A. proteus* could reveal evidence of recombination. Such data are yet lacking for these and the majority of amoeboid protists.

The logical equation 'lack of evidence = asexual' is precarious, but the opposite stance is perhaps equally dangerous. Assuming that all lineages in Amoebozoa are sexual may mean discarding the possibility that alternative means to deal with Muller's ratchet have arisen independently. Microbial eukaryote lineages may well have different strategies, such as lateral gene transfer (LGT) and cyclic polyploidy. Bdelloid rotifers, a clade of asexual microscopic animals, provide the most famous example of an alternative mechanism to avoid

the ratchet: during rehydration following anhydrobiosis (a suspended animation state that allows the organism to survive dehydration), these organisms acquire foreign DNA and reorganize genomic regions [125]. This may well be a remarkable example of an evolutionary approach to reap the benefits of recombination, and could represent one of many strategies that eukaryotes have explored to avoid the deleterious effects of Muller's ratchet. If such an unusual mechanism appeared in Metazoa, comparably non-canonical mechanisms may have evolved among 30+ amoeboid lineages.

Cyclic polyploidy may be another evasion method for avoiding the impact of Muller's ratchet. Ploidy cycles may reduce the mutational load usually associated with high ploidy, and maintain the selective advantages of haploid genetic transmission [17]. Many microbial eukaryotes (amoeboid and others) experiment with ploidy changes that go far beyond the metazoan $n-2n$ (haploid–diploid) fluctuation [24]. For instance, *A. proteus* shows up to $3n$ variation during interphase, suggesting a cycle of polyploidization and return to haploidy before mitosis; and *E. histolytica* shows heterogeneity in nuclear ploidy owing to varying levels of endomitosis—within a population, individual trophozoites exhibit continuous variation from $4n$ to $40n$ [126]. The consequences of these phenomena are still poorly understood, as implications about the dynamics of eukaryotic genomes are only beginning to be explored [24].

An open question is whether LGT through endosymbiotic organisms may supply genetic variability to populations of amoebae. Diverse amoebae (e.g. *Acanthamoeba* spp., *Hartmannella* spp., *Arcella* spp., *Amoeba* spp.) harbour a wide variety of bacterial endosymbionts and viruses during their life cycle [127–130]. The possibility of genetic recombination between amoebae and their multiple cytoplasmic inhabitants has just begun to be studied, as is the case of the giant amoeba-infecting Marseillevirus and Mimivirus that show evidence of chimeric genomes, with fragments of DNA acquired from multiple sources [131,132].

Well-resolved phylogenetic trees provide a framework to investigate possible sexuality and identify truly asexual lineages. *Amoeba proteus* is a member of the Amoebidae clade, for which no evidence for sex has been uncovered. The closely related Arcellinida are probably sexual. Hence, the Amoebidae make an ideal group for deeply searching for signs of sex/asexuality. Documentation of the complete life cycle is difficult, but suitable alternative methods to identify the presence of sex include intense culturing and/or surveying of natural populations to document recombination (as predicted by meiosis) and genetic studies to identify a set of meiosis genes. In this case, there are three possible outcomes: (i) the Amoebidae are indeed sexual and we failed to document sex so far; (ii) the Arcellinida–Amoebidae ancestral was sexual and the Amoebidae became truly asexual independently; or (iii) the Amoebidae use a distinct strategy for evading Muller's ratchet, which might involve extensive LGT and/or ploidy cycles.

We conclude that the generalization of widespread asexuality in amoeboid organisms is superficial and a product of two main forces: (i) an intrinsic practical difficulty in studying microbial organisms, and (ii) the long-held belief that amoeboid organisms are a single unit of evolution, as opposed to a morphological strategy that was adopted by a wide variety of independent lineages. Amoebae are not fundamentally chaste. The timing and flow of events that lead each independent lineage to adopt an asexual or sexual life cycle must be evaluated separately. A multiple-evidence approach, using a phylogenetic framework, gathering evidence on life cycles, genetic information on recombination and/or suits of meiotic genes, will be more efficient in reconstructing the history of eukaryotic sexual life cycles. In line with Calkin's reasoning almost a century ago about the chemical constitution of amoebae [1], there is no reason to doubt that the rules of evolution governing sex in amoeboid organisms agree with those of other living beings. We predict that thorough and careful study of amoeboid organisms will reveal even more unusual ways of performing sex or otherwise exchanging genetic information. When discussing the sex of amoeboid protists, the existing evidence does not evoke chastity but rather the Kama Sutra.

