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Secretive ciliates and putative asexuality in microbial eukaryotes

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Facultative sexuality is assumed to have occurred in the ancestor of all extant eukaryotes, but the distribution and maintenance of sex among microbial eukaryotes is still under debate. In this paper, we address the purported asexuality in colpodean ciliates as an exemplary lineage. Colpodeans are a primarily terrestrial clade thought to have arisen up to 900 MYA and contain one known derived sexual species. We conclude that the putative asexuality of this lineage is an observational artifact. We suggest that the same might hold for other microbial eukaryotes, and that many are secretively sexual as well. Theoretical work from the distantly related plants and animals suggests that both the evolutionary success of ancient asexuals and the reversal of the loss of sex are highly unlikely, further suggesting that colpodeans are secretively sexual. However, it remains to be seen to what extent sexual theories and predictions derived from macro-organismic lineages apply also to microbial eukaryotes.

Putative asexual microbial eukaryotes

Based on the broad distribution of meiotic sex, the ancestor of extant eukaryotes was probably facultatively sexual [1,2]. Although there is a cost to being sexual [3,4], numerous advantages have been proposed for its maintenance in plants and animals (Box 1). While most lineages have remained sexual, but asexual species are found scattered throughout the eukaryotic tree of life, primarily at the tips [4]. When plants and animals are found that are putatively asexual [particularly if they are putatively ancient asexuals (see Glossary)] they are studied intensively, as their existence goes against established theory [3–5]. By contrast, when asexuality is purported in microbial eukaryotes they are ignored or are judged not to be a theoretical problem [6]. But are microbial eukaryotes asexual as often assumed [7–9] and if so, do they pose a problem to our theories and expectations of the distribution and maintenance of sex?

In this paper, we discuss the putative lack of sex in microbial eukaryotes by concentrating on the Colpodea as an exemplary lineage. The Colpodea, one of 11 major ciliate clades, consists of about 200 described species with similar somatic but diverse oral morphologies [10,11] (Figure 1). Like all ciliates, a clade that is sister to the apicomplexans (e.g. *Plasmodium* sp., the causative agent of malaria) and

dinoflagellates (e.g. *Symbiodinium* sp., the photosynthetic symbiont of corals), colpodean ciliates posses two types of nuclei within each cell: a 'germline' micronucleus and a 'somatic' macronucleus [12]. Colpodean ciliates are found in numerous habitats, some are fungivores, and at least one species has a multicellular slime mold-like life stage [10]. Much is known about their biology through morphological and molecular analyses [10,11,13–15].

Sex in colpodeans has been observed only in *Bursaria truncatella*, a relatively large species that can be up to 0.5 mm in length, and preys on other ciliates [10,16]. The extent of sexuality in the rest of the colpodeans is debated; Foissner [10] proposed that they are asexual, whereas Dunthorn *et al.* [13] suggested that they are secretively sexual.

In this article we first present the observational evidence for and against sexuality in the Colpodea, then discuss their putative lack of sex in relation to the theoretical problems of reversing the loss of complex traits and ancient asexuality. We conclude that most, if not all, colpodean ciliates are likely to be secretively sexual, particularly if macro-organismic expectations of sexuality apply to microbial eukaryotes. The arguments here are applicable to other putatively asexual microbial eukaryotes, many of which are also likely to be secretively sexual.

The powerlessness of observation

Most of the evidence for the purported asexuality in colpodean ciliates derives from the lack of observing sex. But sex is easily missed for three reasons: sex in ciliates is facultative, sex might not occur because of inappropriate laboratory conditions, and there are no obvious morphological features for sex in these ciliates.

Observing sex in colpodean ciliates in specific and in all ciliates generally, is not easy because sex has remained facultative [17]. The probability, then, that a researcher will observe sex occurring can be extremely low, depending

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Glossary

Ancient asexuals: a group of species that have remained asexual over longer periods of evolutionary or even geological time than is predicted by theory.
Eukaryote: the group of organisms diagnosed by the presence of a nucleus.
Microbial eukaryote: eukaryotes that are not plants, animals or fungi. Also known as protists, protoctistas, algae and protozoa.
Molecular clock: estimate of the time of divergence among taxa using a molecular phylogeny and one or more calibration points such as fossils.
Reproduction: creation of offspring cells or individuals. Can be independent of sex.
Sex: meiosis, followed by the fusion of meiotic products from different individuals. This working definition restricts sex to eukaryotes.

