Comparative Genomics Supports Sex and Meiosis in Diverse Amoebozoa

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Abstract

Sex and reproduction are often treated as a single phenomenon in animals and plants, as in these organisms reproduction implies mixis and meiosis. In contrast, sex and reproduction are independent biological phenomena that may or may not be linked in the majority of other eukaryotes. Current evidence supports a eukaryotic ancestor bearing a mating type system and meiosis, which is a process exclusive to eukaryotes. Even though sex is ancestral, the literature regarding life cycles of amoeboid lineages depicts them as asexual organisms. Why would loss of sex be common in amoebae, if it is rarely lost, if ever, in plants and animals, as well as in fungi? One way to approach the question of meiosis in the "asexuals" is to evaluate the patterns of occurrence of genes for the proteins involved in syngamy and meiosis. We have applied a comparative genomic approach to study the occurrence of the machinery for plasmogamy, karyogamy, and meiosis in Amoebozoa, a major amoeboid supergroup. Our results support a putative occurrence of syngamy and meiotic processes in all major amoebozoan lineages. We conclude that most amoebozoans may perform mixis, recombination, and ploidy reduction through canonical meiotic processes. The present evidence indicates the possibility of sexual cycles in many lineages traditionally held as asexual.

Key words: amoebae, Amoebozoa, meiosis, mixis, sex.

Introduction

Sex is an inherent part of the "textbook" eukaryotic life cycle. Current genetic and phylogenetic evidence suggests that sex is ancestral to all eukaryotes. Additionally, sexual processes are too complex to have evolved several times independently (convergences in this case are unlikely because the same machinery in employed in all characterized groups) and the existence of any truly asexual eukaryotic group can only be explained by secondary loss of sex (Speijer et al. 2015). Several eukaryotic lineages are traditionally considered to be asexual as no sexual process has been reported for them. However, lack of evidence is not evidence of absence. Because these are microbial organisms, there may be inherent difficulties of observing certain lineages engaging in sexual processes in laboratory (different mating types are not present in clonal cultures; necessary stimuli are not present; among others), leading to observation artifacts (Dunthorn and Katz 2010). Despite the existence of some self-compatible (homothallic) lineages, several model organisms are self-incompatible (heterothallic) and their cells will only fuse if appropriate mating types are present. This is the case in *Dictyostelium discoideum*, which exhibits three mating types (Bloomfield et al. 2010) and in several fungi as *Candida*, *Saccharomyces*, *Ustilago*, *Aspergillus*, which may have two, three, or four mating types (Lee et al. 2010). Some species require specific stimuli to initiate sexual processes as demonstrated for inducing mating in the choanoflagellate *Salpingoeca rosetta* upon release of chondroitinase by the marine bacteria *Vibrio fischerii* (Woznica et al. 2017).

Mating and meiosis are implied in *bona fide* sexual eukaryotic life cycles (Carr et al. 2010; Lahr et al. 2011). Cell fusion (plasmogamy or syngamy) normally involves two cells that function as gametes. Gamete compatibility is dependent on mating types and is molecularly regulated. Gametes of a single mating type of green alga *Chlamydomonas reinhardtii* express the fusogen HAP2 that participates in cell membrane fusion (Liu et al. 2015). In *C. reinhardtii* and *Plasmodium* (the malaria parasite), GEX1 is implied in karyogamy and meiosis

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as well (Ning et al. 2013). Both HAP2 and GEX1 were demonstrated to be present in most eukaryotic lineages and can be used as evidence of sex (Speijer et al. 2015). The complex meiotic process and its characteristic events such as bouquet formation (Scherthan 2001), synaptonemal complex (SC) assembly (Zickler and Kleckner 1999), and the occurrence of crossing over between homologous chromosomes (Lynn et al. 2007) are meiosis specific events that are highly conserved in eukaryotes. Part of the specific machinery responsible for such processes is phylogenetically conserved, performs the same functions in distantly related model organisms and is detectable in most groups (Malik et al. 2008) (fig. 1). The detection of the occurrence of a conserved gene set specific or required to meiosis was proposed as an approach to investigate putative sexual processes in putative asexuals (Schurko and Logsdon 2008). Positive results would indicate that a given organism is either sexual or is an evolutionarily recent asexual (Villeneuve and Hillers 2001; Ramesh et al. 2005; Schurko and Logsdon 2008). Some studies indicate that meiotic genes are ancestral to all eukaryotes as even early diverging lineages as *Trichomonas vaginalis* present them (Malik et al. 2008). Similarly, some groups whose sexual cycles are unknown or only recently discovered present meiosis-specific proteins (MSP), such as choanoflagellates, Glomeromycota fungi, amoebozoan parasite *Entamoeba invadens*, heterolobosean amoeba *Naegleria gruberi*, several ciliates, dinoflagellate *Symbiodinium* sp., diatoms *Pseudo-nitzschia* and *Seminavis*, and Trebouxiophyceae green algae (Carr et al. 2010; Fritz-Laylin et al. 2010; Halary et al. 2011; Ehrenkaufer et al. 2013; Chi, Mahé, et al. 2014; Chi, Parrow, et al. 2014; Fučíková et al. 2015; Patil et al. 2015).