REFERENCES

1 Calkins, G. N. 1916 General biology of the protozoan life cycle. *Am. Nat.* **50**, 257–270. (doi:10.1086/279539)

2 Haeckel, E. H. P. A. 1866 *Generelle morphologie der organismen. Allgemeine grundzüge der organischen formen-wissenschaft, mechanisch begründet durch die von charles darwin reformirte descendenztheorie.* Berlin, Germany: G. Reimer.

3 Margulis, L. & Schwartz, K. 1988 *Five Kingdoms: an illustrated guide to the phyla of life on earth*, 2nd edn. New York, NY, W. H.: Freeman and Company.

4 Whittaker, R. H. 1969 New concepts of Kingdoms of organisms. *Science* **163**, 150–160. (doi:10.1126/science.163.3863.150)

5 Corliss, J. O. 1984 The Kingdom Protista and its 45 phyla. *Biosystems* **17**, 87–126. (doi:10.1016/0303-2647(84)90003-0)

6 Mayr, E. 1964 *Systematics and the origin of species: from the viewpoint of a zoologist (with a new introduction by the author)*. New York, NY: Dover Publications, Inc.

7 Maynard Smith, J. 1978 *The evolution of sex*. Cambridge, UK: Cambridge University Press.

8 Bell, G. 1988 *Sex and death in protozoa: the history of an obsession*. Cambridge, UK: Cambridge University Press.

9 Adl, S. M. *et al.* 2005 The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* **52**, 399–451. (doi:10.1111/j.1550-7408.2005.00053.x)

10 Baldauf, S. L. 2003 The deep roots of eukaryotes. *Science* **300**, 1703–1706. (doi:10.1126/science.1085544)

11 Cavalier-Smith, T. 1998 A revised six-kingdom system of life. *Biol. Rev. Camb. Phil. Soc.* **73**, 203–266. (doi:10.1017/S0006323198005167)

12 Keeling, P. J., Burger, G., Durnford, D. G., Lang, B. F., Lee, R. W., Pearlman, R. E., Roger, A. J. & Gray, M. W. 2005 The tree of eukaryotes. *Trends Ecol. Evol.* **20**, 670–676. (doi:10.1016/j.tree.2005.09.005)

13 Parfrey, L. W., Grant, J., Tekle, Y. I., Lasek-Nesselquist, E., Morrison, H. G., Sogin, M. L., Patterson, D. J. & Katz, L. A. 2010 Broadly sampled multigene analyses yield a well-resolved eukaryotic tree of life. *Syst. Biol.* **59**, 518–533. (doi:10.1093/sysbio/syq037)

14 Bonner, J. T. 1998 The origins of multicellularity. *Integr. Biol. Issues News Rev.* **1**, 27–36. (doi:10.1002/(SICI)1520-6602(1998)1:1<27::AID-INBI4>3.0.CO;2-6)

15 Dacks, J. & Roger, A. J. 1999 The first sexual lineage and the relevance of facultative sex. *J. Mol. Evol.* **48**, 779–783. (doi:10.1007/PL00013156)

16 Schwander, T. & Crespi, B. J. 2009 Twigs on the tree of life? Neutral and selective models for integrating macroevolutionary patterns and microevolutionary processes in the analysis of asexuality. *Mol. Ecol.* **18**, 28–42. (doi:10.1111/j.1365-294X.2008.03992.x)

17 Kondrashov, A. S. 1997 Evolutionary genetics of life cycles. *Annu. Rev. Ecol. Syst.* [*Review*] **28**, 391–435.

18 Weismann, A. 1889 *Essays on heredity and kindred biological subjects.* Oxford, UK: Oxford University Press.

19 Muller, H. J. 1932 Some genetic aspects of sex. *Am. Nat.* **66**, 118–138. (doi:10.1086/280418)

20 Muller, H. J. 1964 The relation of recombination to mutational advance. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* **1**, 2–9. (doi:10.1016/0027-5107(64)90047-8)