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Box 1. Sex and its maintenance in macro-organisms

There are many definitions of sex. In this paper, we have restricted 'sex' to the definition used by Normark *et al.* [5]: 'meiosis followed by the fusion of meiotic products from different individuals'. Sex and reproduction are often intimately linked in macro-organisms, but new cells or progeny are not produced during sex in all eukaryotes. Recombination in archaea and bacteria is not covered under this definition, as it is non-meiotic [54,55].

Numerous theories exist for the maintenance of sex in macro-organisms. These include theories that deal with changing environments (e.g. Red Queen, Tangled Bank) and the accumulation and elimination of harmful mutations (e.g. Muller's ratchet, Kondrashov's hatchet), which are extensively reviewed elsewhere [3,4,35,42–46,56,57]. Most macro-organismic species are sexual during their life cycle or after a certain number of generations. The loss of sex is often thought to lead to rapid extinction with little chance of speciation [3,5,42].

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on its rate in nature, which can vary dramatically among species [18,19]. This rate can be further decreased over time in the laboratory [20]. Reproduction in ciliates is via asexual cell division that can continue uninterrupted for thousands of generations, although the rate of division decreases over time without a sexual event [11,21]. In ciliates, sex occurs during conjugation, with haploid micronuclei products being mutually exchanged between complementary cells [11]. Sex is assumed to occur in almost all ciliate clades, although details and direct observations for most species are lacking [11]. There are known derived asexual strains of sexual species in other ciliate clades that have lost their micronuclei and are thus unable to conjugate [11,21]. Although most colpodean ciliates will divide in laboratory cultures, they have never been observed to have sex, despite the many observations that have taken place over many years (with the exception of *B. truncatella*) [10]. This might be because the facultative events of sex are so rare that they are easily missed. Nevertheless, all of these observations could have overlooked that one rare and facultative event between two lonely, but secretively sexual cells.

Observing sex in colpodeans is not necessarily easy because laboratory culture conditions might not be appropriate. For colpodeans, and even for most ciliates, we do not know what the right conditions for inducing sex are, and until we mimic their environment we might not observe their secretive sexuality. Like the Colpodea, the fungal pathogen *Aspergillus fumigatus* was long thought to be asexual because there were no laboratory observations of sex. Only when *A. fumigatus* was finally cultured in conditions that mimicked its natural environment was a sexual life-cycle stage demonstrated [22].