Traditionally considered asexuals, amoeboid organisms are scattered in several eukaryotic lineages, for example, Rhizaria, Excavata, Stramenopiles, Opisthokonta, and Amoebozoa (Lahr et al. 2011). Among them, Amoebozoa is a very ancient (>750 Ma old; Porter and Knoll 2000) monophyletic assemblage of diverse amoebae and amoeboflagellates (see Kang et al. 2017). Some important human pathogens such as *Entamoeba histolytica* and *Acanthamoeba castellanii* as well as the model organism *D. discoideum* are amoebozoans. Phylogenetically, the lineage is closer to Obazoa (the group that includes animals and fungi) than to any other eukaryotic supergroup (Brown et al. 2013). Due to the lack or rarity of observable sexual processes, most amoebozoans are considered "asexuals." The emended description of Amoebozoa does not mention sex or meiosis in the group (Cavalier-Smith 1998). Most literature on Amoebozoa (or some of its groups) refers to them as "presumably asexual," "sexual or asexual" (sexual referring to Myxogastria and Dictyosteliida) or simply does not mention sexual processes at all (Smirnov 2005; Smirnov et al. 2011; Adl et al. 2012; Cavalier-Smith et al. 2015). Kang et al. (2017) point out a handful lineages out of the entire amoebozoan diversity as sexual (three members of Tubulinea, Myxogastria, Dictyostelia, and only *Sappinia* inside Discosea) basically depicting the whole diversity of Discosea as asexual. However, Myxogastria and Dictyostelia represent exceptions among amoebozoan lineages as their life cycles are well known and their sexual processes (including details of syngamy and meiosis) have been described, including occurrence of SC, a meiosis-specific structure, in cysts or spores of *Physarum*, *Dictyostelium*, *Echinostelium*, *Ceratiomyxa*, and *Microglomus* (Aldrich 1967; Furtado and Olive 1971; Haskins et al. 1971; Erdos et al. 1972, 1975; Szabo et al. 1982; Olive et al. 1983). Furthermore, microscopic evidence suggests the occurrence of meiosis in Tubulinea based on the observation of SC in *Arcella* (Mignot and Raikov 1992); *Paraquadrula* was convincingly demonstrated to perform plasmogamy and karyogamy with subsequent cyst formation (Lüftenegger and Foissner 1991); *Copromyxa* was also observed to fuse and encyst in a putative sexual process (Brown et al. 2011). Among Discosea, *Sappinia* makes a bicellular cyst, where sexual processes are hypothesized to happen (Brown et al. 2007; Walochnik et al. 2010). *Cochliopodium* was also proposed to have sexual processes base on described fusions of cells and karyogamy (Wood et al. 2017). *Echinosteliopsis* produces two kinds of spores with different germination rates, what may be interpreted as evidence for sexual processes, in this case, meiosis, even though the author himself asserted that no evidence for sex could be found then (Reinhardt 1968). Among Archamoebae, transcriptomic and microscopic evidence strongly suggest the occurrence of meiosis in *Entamoeba invadens* during the encystation process, when meiotic genes are up-regulated in the first hours after cyst formation resulting in a mature cyst with four nuclei (Ehrenkaufer et al. 2013).

The current general understanding depicts amoebozoan groups mostly as asexuals despite scattered evidence on the contrary. The issue of sex in Amoebozoa was addressed once before through bioinformatics (Tekle et al. 2017). The authors aimed to evaluate the presence of meiosis-related proteins in several Amoebozoan lineages. However, the molecular machinery for plasmogamy and karyogamy was not investigated, and the large and diverse lineage of Tubulinea was severely undersampled. Here, we assess the occurrence of proteins associated to both syngamy and meiosis across all Amoebozoa lineages. We employ a comparative genomics analysis based on molecular genomic and transcriptomic data obtained from a wide phylogenetic sampling of the group (a data set of 52 taxa).

Materials and Methods

We have sampled 52 different amoebozoan species covering the whole known diversity of this super group, including both species known to perform sexual cycles as well as those with unknown sexual processes. All data were obtained exclusively from public databases. *Entamoeba histolytica*, *Dictyostelium discoideum*, *Polysphondylium pallidum* are represented by