21 Judson, O. P. & Normark, B. B. 1996 Ancient asexual scandals. *Trends Ecol. Evol.* **11**, A41–A46. (doi:10.1016/0169-5347(96)81040-8)

22 Otto, S. P. 2009 The evolutionary enigma of sex. *Am. Nat.* **174**(Suppl. 1), S1–S14. (doi:10.1086/599084)

23 Ross, L., Pen, I. & Shuker, D. M. 2010 Genomic conflict in scale insects: the causes and consequences of bizarre genetic systems. *Biol. Rev. Camb. Phil. Soc.* **85**, 807–828. (doi:10.1111/j.1469-185x.2010.00127.x)

24 Parfrey, L. W., Lahr, D. J. G. & Katz, L. A. 2008 The dynamic nature of eukaryotic genomes. *Mol. Biol. Evol.* **25**, 787–794. (doi:10.1093/molbev/msn032)

25 Dunthorn, M. & Katz, L. A. 2010 Secretive ciliates and putative asexuality in microbial eukaryotes. *Trends Microbiol.* **18**, 183–188. (doi:10.1016/j.tim.2010.02.005)

26 Anderson, O. R. 1981 *Radiolaria.* Berlin, Germany: Springer Verlag.

27 Smith, R. J., Kamiya, T. & Horne, D. J. 2006 Living males of the 'ancient asexual' Darwinulidae (Ostracoda: Crustacea). *Proc. R. Soc. Lond. B* **273**, 1569–1578. (doi:10.1098/rspb.2005.3452)

28 Croll, D. & Sanders, I. R. 2009 Recombination in *Glomus intraradices*, a supposed ancient asexual arbuscular mycorrhizal fungus. *BMC Evol. Biol.* **9**, 13.

29 O'Gorman, C. M., Fuller, H. T. & Dyer, P. S. 2009 Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457**, 471–474. (doi:10.1038/nature07528)

30 Pontecorvo, G. 1956 The parasexual cycle in Fungi. *Annu. Rev. Microbiol.* **10**, 393–400. (doi:10.1146/annurev.mi.10.100156.002141)

31 Birky, C. W. 2010 *Giardia* sex? Yes, but how and how much? *Trends Parasitol.* **26**, 70–74. (doi:10.1016/j.pt.2009.11.007)

32 Pawlowski, J. & Burki, F. 2009 Untangling the phylogeny of amoeboid protists. *J. Eukaryot. Microbiol.* **56**, 16–25.

33 Patterson, D. J. 1999 The diversity of eukaryotes. *Am. Nat.* [*Review*]. **154**, S96–S124.

34 Hurst, L. D., Hamilton, W. D. & Ladle, R. J. 1992 Covert sex. *Trends Ecol. Evol.* **7**, 144–145.

35 Cavalier-Smith, T. 2002 The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. *Int. J. Syst. Evol. Microbiol.* **52**, 297–354.

36 Bonner, J. T. 1944 A descriptive study of the development of the slime mold *Dictyostelium discoideum*. *Am. J. Bot.* **31**, 175–182.

37 Bonner, J. T. 1947 Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.* **106**, 1–26.

38 Tekle, Y. T., Grant, J., Anderson, O. R., Nerad, T. A., Cole, C. C., Patterson, D. J. & Katz, L. A. 2008 Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. *Mol. Phyl. Evol.* **47**, 339–352.

39 Shadwick, L. L., Spiegel, F. W., Shadwick, J. D. L., Brown, M. W. & Silberman, J. D. 2009 Eumycetozoa = Amoebozoa? SSU-rDNA phylogeny of protosteloid slime molds and its significance for the amoebozoan supergroup. *PLoS ONE* **4**, e6754.

40 Erdos, G. W., Raper, K. B. & Vogen, L. K. 1973 Mating types and macrocyst formation in *Dictyostelium discoideum*. *Proc. Natl Acad. Sci. USA* **70**, 1828–1830.

41 Erdos, G. W., Raper, K. B. & Vogen, L. K. 1975 Sexuality in cellular slime mold *Dictyostelium giganteum*. *Proc. Natl Acad. Sci. USA* **72**, 970–973.