Observing sex in colpodean ciliates is also not necessarily easy because there is a lack of what Schurko *et al.* [23]
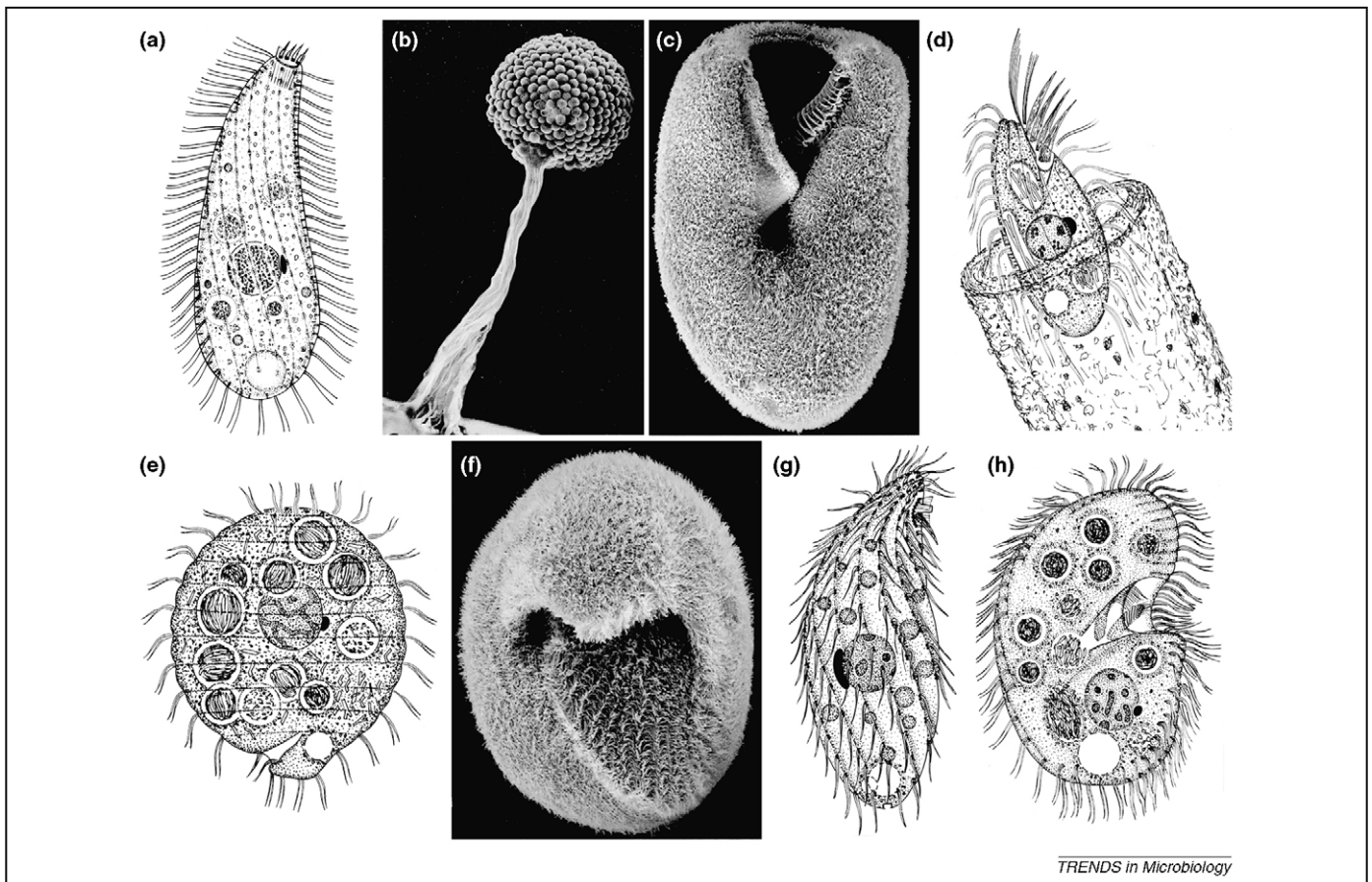

Figure 1. Morphological diversity within the putative asexual Colpodea ciliates. **(a)** *Platyophrya vorax* (right lateral view). **(b)** *Sorogena stoianovitchae*, slime mold-like multicellular sorocarp. **(c)** *Bursaria truncatella* (ventral view) the only known sexual species in the Colpodea. **(d)** *Cyrtolophosis mucicola* (left lateral view) in a mucilaginous dwelling-tube. **(e)** *Ilsiella palustris* (dorsal view) with posterior oral structure. **(f)** *Bresslauides discoideus*, (oblique apical view). **(g)** *Grossglockneria acuta* (right lateral view), oral structure modified into a tube used to perforate and feed on fungi. **(h)** *Colpoda cucullus* (right lateral view). Pictures by Professor Dr Wilhelm Foissner.

describe as 'organismal signs of sex', such as sex-specific morphologies and organs, or gender differences, as found in plants and animals. In macro-organisms, if functional organismal signs of sex are observed in an individual it is safe to assume that it belongs to a sexual species, although there are exceptions [23]. Even if organismal signs of sex do exist in nature, they might not be easy to find; for example, darwinulid ostracods were long thought to be asexual until males were found after more than a century of searching [24]. Most microbial eukaryotes such as colpodeans lack morphological signs of sex, and although there might be differences in the mating types of ciliates [25], these differences are not outwardly visible. There is one potential sign of sex in colpodeans: they contain germline micronuclei [10], although these might be incapable of meiotic division. Because there is little reason for an asexual ciliate to maintain micronuclei, as they are only used for sex and for modeling the next generation's somatic nucleus [12], the colpodeans might be secretively sexual.