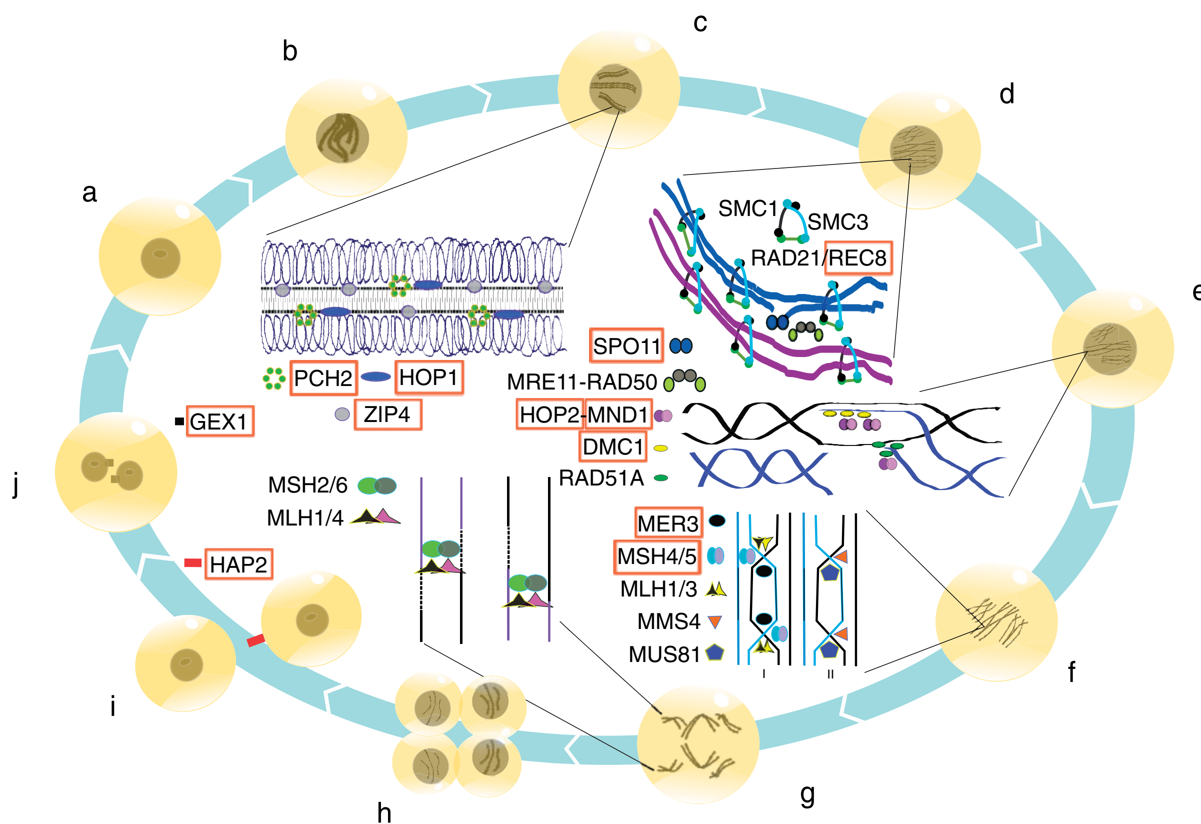

Fig. 1.—Life cycle highlighting main processes happening upon meiosis and plasmogamy/karyogamy: (*a*) duplication of DNA during interphase (synthesis phase); (*b*) meiosis-specific bouquet formation, promoted by BQT1 and BQT2 in *Schizosaccharomyces pombe* (Scherthan 2001; Chikashige et al. 2006); (*c*) the assembly of synaptonemal complex (Zickler and Kleckner 1999; Fraune et al. 2012) involves many meiosis-specific structural proteins, some of them high conserved, PHC2 and HOP1 (Anuradha and Muniyappa 2004; Farmer et al. 2012) and ZMM complex protein ZIP4/SPO22 (Lynn et al. 2007); (*d*) sister chromatids are kept close together by cohesin complexes, composed by SMC1, SMC3, RAD21 or its meiotic paralog REC8 (Uhlmann et al. 1999; Haering and Nasmyth 2003; Revenkova and Jessberger 2005; Peters et al. 2008), which keep together sister chromatids until anaphase II when they are finally cleaved by separases (Nasmyth 2005); double-strand breaks are introduced onto DNA by SPO11 and TopoVIB-like proteins working as dimers or tetramers (Malik et al. 2007; Keeney 2008; Robert et al. 2016); before the activation of the homologous recombination machinery SPO11 is removed and DNA strands are processed (resection) by MRN complex (MRE11, RAD50 and NBS1) resulting in the single 3′ strand used for invasion of the homologous chromosome, where it is extended by a DNA polymerase forming a D-loop (Borde 2007; Williams et al. 2007; Berchowitz and Copenhaver 2010); (*e*) homologous recombination mediated by RAD51A and its meiotic paralog DMC1, HOP2 and MND1 (Petukhova et al. 2005; Lin et al. 2006); (*f*) chiasmata contain double-Holliday junctions, which can be resolved in order to promote cross-overs by two main pathways: the main interference bearing pathway I, which involves MER3, MSH4-5, MLH1-3, EXO1, and SGS1 (Wang et al. 1999; Nakagawa and Kolodner 2002; Snowden et al. 2004; Zakharyevich et al. 2012) and pathway II, which involves MUS81 and MMS4 (de los Santos et al. 2003; Higgins et al. 2008); the correct assortment of chromosomes depends on the occurrence of cross-overs (Chakraborty et al. 2017); both pathways work at the same time, but pathway I is responsible for most cross-overs in *Saccharomyces* and *Arabidopsis*; however, some organisms relay completely on pathway II for cross-over resolution (*Schizosaccharomyces pombe* and *Tetrahymena thermophila*) (de los Santos et al. 2003; Higgins et al. 2008; Lukaszewicz et al. 2013); (*g*) the mismatches formed are corrected by the nuclear mismatch repair system composed basically by MSH2-6 and MLH1-PMS1 (in yeast) (Wang et al. 1999); (*h*) canonical meiosis results in four haploid cells; (*i*) Gametes of a single mating type express the transmembrane HAP2, that facilitates cell membrane fusion (Wong and Johnson 2010; Liu et al. 2015); (*j*) GEX1 is a nuclear membrane protein involved in karyogamy (Ning et al. 2013). Proteins considered to be meiosis-specific are highlighted with a red box.