42 Macinnes, M. A. & Francis, D. 1974 Meiosis in *Dictyostelium mucoroides*. *Nature* **251**, 321–324. (doi:10.1038/251321a0)

43 Fiore-Donno, A. M., Berney, C., Pawlowski, J. & Baldauf, S. A. 2005 Higher-order phylogeny of plasmodial slime molds (Myxogastria) based on elongation factor 1-a and small subunit rRNA gene sequences. *J. Euk. Microbiol.* **52**, 201–210.

44 Martin, G. W. & Alexopoulos, C. J. 1969 *The myxomycetes.* Iowa City, IA: University of Iowa Press.

45 Collins, O. R. & Tang, H. C. 1977 New mating types in *Physarum polycephalum*. *Mycologia* **69**, 421–423.

46 Michel, R., Wylezich, C., Hauroder, B. & Smirnov, A. 2006 Phylogenetic position and notes on the ultrastructure of *Sappinia diploidea* (Thecamoebidae). *Protistology* **4**, 319–325.

47 Goodfellow, L. P., Belcher, J. H. & Page, F. C. 1974 A light- and electron-microscopical study of *Sappinia diploidea*, a sexual amoeba. *Protistologica* **2**, 207–216.

48 Wenrich, D. H. 1954 Sex in Protozoa: a comparative review. In *Sex in microorganisms: a symposium* (ed. D. H. Wenrich), p. 362. Washington, DC: AAAS.

49 Brown, M. W., Spiegel, F. W. & Silberman, J. D. 2007 Amoeba at attention: phylogenetic affinity of *Sappinia pedata*. *J. Eukaryot. Microbiol.* **54**, 511–519.

50 Brown, M. W., Spiegel, F. W. & Silberman, J. D. 2009 Phylogeny of the 'forgotten' cellular slime mold, *Fonticula alba*, reveals a key evolutionary branch within Opisthokonta. *Mol. Biol. Evol.* **26**, 2699–2709.

51 Spiegel, F. W. & Olive, L. S. 1978 New evidence for validity of *Copromyxa protea*. *Mycologia* **70**, 843–847.

52 Mignot, J.-P. & Raikov, I. B. 1992 Evidence for meiosis in the testate amoeba *Arcella*. *J. Eurkaryot. Microbiol.* **39**, 287–289.

53 Moses, M. J. 1969 Structure and function of the synaptonemal complex. *Genetics* **61**(Suppl. 1), 41–51.

54 Lahr, D. J., Nguyen, T. B., Barbero, E. & Katz, L. A. 2011 Evolution of the actin gene family in testate lobose amoebae (Arcellinida) is characterized by 2 distinct clades of paralogs and recent independent expansions. *Mol. Biol. Evol.* **28**, 223–236. (doi:10.1093/molbev/msq200)

55 Lüftenegger, G. & Foissner, W. 1991 Morphology and biometry of twelve soil testate amoebae (Protozoa,

Rhizopoda) from Australia, Africa, and Austria. *Bull. Br. Mus. Nat. Hist.* (*Zoology*) **57**, 1–16.

56 Meisterfeld, R. 2002 Order Arcellinida Kent, 1880. In *The illustrated guide to the Protozoa* (eds J. J. Lee, G. F. Leedale & P. Bradbury), pp. 827–860, 2nd edn. Lawrence, KS: Allen Press.

57 Dangeard, L. 1937 Memoirs sur le *Difflugia globulosa Dujardin*. *Le Botaniste* **28**, 229–274.

58 Rhumbler, H. 1898 Zelleib-, schalen-, und kern-verschmelzungen bei den rhizopoden und deren wahrscheinliche beziehungen zu phylogenetischen vorstufen der metazoenbefruchtung. 2. *Biologisches Centralblatt* **18**, 33–38.

59 Heger, T. J., Mitchell, E. A. D., Ledeganck, P., Vincke, S., Van de Vijver, B. & Beyens, L. 2009 The curse of taxonomic uncertainty in biogeographical studies of free-living terrestrial protists: a case study of testate amoebae from Amsterdam island. *J. Biogeogr.* **36**, 1551–1560.

60 Lahr, D. J. G. & Lopes, S. 2009 Evaluating the taxonomic identity in four species of the lobose testate amoebae genus *Arcella* Ehrenberg, 1832. *Acta Protozoolog.* **48**, 127–142.