Given these difficulties in observing sex in colpodean ciliates, the lack of observation does not mean that sex is not occurring. A lack of data is not evidence for the hypothesis of ancient asexuality. There is a history of microbial eukaryotes being long thought to be asexual because of the powerlessness of observation, which were later to be shown either to have sex or the genetic signatures of sex, such as *Giardia duodenalis* [26], *Leishmania major* [27], *Naegleria lovaniensis* [28,29] and *Trichomonas vaginalis* [30]. Putative asexuality in colpodean ciliates (and in other microbial eukaryotes in which there have been a lack of observations of sex) might represent a similar situation: given enough time and requisite conditions their secretive sexuality might be observed.

An improved phylogenetic framework

While acknowledging that observational artifacts might be the root of putative asexuality, an improved phylogenetic framework can allow us to understand the distribution and maintenance of sexuality better in larger groups of microbial eukaryotes. Using earlier estimates of the eukaryotic tree of life, Dacks and Rogers [1] suggested that, based on the distribution of known sexual lineages, facultative sexuality is probably the ancestral condition for all eukaryotes. With the data from recent molecular work that includes an increased number of sampled taxa and increased resolution of relationships, we can make further and more exact inferences about the distribution of sex within and among microbial eukaryotes such as colpodean ciliates, beyond issues relating to observational artifacts.
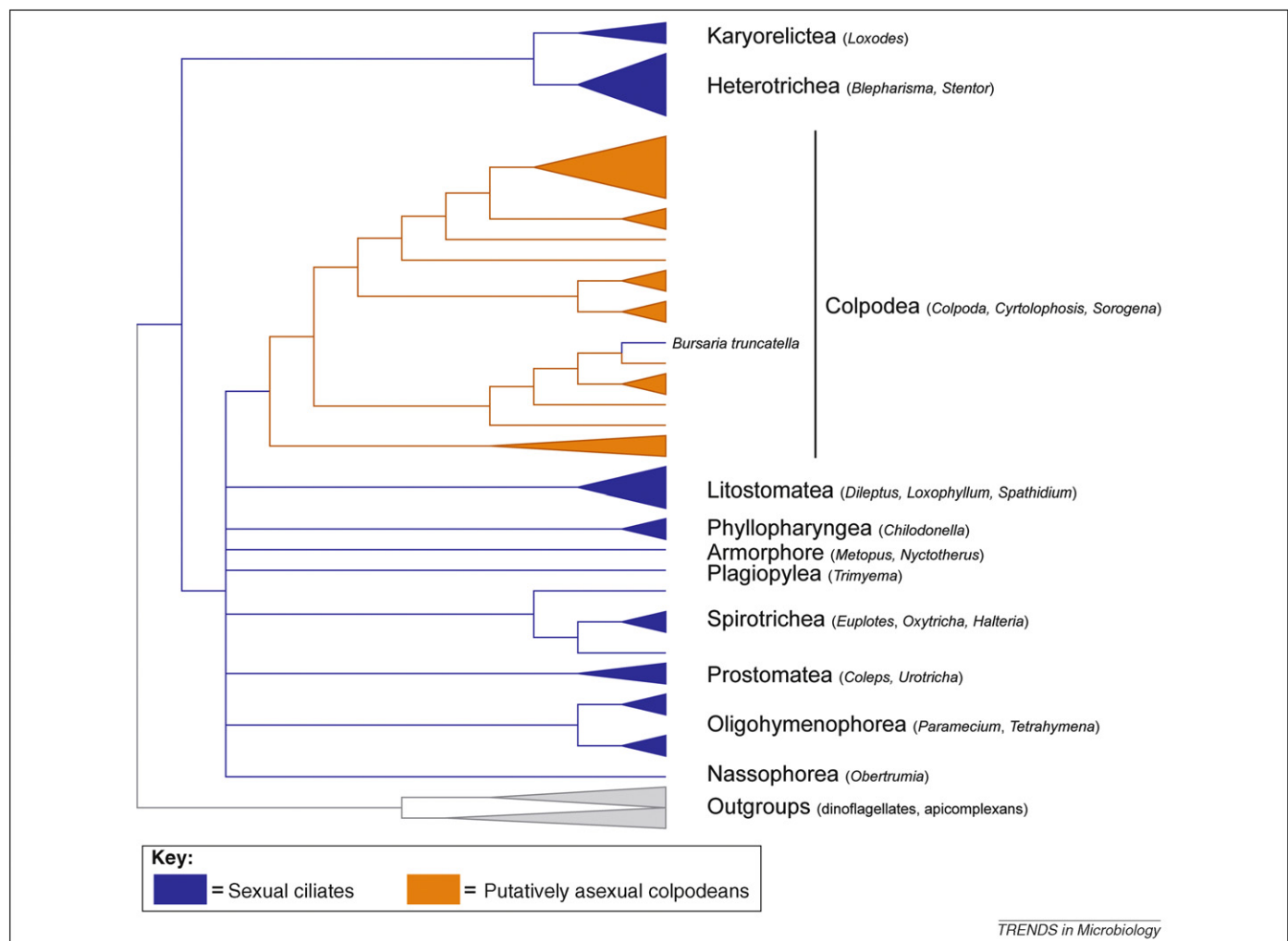