genomic sequences obtained from public databases. All other species are represented by transcriptomic data and are derived from sequences which have been deposited in NCBI, mostly under BioProject PRJNA380424 among others (supplementary table 1, Supplementary Material online). Raw sequence data were subjected to TRIMMOMATIC (Bolger et al. 2014) for cleaning and trimming of adaptors for posterior assembly with TRINITY (Grabherr et al. 2011). Translation of nucleotide sequences was performed by Transdecoder (https://github.com/TransDecoder/TransDecoder/wiki; Last accessed November 14, 2018) in order to establish protein data sets used for further analyses.

Sequences of meiotic proteins characterized in model organisms (*H. sapiens*, *S. cerevisiae*, *A. thaliana*, and others)

serving as guides for trees were obtained from GenBank. Sequences from diverse Archaea and Bacteria strategically sampled were used as outgroups for trees, the proteomes being obtained from GenBank as well. Outgroups are important to determine more easily different paralogs in the analyses. In order to build profiles for the search of candidate sequences model organism sequences were aligned using the *mafft-linsi* tool of MAFFT (Katoh and Standley 2013). Alignments thus obtained were employed for the construction of profiles with *hmmbuild* tool of HMMER (Eddy 2011). The only exception was the profile for GEX1/KAR5 because this protein is not well conserved. For this, we constructed a HMMER profile according to (Ning et al. 2013). All amoebozoan proteomes either from genomic or transcriptomic sources were combined in a single database and screened with protein profiles using *hmmsearch* tool of HMMER. Best hits (e-value < e-6 for most proteins and e-value < 0.001 for GEX1) were extracted from the local database using the tool HMMER *esl-sfetch* for further processing. As the simple occurrence of similar or homologous sequences is not enough to determine a candidate sequence, all sequences obtained were subjected to phylogenetic reconstruction to confirm *bona fide* orthologs. For this, sequences from a strategic sampling that could provide both wide phylogenetic coverage and outgroups were provided. We aligned matrices using default *mafft* tool from MAFFT; multiple sequences alignments (MSA) were subjected to trimming using BMGE (Criscuolo and Gribaldo 2010) with relaxed parameters as matrix BLOSUM30 given the divergent feature of the sequences and all steps inspected visually. The trimmed MSA files were used as input for phylogenetic reconstructions with IQ-TREE (Nguyen et al. 2015). The substitution models were evaluated and set automatically by ModelFinder based on the input data (Kalyaanamoorthy et al. 2017) and 1000 ultrafast bootstrap (Hoang et al. 2018). All candidate orthologs were compiled to a single table used as input for the Coulson Plot Generator (Field et al. 2013) in order to make the results easier to understand and expose possible evolutionary patterns.

Results

The present study was proposed in order to investigate the molecular machinery required for syngamy and meiosis in most of the known diversity of Amoebozoa. The data obtained from amoebozoan lineages were organized and interpreted based on the most recent comprehensive phylogenomic reconstruction of the evolutionary relationships of the group according to Kang et al. (2017). Genes required for plasmogamy, karyogamy, and main meiotic steps, either specific or not, were analyzed using a phylogenetics approach. In general, every protein surveyed yielded positive results for most amoebozoan groups including the "asexual" model organisms *Acanthamoeba* and *Amoeba proteus* which present most of proteins associated to sexual processes (fig. 2 and supplementary table 2 and supplementary Alignments and Trees, Supplementary Material online). All proteins surveyed were identified in the three major amoebozoan lineages Tubulinea, Evosea, and Discosea, except for REC8 (not detected in Evosea). The most parsimonious interpretation would be that all of these genes were present in the amoebozoan ancestor. On an average, each MSP was detected in ~44% of the samples, while each non-MSP were detected in 75% of the samples. Considering that most of the data obtained is derived from transcriptomes, the occurrence of MSP was expected to be lower than other non-MSP which are involved in general DNA metabolism regardless of the life cycle stage and are continuously expressed. Nevertheless, the proteins HOP2 and MND1 were each detected in 90% of the samples. The proteins used in the present study were grouped according to their function in functional groups: syngamy (HAP2 and GEX1), sister chromatid cohesion (SMC1, SMC3, RAD21, and REC8), introduction of double-stranded breaks (DSB) (SPO11, MRE11, and RAD50), pairing and synaptonemal complex (SC) (HOP1, PCH2, and ZIP4), homologous recombination (HR) (DMC1, RAD51A, HOP2, and MND1), crossing-over and its resolution through pathway I (MER3, MSH4-5, MLH1, MLH3, and EXO1) and pathway II (MUS81 and MMS4), and gene conversion by mismatch repair of the resulting heteroduplexes (MSH2, MSH6, MLH1, PMS1-2) (fig. 2).