61 Seravin, L. N. & Goodkov, A. V. 1984 Possible forms of agamic genetic interactions in protists and ways of establishment of the sexual process. *Tsitologiya* **26**, 1224–1236.

62 Seravin, L. N. & Goodkov, A. V. 1984 The main types and forms of agamic cell fusion in Protozoa. *Tsitologiya* **26**, 123–131.

63 Smirnov, A. V. & Goodkov, A. V. 1999 An illustrated list of basic morphotypes of Gymnamoebia (Rhizopoda, Lobosea). *Protistology* **1**, 20–29.

64 Dykova, I., Fiala, I., Peckova, H. & Dvorakova, H. 2008 Phylogeny of Flabellulidae (Amoebozoa: Leptomyxida) inferred from SSU rDNA sequences of the type strain of *Flabellula citata* Schaeffer, 1926 and newly isolated strains of marine amoebae. *Folia Parasitol.* **55**, 256–264.

65 Sargeaunt, P. G., Jackson, T. F., Wiffen, S. R. & Bhojnani, R. 1988 Biological evidence of genetic exchange in *Entamoeba histolytica*. *Trans. R. Soc. Trop. Med. Hyg.* **82**, 862–867.

66 Blanc, D., Nicholls, R. & Sargeaunt, P. G. 1989 Experimental production of new zymodemes of *Entamoeba histolytica* supports the hypothesis of genetic exchange. *Trans. R. Soc. Trop. Med. Hyg.* **83**, 787–790.

67 Loftus, B. *et al.* 2005 The genome of the protist parasite *Entamoeba histolytica*. *Nature* **433**, 865–868.

68 Ramesh, M. A., Malik, S.-B. & Longsdon, J. M. 2005 A phylogenomic inventory of meiotic genes: evidence for sex in *Giardia* and an early eukaryotic origin of meiosis. *Curr. Biol.* **15**, 185–191.

69 Stanley, J. S. L. 2005 The *Entamoeba histolytica* genome: something old, something new, something borrowed and sex too? *Trends Parasitol.* **21**, 451.

70 Angell, R. W. 1976 Observations on *Trichosphaerium platyxyrum* sp. n. *J. Protozool.* **23**, 357–364.

71 Schuster, F. L. 1976 Fine structure of the schizont stage of the testate marine amoeba, *Trichosphaerium* sp. *J. Euk. Microbiol.* **23**, 86–93.

72 Schaudinn, F. R. 1899 Untersuchungen uber den generationswechsel von *Trichosphaerium sieboldi*. *Sch. Abh. Konigl. Preuss. Akd. Wiss. Berlin* **1**(Suppl.), 1–93.

73 Whatley, J. & Chapman-Andresen, C. 1990 Phylum Karyoblastea. In *Handbook of Protoctista* (eds L. Margulis, J. O. Corliss, M. Melkonian & D. Chapman), pp. 167–185. Boston, MA: Jones and Bartlett Publishers.

74 Ekelund, F. & Patterson, D. J. 1997 Some heterotrophic flagellates from a cultivated soil in Australia. *Arch. Protistenk.* **148**, 461–478.

75 Foissner, W. 1991 Diversity and ecology of soil flagellates. In *The biology of free-living heterotrophic flagellates*

(eds D. J. Patterson & J. Larsen), pp. 93–112. Oxford, UK: Clarendon Press.

76 Bass, D., Chao, E. E. Y., Nikolaev, S., Yabuki, A., Ishida, K. I., Berney, C., Pakzad, U., Wylezich, C. & Cavalier-Smith, T. 2009 Phylogeny of novel naked filose and reticulose Cercozoa: Granofilosea cl. n. and Proteomyxidea revised. *Protist* **160**, 75–109.

77 Bowser, S. S. & Travis, J. L. 2002 Reticulopodia: structural and behavioral basis for the suprageneric placement of granuloreticulosan protists. *J. Foraminiferal Res.* **32**, 440–447.

78 Goldstein, S. T. 1999 Foraminifera: a biological overview. In *Modern foraminifera* (ed. B. K. Sen Gupta), pp. 37–56. Dordrecht, The Netherlands: Kluwer.