Figure 2. Distribution of sex in ciliates. The 11 major ciliate taxa are shown, along with their commonly known subclades. Most of these clades are largely sexual (blue). The Colpodea are putatively asexual (orange), except for one derived sexual species, *B. truncatella.* Modified from Dunthorn *et al.* [14] and Lynn [11].

Reversing the loss of sex?

Recent molecular phylogenetic work has shown that the one known sexual colpodean species, *B. truncatella*, nests within the larger clade of putatively asexual colpodeans [13] (Figure 2). If colpodeans are truly asexual, then we would have to hypothesize a reversal of the loss of sex after many millions of years of asexuality.

The general ability of a species to reverse the loss of any complex characteristic is open to debate [31–34]. Complex characteristics can either be lost phenotypically or genotypically; the ability to regain a characteristic depends on which of these levels was involved and the amount of time intervening between loss and reversal [31]. Can the loss of sex be reversed? It is a formal possibility that sex might have been lost and regained along lineages throughout the eukaryotic tree of life, although we would not know this given the current distribution of sex in extant species [35]. We do know of two putative cases of regaining sexuality, both at relatively shallow nodes. In multiple populations of the plant *Hieracium pilosella* [36], reversal from asexuality to sexuality entailed returning to homozygosity (and tetraploidy) for a recessive allele [36]. In this case, although the phenotype of sex was lost, sexual genes remained in the population. In oribatid mites, the case for reversing the loss of sex depends on likelihood character state reconstructions [37]. However, these reconstructions could be fundamentally flawed because of incorrect model specifications of both ancestral states and transitions rates, leading to the false acceptance of reversal [32]. The case of regaining sex in oribatid mites is thus ambiguous, and might merely represent multiple independent losses of sex. There is also no current evidence on whether genes involved in sex have been lost in oribatid mites. We also note that had Domes *et al.* [37] applied their method to other macro-organisms (especially in other insect clades) they might have increased the number of putative cases of reversing the loss of sex, although these additional likelihood character state reconstructions would also be fundamentally flawed because of the same model problems.

If genes involved in meiosis or other sexual processes are shown to be retained in the putative asexual relatives of *B. truncatella*, then a case for reversing the loss of sex in colpodean ciliates might be made, although the problem of reactivation of silenced genes increases over time as they could be mutated or lost [5,31,38]. However, retention of sex genes is more consistent with the retention of secretive sexuality. If we assume that both the phenotype and the genotype of sex were lost in the colpodeans, then our expectations for reversing the loss of sex would be much smaller to none. On the other hand, the problem of hypothesizing the reversal of the loss of sex in colpodeans is removed if they are secretively sexual.