We could assess the presence of the orthologs associated with syngamy in several lineages distributed among Tubulinea, Evosea, and Discosea. Noteworthy, the fusogen HAP2 may not be easily detected in transcriptomic data due to its probable low expression levels in specific mating types only and due to the observation that GEX1 is broadly but only fairly conserved among eukaryotes (Ning et al. 2013). They could be detected in most genomic data (*Dictyostelium*, *Polysphondylium*, and *Acanthamoeba*). However, both forms are absent and seemingly lost in the *Entamoeba* lineage. The detection of both forms is a strong evidence of mixing dependent of an ancestral system of gamete recognition and fusion, implying the existence of different mating types. Regarding the main meiotic steps, the proteins implied in sister chromatid cohesion, promoted by the cohesin complex subunits, are widely present in Amoebozoa. REC8 was lost in *Dictyostelium* and *Entamoeba*, but it is present in Tubulinea and Discosea and, based on our results, it appears to have been lost in some ancestor of Evosea which concentrates most genomic data sets available. Previous studies failed to detect REC8 among protists and this protein is basically known only from plants, animals, and fungi (Malik et al. 2008; Schurko and Logsdon 2008). The proteins involved in the introduction of DSB are distributed among the whole diversity of the group. Strikingly, *Dictyostelium* and *Polysphondylium* have lost SPO11, previously reported by Bloomfield (Bloomfield 2018). These organisms are some of the few amoebozoans with well-known sexual cycles (Erdos et al. 1973; Francis 1975). Distantly