79 Arnold, Z. M. 1972 Observations on the biology of the protozoan *Gromia oviformis* Dujardin. Berkley, CA: University of California Press.

80 Matz, M. V., Frank, T. M., Marshall, N. J., Widder, E. A. & Johnsen, S. 2008 Giant deep-sea protist produces bilaterian-like traces. *Curr. Biol.* **18**, 1849–1854. (doi:10.1016/j.cub.2008.10.028)

81 Pina-Ochoa, E. *et al.* 2010 Widespread occurrence of nitrate storage and denitrification among Foraminifera and Gromiida. *Proc. Natl Acad. Sci. USA* **107**, 1148–1153. (doi:10.1073/pnas.0908440107)

82 Tolonen, K., Warner, B. G. & Vasander, H. 1992 Ecology of testaceans (Protozoa, Rhizopoda) in mires in southern Finland. I. Autecology. *Arch. Protistenk.* **142**, 119–138.

83 Mitchell, E. A. D., Charman, D. J. & Warner, B. G. 2008 Testate amoebae analysis in ecological and paleo-ecological studies of wetlands: past, present and future. *Biodivers. Conserv.* **17**, 2115–2137. (doi:10.1007/s10531-007-9221-3)

84 Wylezich, C., Meisterfeld, R., Meisterfeld, S. & Schlegel, M. 2002 Phylogenetic analyses of small subunit ribosomal RNA coding regions reveal a monophyletic lineage of euglyphid testate amoebae (Order Euglyphida). *J. Eukaryot. Microbiol.* **49**, 108–118. (doi:10.1111/j.1550-7408.2002.tb00352.x)

85 Lara, E., Heger, T. J., Mitchell, E. A. D., Meisterfeld, R. & Ekelund, F. 2007 SSU rRNA reveals a sequential increase in shell complexity among the euglyphid testate amoebae (Rhizaria: Euglyphida). *Protist* **158**, 229–237. (doi:10.1016/j.protis.2006.11.006)

86 Heger, T. J., Mitchell, E. A. D., Todorov, M., Golemansky, V., Lara, E., Leander, B. S. & Pawlowski, J. 2010 Molecular phylogeny of euglyphid testate amoebae (Cercozoa: Euglyphida) suggests transitions between marine supralittoral and freshwater/terrestrial environments are infrequent. *Mol. Phylogenet. Evol.* **55**, 113–122. (doi:10.1016/j.ympev.2009.11.023)

87 Reukauf, E. 1912 Zur encystierung von *Euglypha alveolata*. *Zoolog. Anzeiger* **39**, 372–375.

88 Penard, E. 1902 *Faune rhizopodique du bassin du léman*. Geneva, Switzerland: Henry Kündig.

89 Awerintzew, S. 1906 Die süsswasser Rhizopoden. I and II. *Trudui S.-Peter. Oscch* **36**, 351.

90 Blochmann, F. 1887 Zur kenntniss der fortpflanzung von *Euglypha alveolata* Dujardin. *Morphologische Jahrbuch* **13**, 173–183.

91 Cash, J., Wailes, G. H. & Hopkinson, J. 1915 *The British freshwater Rhizopoda and Heliozoa: Rhizopoda part 3*. London, UK: The Ray Society.

92 Chardez, D. 1965 On the particular mode and little understood reproduction in aquatic thecamoebae (Rhizopoda; Testacea.). *Bull. Inst. Agron. Sta. Rech. Gembloux* **33**, 26–34.

93 Valkanov, A. 1962 *Euglyphella delicatula* n. g., n. sp. (Rhizopoda Testacea) und ihre kopulation. *Dokl. Bolgarkoi Akad. Nauk* **15**, 207–209.

94 Schonborn, W. & Peschke, T. 1990 Evolutionary studies on the *Assulina-Valkanovia* complex (Rhizopoda, Testaceafilosia) in sphagnum and soil. *Biol. Fertil. Soils* **9**, 95–100. (doi:10.1007/BF00335790)

95 Valkanov, A. 1962 Über die kopulation der testaceen (Rhizopoda Testacea). *Dokl. Bolgarskoi Akad. Nauk* **15**, 305–308.