Ancient asexuality?

Phylogenetic analyses coupled with molecular clock estimates suggest that colpodean ciliates would be extremely ancient asexuals (up to 900 MYA) [39]. There are also fossils of derived lineages in 93 MYA-old amber [40]. Colpodeans are thus at least as old, or perhaps even an order of magnitude older, than the putatively ancient asexual bdelloid rotifers that date to about 100 MYA [41].

There are other putative ancient asexuals lineages of plants and animals. Although asexuals are known to exist at the tips of the tree of life of macro-organisms, ancient asexuals are generally thought to be unlikely because asexuality is believed to lead to rapid extinction [3,5,42]. For example, without sex, macro-organisms might become more susceptible to increased mutational load and retrotransposon invasion/expansion, might not be able to adapt to a changing environment, or might not be able to escape predators and parasites [3,4,43–46]. Most claims of ancient asexuals have not been supported [5,47], except possibly those for the bdelloid rotifers [41,48–52]. However, this low expectation of ancient asexuality is based on theory and on observations of macro-organisms [5]. If these low expectations of ancient asexuality in macro-organisms do not apply to colpodean ciliates, then a case for asexuality in colpodeans can be made. For example, it is possible that large effective population sizes combined with non-canonical genetic systems could potentially allow these ciliates to remain asexual over long periods of evolutionary or geological time, beyond that predicted from plants and animals. However, if the expectations from macro-organisms do apply, then colpodean ciliates are likely to be secretively sexual.

Concluding remarks and outstanding questions

Most of our theoretical and empirical understanding of evolution derives from our familiarity and observations of plants and animals; our understanding of the distribution and maintenance of sex is just one example. Although originally thought to be closely related, plants and animals are on disparate branches of the eukaryotic tree of life [53]. Hence, as theoretical work on sex applies to both of these non-sister groups, this suggests that the theory might apply more broadly to other eukaryotic lineages. However, if additional research indicates that our theories do not apply to microbial eukaryotes, then our basis for understanding evolution would be restricted to distantly related macro-organisms in terminal branches in the eukaryotic tree of life – a very limited view indeed.

Despite macro-organismic theory, many microbial eukaryotes are often assumed to be asexual. Undoubtedly some of them truly are, although their age and distribution is unknown. But for the rest, the way in which most species in the laboratory are observed could prevent us from actually observing their secretive sex. For most microbial eukaryotes we are far from understanding aspects such as the facultative nature and timing of their sexual phases, the appropriate laboratory conditions to induce sex, and the organismal signs of sex at the morphological, biochemical and molecular levels. Given these problems, putative asexuality in our exemplary clade of colpodean ciliates is likely to be an observational artifact. This powerlessness of observation might also apply to all other putative asexual microbial eukaryotes, and many of these are secretively sexual as well.

For those microbial eukaryotes that are either anciently asexual and/or have known derived sexual species demonstrated by molecular phylogenetic analyses, a further claim against putative asexuality can be made using macro-organismic theory, such as in colpodean ciliates.

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Do these expectations also apply to microbial eukaryotes? We do not yet know. A more generalized understanding of ancient asexuality that takes into account microbial eukaryotes, and a better understanding of the possibility to regain the loss of sex in both macro-organisms and microbial eukaryotes, is needed. If our macro-organismic theories of the low expectations of ancient asexuals and the problems of reversing the loss of sex are shown not to apply, then colpodean ciliates and other microbial eukaryotes might be asexual, but if these expectations do apply, then we would expect most to be secretively sexual.

Beyond taking into account observational biases and developing a broader theoretical understanding of the distribution and maintenance of sex in microbial eukaryotes, there are numerous potential tests that can be performed to look for genomic signatures of sex or asexuality (which are reviewed elsewhere [5,23]). For example, the inventory of meiosis-related genes [30] or transposable elements [43] can be assessed. Any of these potential tests can and should be applied to colpodean ciliates and other putative asexual microbial eukaryotes as the next step in understanding the distribution of sex among diverse microbial eukaryotes.

Acknowledgements

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