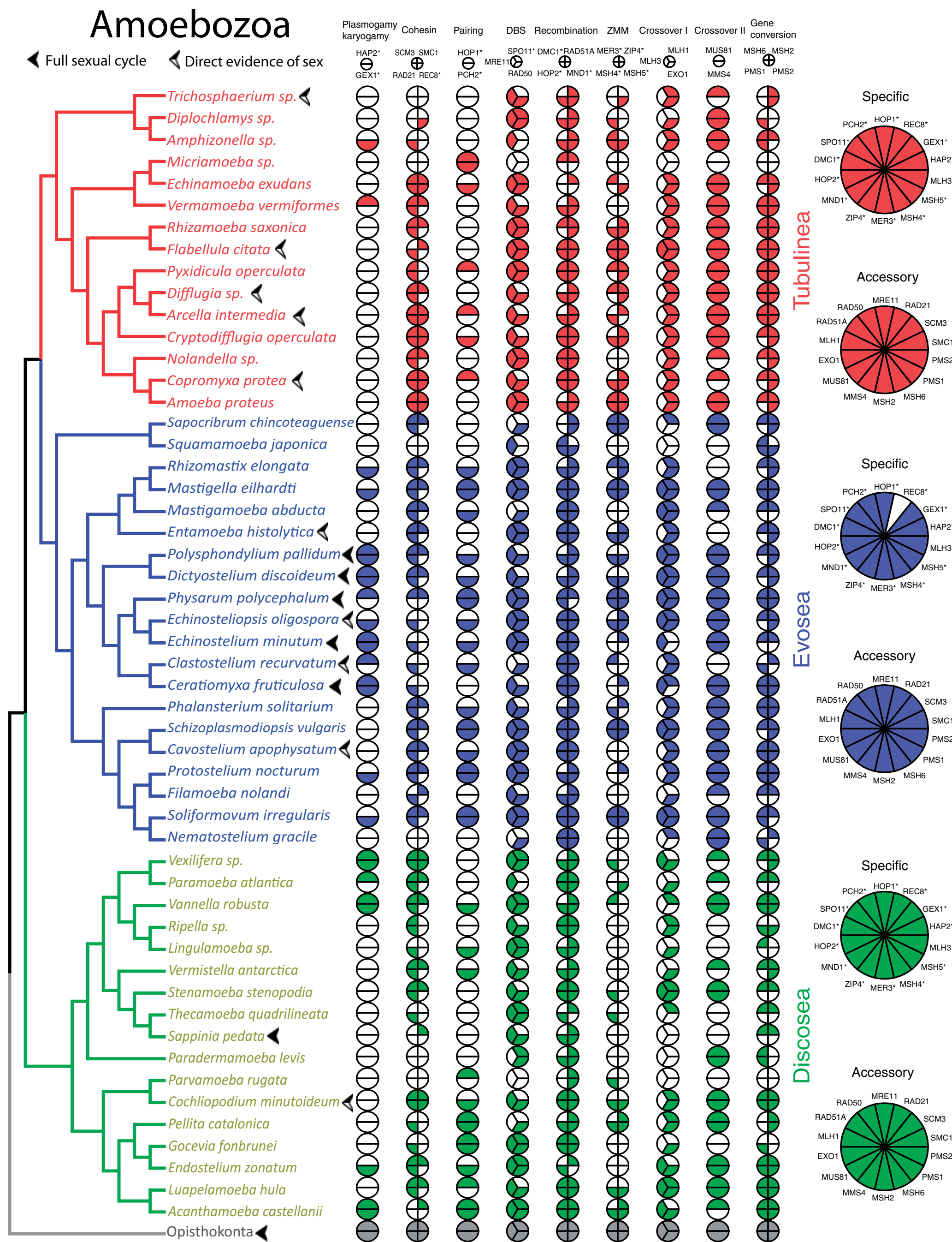

Fig. 2.—Distribution of proteins required for syngamy, karyogamy, and the main meiotic steps in most of the known amoebozoan diversity based in genomic and transcriptomic data, organized and distributed according to the most recent and comprehensive phylogenomic reconstruction of evolutionary relationships in the group according to Kang et al. (2017). All the proteins detected by this analyses were clustered according to functional groups: syngamy: HAP2 and GEX1; sister chromatid cohesion: SMC1, SMC3, RAD21, and REC8; Homologs pairing: HOP1 and PCH2; introduction of double-strand breaks (DSB): SPO11, MRE11, and RAD50; homologous recombination (HR): DMC1, RAD51A, HOP2, MND1; ZMM complex: MER3, ZIP4, MSH4-5; interference bearing crossover resolution pathway I: MLH1, MLH3, and EXO1; crossover resolution pathway II: MUS81 and MMS4; Gene conversion: MSH2, MSH6, PMS1-2 (also known as MLH2 and MLH4 in some sources). Proteins considered to be meiosis-specific are marked with *. All the proteins that could be detected here are marked by color filling of the corresponding section of the circle. Empty sections (white) represent proteins that are absent from analyzed data sets; such absences represent losses only for *Dictyostelium discoideum, Polysphondylium pallidum* and *Entamoeba histolytica* for those are the only species with whole genomes available. Other absences do not necessarily represent losses, as they just could not be detected in the present analysis. Black arrowheads indicate species or lineages with full sexual life cycles already described, while black and white arrowheads indicate the groups with direct evidence supporting sexual life cycles (plasmogamy, karyogamy, synaptonemal complex and so on). The graphics on the right side represent a compilation of the occurrence of all meiosis-related proteins in the three main known lineages inside Amoebozoa.

related *Acanthamoeba*, *Amoeba proteus, Physarum*, and *Entamoeba* present the whole set of DSB genes. Some data sets present more than one copy of SPO11, as in the case of *Pyxidicula*, *Entamoeba*, *Physarum*, *Acanthamoeba*, and others (supplementary table 2, Supplementary Material online), but it is not possible to determine with certainty whether such a duplication is ancestral or not (supplementary trees, Supplementary Material online). Proteins associated with pairing of chromosomes and SC assembly occur in most amoebozoan groups with losses of HOP1 and ZIP4 in *Dictyostelium* and *Polysphondylium* and HOP1 and PCH2 in *Entamoeba*. The presence of SC proteins in *Arcella* and *Physarum* corroborates ultrastructural data from literature (Aldrich 1967; Mignot and Raikov 1992). ZIP4 was identified in the mixogastrid *Echinostelium*, another genus whose SC has been demonstrated through electronic microscopy (Haskins et al. 1971). Presence of both HOP1 and ZIP4 in *Protostelium* and *Acanthamoeba* among others is a strong evidence for occurrence of SC in these groups as well. The machinery for meiotic HR is ubiquitous among amoebozoan lineages. However, the meiosis specific DMC1 was lost in *Dictyostelium* and *Polysphondylium*. The double strand invasion is stabilized by another set of MSP, namely MER3, MSH4, and MSH5 (components of yeast ZMM complex). Members of the ZMM complex are widely present in representatives of most amoebozoan lineages indicating a possible maintenance of meiosis specific interference pathway I as the main pathway to resolve crossovers. Additionally, pathway I resolution proteins MutLγ (MLH1–MLH3) and EXO1 are present as well. Class II crossover pathway proteins MUS81 and MMS4 could also be detected in most lineages (seemingly lost in *Entamoeba*). The machinery involved in nuclear DNA mismatch repair and gene conversion is also present basically in all lineages.