96 Iudina, T. A. & Sukhanova, K. M. 2000 The testate amoeba *Corythion delamarei*: cell biology and life cycle. *Tsitologiya* **42**, 613–623.

97 Sukhanova, I. N. & Cheban, E. A. 1990 Heterotrophic phytoplankton of the Black-Sea in the early spring period of the phytocen development. *Okeanologiya* **30**, 979–986.

98 Cavalier-Smith, T. & Chao, E. E. Y. 2003 Phylogeny and classification of phylum Cercozoa (protozoa). *Protist* **154**, 341–358. (doi:10.1078/143446103322454112)

99 Archibald, J. M. 2009 The puzzle of plastid evolution. *Curr. Biol. CB* **19**, R81–R88. (doi:10.1016/j.cub.2008.11.067)

100 Grell, K. G. 1990 Indications of sexual reproduction in the plasmodial protist *Chlorarachnion reptans* Geitler. *Zeitsch. Naturforsch. C J. Biosci.* **45**, 112–114.

101 Beutlich, A. & Schnetter, R. 1993 The life-cycle of *Cryptochlora perforans* (Chlorarachniophyta). *Bot. Acta* **106**, 441–447.

102 Braselton, J. P. 2002 Phylum Plasmodiophora (Plasmodiophoromycota). In *The illustrated guide to the Protozoa* (eds J. J. Lee, G. F. Leedale & P. C. Bradbury), 2nd edn, pp. 1342–1345. Lawrence, KS: Society of Protozoologists.

103 Raikov, I. B. 1982 *The protozoan nucleus: morphology and evolution*. Wien, Austria: Springer-Verlag.

104 Grell, K. G. & Ruthmann, A. 1964 Uber die karyologie des radiolars *Aulacantha scolymantha* und die feinstruktur seiner chromosomen. *Chromosoma* **15**, 185–211 (doi:10.1007/BF00285729)

105 Febvre, C., Febvre, J. & Michaels, A. F. 2002 Class Acantharea. In *The illustrated guide to the Protozoa* (eds J. J. Lee, G. F. Leedale & P. C. Bradbury), 2nd edn, pp. 1055–1084. Lawrence, KS: Society of Protozoologists.

106 Anderson, O. R., Nigrini, C., Boltovskoy, D., Takahashi, K. & Swanberg, N. R. 2002 Class Polycystina. In *The illustrated guide to the protozoa* (eds J. J. Lee, G. F. Leedale & P. C. Bradbury), 2nd edn, pp. 994–1022. Lawrence, KS: Society of Protozoologists.

107 Woodcock, H. M. & Lapage, G. 1915 Observations on the life-cycle of a new flagellate, *Helkesimastix faecicola*, n. g., n. sp.: together with remarks on the question of syngamy in the trypanosomes. *Proc. R. Soc. Lond. B* **88**, 353–370. (doi:10.1098/rspb.1915.0001)

108 Woodcock, H. M. 1916 Observations on coprozoic flagellates. *Phil. Trans. R. Soc. Lond. B* **207**, 375–412. (doi:10.1098/rstb.1916.0008)

109 Shirkina, N. I. 1987 Biological features in some flagellates of the genus *Cercomonas* (Rhizomastigida, Cercomonadidae). *Zoolog. Z.* **66**, 485–489.

110 Karpov, S. A. 1997 Cercomonads and their relationship to the myxomycetes. *Arch. Protistenkunde* **148**, 297–307.

111 Hulsmann, N. 1993 *Lateromyxa gallica* n. g. n. sp. (Vampyrellidae)—a filopodial ameboid protist with a novel life-cycle and conspicuous ultrastructural characters. *J. Eukaryot. Microbiol.* **40**, 141–149. (doi:10.1111/j.1550-7408.1993.tb04894.x)

112 Ropstorf, P., Hulsmann, N. & Hausmann, K. 1993 Karyological investigations on the vampyrellid filose amoeba *Lateromyxa gallica* Hulsmann 1993. *Eur. J. Protistol.* **29**, 302–310.