Discussion

Earlier debate about sex was focused on maintenance of variability as an important adaptive characteristic provided by sexual processes. Lineages performing sexual cycles would, for instance, have an advantage at surviving parasitic infections under “arms race” scenarios as hypothesized in the “Red Queen hypothesis” (van Valen 1973). Meiosis is stable throughout the evolutionary history of eukaryotes, also explained by its importance for genome stability through control of transposons (Borgognone et al. 2017). For example, occurrence of meiosis is central for controlling of transposable elements in the filamentous fungus *Neurospora crassa* through meiotic silencing by unpaired DNA (Shiu et al. 2001). Sexual processes as a phenomenon is accepted to be a defining eukaryotic characteristic ancestral to all groups. The few documented asexual groups are restricted to mostly triploid or hybrids hybrid subpopulations of sexual species and lineages recently asexual as in the case of *Taraxacum* (dandelions) and up to 10% of ferns (van Dijk and Bakx-Schotman 2004; Dyer et al. 2012), to which the bdelloid rotifers seem to represent a remarkable exception, as an asexual order of small metazoans, bestowing upon them the title of “evolutionary scandal” (Smith 1986; Judson and Normark 1996). The first comprehensive analysis of the genome of the bdelloid *Adineta vaga* showed that its structure is incompatible with conventional meiosis (Flot et al. 2013). Additionally, bdelloid rotifers are the only metazoan group lacking LINE-like and gypsy-like reverse transcriptases, which seem to be related to sexual processes (Arkhipova and Meselson 2000). Debortoli et al. (2016) proposed that most genetic exchange in bdelloids is probably due to lateral gene transfer rather than to meiotic sex.

Spiegel (2011) argues that the canonical biological view of sex is biased toward animals (zoocentric) and that sex is not always reproductive. Moreover, he posits that Myxogastria have well described life cycles and sexual processes because they were more extensively studied (they were previously considered Fungi) than other amoebozoan groups. Thus, if loss of sex is a rare occurrence in the more well-studied groups, namely animals, plants, and fungi, why would the majority of amoebozoans be so easily accepted as asexual? The key to that problem lies on dispensing more attention to poorly understood lineages. There is a growing interest on this subject exemplified by *Entamoeba*, *Sappinia*, *Copromyxa*, and others groups (Brown et al. 2007, 2011; Ehrenkaufer et al. 2013). In theory, the mere presence of fully sexual lineages nested inside Amoebozoa (fig. 2) is per se a demonstration of sex as an ancestral character to the whole group as it is highly unlikely that sex would be lost and would evolve again only in Myxogastria and Dictyosteliida. The scenario of many amoebozoan groups losing sex independently is also unlikely because it is not parsimonious. The scenario of an asexual ancestor to all amoebozoans is not acceptable at all as this hypothesis would require sex and meiosis to evolve again inside the group and this would not be parsimonious either. Our assessment of the presence of the whole meiosis-specific machinery in a broad range of diverse Amoebozoa supports the hypothesis of the widespread sexual cycles in the group and agrees with the idea of sex as an ancestral character.

A recent study detected the presence of meiotic genes in Amoebozoa concluding that amoebozoans are ancestrally and “secretly” sexual (Tekle et al. 2017). As we already discussed, Amoebozoa could be proposed to be ancestrally sexual based solely on the position of fully sexual lineages nested inside Amoebozoa if we assume that the topology of the tree produced by Kang et al. (2017) is a good approximation of the real phylogenetic relationships of the lineages inside this super-group. As such, a mere confirmation of presence of MSPs simply corroborates the hypothesis of amoebozoans being ancestrally sexual. However, the patterns of occurrence may shed light into evolution of sex in the group, while additionally indicating putative presence of sexual processes in lineages assumed to be asexual based on lack of

observations of otherwise. However, some issues regarding the study of Tekle et al. (2017) are concerning as most positives results are not of MSP, but rather only meiosis-*related* proteins. Additionally, plasmogamy and karyogamy were not surveyed, even though they are integral parts of any *bona fide* sexual cycle. We demonstrated the presence of both plasmogamy and karyogamy proteins in several amoebozoans and focused directly on MSP, a result supporting the occurrence of sexual processes in the group. Tekle and collaborators also did not survey the highly conserved MSPs ZIP4 and PCH2 while searching for the nonconserved ZIP1 and RED1, which led to the expected negative results. They also failed to detect HOP1 (except for *Physarum*) in their data sets. Thus, results lacking the SC-associated proteins HOP1, ZIP4, PCH2, RED1, and ZIP1 are an artifact of their approach. The authors also dismissed the very occurrence of SC in Amoebozoa, despite previous reliable microscopic documentation for some groups, and proposed a putative "novel crossover pathway" for amoebozoans without evidence. We demonstrated the presence of both HOP1 and ZIP4 in several lineages, what is consistent with occurrence of SC as revealed earlier by ultrastructural documentation. Another major issue with their results is their assumption that "Mycetozoa" (we assume here that this taxon refers to Myxogastria sensu Adl et al. 2012) lost SPO11 and that they may have another mechanism to initiate meiotic recombination, seemingly in a SPO11-independent pathway, again without evidence and based on another artifact. While it is likely that dictyosteliids lack SPO11, this is not true for other groups as our results demonstrate clearly the presence of SPO11 in Myxogastria (e.g., in *Physarum*) and other related groups within Evosea. We also greatly expanded sampling with a total of 52 taxa here against 29 there (for more details, see supplementary table 3, Supplementary Material online). Their poor taxon sampling issue is more pronounced in Tubulinea: Tekle and collaborators sampled only three species with seemingly poor data sets leading to a complete absence of any positive results for MSP in Tubulinea, while we provided robust positive results for several MSP in 15 different species of Tubulinea, in a clear demonstration of another artifact resulting from their approach. Other problematic issues include their methods, as most MSP are results of gene duplication events, it is necessary to discriminate between different paralogs upon reconstruction of each MSP family. That is not what one can observe in Tekle et al. (2017), as paralogs of protein families were analyzed separately in different reconstructions (e.g., MND1-HOP2, RAD51A-DMC1, and MSH4-MSH5), which yielded poorly recovered trees that cannot be used to ascertain the presence of MSP in the surveyed lineages. Moreover, due to poor taxon sampling and limited methodological power, a key MSP, REC8, was not detected, while present in our analysis. In the present study we present many more positive results for cell fusion and meiotic machinery in the group and we have the opportunity to offer new perspectives to understand the biology of Amoebozoa by pointing out artifacts generated by Tekle et al. (2017).