113 Nikolaev, S. I., Berney, C., Fahrni, J. F., Bolivar, I., Polet, S., Mylnikov, A. P., Aleshin, V. V., Petrov, N. B. & Pawlowski, J. 2004 The twilight of Heliozoa and rise of Rhizaria, an emerging supergroup of amoeboid eukaryotes. *Proc. Natl Acad. Sci. USA* **101**, 8066–8071. (doi:10.1073/pnas.0308602101)

114 Mikrjukov, K. A. & Patterson, D. J. 2001 Taxonomy and phylogeny of Heliozoa. III. Actinophryids. *Acta Protozoolog.* **40**, 3–25.

115 Simpson, A. G. B. 2003 Cytoskeletal organization, phylogenetic affinities and systematics in the contentious taxon Excavata (Eukaryota). *Int. J. Syst. Evol. Microbiol.* **53**, 1759–1777. (doi:10.1099/ijs.0.02578-0)

116 Droop, M. R. 1961 Evidence for a sexual phase in life-cycle of an amoeba. *Nature* **192**, 281. (doi:10.1038/192281a0)

117 Fritz-Laylin, L. K. *et al.* 2010 The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* **140**, 631–642. (doi:10.1016/j.cell.2010.01.032)

118 Perkins, F. O. & Amon, J. P. 1969 Zoosporulation in *Labyrinthula* sp.—an electron microscope study. *J. Protozoolog.* **16**, 235–257.

119 Moens, P. B. & Perkins, F. O. 1969 Chromosome number of a small protist—accurate determination. *Science* **166**, 1289–1291.

120 Sumbali, G. 2005 *The fungi* (ed. B. M. Johri). Middlesex, UK: Alpha Science International.

121 Hamilton, W. D. 2001 *Narrow roads of gene land, vol. 2: the evolution of sex*. Oxford, UK: Oxford University Press.

122 Felsenstein, J. 1974 The evolutionary advantage of recombination. *Genetics* **78**, 737–756.

123 Porter, S. M. & Knoll, A. H. 2000 Testate amoebae in the neoproterozoic era: evidence from vase-shaped microfossils in the Chuar group, Grand Canyon. *Paleobiology* **26**, 360–385. (doi:10.1666/0094-8373(2000)026<0360:TAITNE>2.0.CO;2)

124 Sen Gupta, B. K. 1999 Introduction to modern Foraminifera. In *Modern Foraminifera* (ed. B. K. Sen Gupta), pp. 1–6. Dordrecht, The Netherlands: Kluwer.

125 Gladyshev, E. A. & Arkhipova, I. R. 2010 Genome structure of bdelloid rotifers: shaped by asexuality or desiccation? *J. Hered.* **101**(Suppl. 1), S85–S93. (doi:10.1093/jhered/esq008)

126 Lohia, A. 2003 The cell cycle of *Entamoeba histolytica*. *Mol. Cell. Biochem.* **253**, 217–222. (doi:10.1023/A:1026055631421)

127 Török, J. K., Pollák, B., Heéger, Z., Csikós, G. & Márialigeti, K. 2008 First evidence of bacterial endocytobionts in the lobose testate amoeba *Arcella* (Amoebozoa, Arcellinida). *Protistology* **5**, 303–312.

128 Corsaro, D. & Venditti, D. 2009 Detection of chlamydiae from freshwater environments by PCR, amoeba coculture and mixed coculture. *Res. Microbiol.* **160**, 547–552. (doi:10.1016/j.resmic.2009.08.001)

129 Jeon, K. W. 2004 Genetic and physiological interactions in the amoeba–bacteria symbiosis. *J. Eukaryot. Microbiol.* **51**, 502–508. (doi:10.1111/j.1550-7408.2004.tb00277.x)

130 Greub, G. & Raoult, D. 2004 Microorganisms resistant to free-living amoebae. *Clin. Microbiol. Rev.* **17**, 413–433. (doi:10.1128/CMR.17.2.413-433.2004)

131 Boyer, M. *et al.* 2009 Giant marseillevirus highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. *Proc. Natl Acad. Sci. USA* **106**, 21 848–21 853. (doi:10.1073/pnas.0911354106)

132 Moreira, D. & Brochier-Armanet, C. 2008 Giant viruses, giant chimeras: the multiple evolutionary histories of mimivirus genes. *BMC Evol. Biol.* **8**, 12. (doi:10.1186/1471-2148-8-12)