Occurrence of sexual life cycles can be assessed by indirect evidence as quantification of recombination through population genetics (Cooper et al. 2007). Accumulated evidence points to occurrence of meiotic reduction of ploidy (canonical meiosis) and sexual activity in at least some amoebozoan groups (fig. 2). The spores formed by Myxogastria, macrocysts in dictyostelids, and cysts in *Sappinia*, *Copromyxa*, and *Entamoeba* seem to be strictly associated to plasmogamy, karyogamy, and meiosis. Upon encystation, *Entamoeba* upregulates meiosis-specific genes $\sim$8 h after cyst formation in a process that will culminate in the formation of four nuclei (Ehrenkaufer et al. 2013). The formation of macrocysts in *Dictyostelium* also involves meiotic reduction (Erdos et al. 1975). In general, cyst formation (and spores in myxogastrids) is often part of sexual processes, stimulated by some kind of stress as desiccation, exit from host (in parasites), temperature changes, or other factors.

The existence of a mating type system has direct implications for observing meiosis in amoebae, as clonal cultures (which is often the norm in protistological laboratories) will not exhibit any signs of sexual processes, leading to the observation that a given organism is asexual. The cell fusogen HAP2, as well as GEX1, were not detected in *Entamoeba* genomes, and seem to be lost in this genus. This loss does not imply these organisms have lost capacity of mixing (fungi have lost the fusogen and have sexual cycles; Speijer et al. 2015), but rather that they may have lost the mating type system and could be homothallic. Accordingly, selfing or unisexual mating happens in parasites, explaining their apparent clonal structure (Feretzaki and Heitman 2013). But mixing alone does not support a canonical sexual cycle, as parasexual processes may happen afterward. *Candida albicans*, which grows as diploid cells of two different mating types, can fuse to form a tetraploid cell, which returns to a diploid state through loss of chromosomes (Bennett and Johnson 2003). Thus, the co-occurrence of fusogens and MSP provide stronger evidence for sexual processes in a given lineage. We have detected genes for the proteins required for syngamy and every meiotic step for the entirety of Amoebozoa, challenging the common conception that amoebae are "asexual" organisms. Most groups present a full cohesin complex and its meiotic variant. The occurrence of pachytene check regulation PCH2 and SC are also conserved in the group. The machinery responsible for the introduction of DSB and resection of the broken ends are present in most lineages with the exception of a very specific loss of SPO11 in dictyostelids. Such a loss is intriguing since losses of SPO11 are not known outside dictyostelids as this topoisomerase was detected in previous works with all candidate asexual protists surveyed (Ramesh et al. 2005; Carlton et al. 2007; Fučíková et al. 2015). Given that dictyostelids are known to have sexual cycles, they are probably relying on another pathway to introduce DBS onto

chromosomes. Alternative mechanisms for DBS have been described for fission yeast *Schizosaccharomyces* and *Caenorhabditis* (Farah et al. 2005; Pauklin et al. 2009), suggesting that there must be alternative processes in dictyostelids.

Among amoebozoans the meiosis-specific HR machinery containing DMC1, HOP2, and MND1 is conserved, which is another strong evidence for canonical meiosis in the group. The SC, ultrastructurally reported in both Tubulinea and throughout Evosea, are molecularly supported by our approach as the conserved proteins HOP1, PCH2, and ZIP4 are present and widespread. Amoebozoans probably proceed with meiotic recombination through stabilization of the initial double strand invasions promoted by interference bearing ZMM complex formed by ZIP4, MER3, MSH4, and MSH5. The simultaneous occurrence of the machinery associated to both crossover pathways in the group suggests a scenario similar to some model organisms as both pathways work during meiosis in *Saccharomyces* and *Arabidopsis* (de los Santos et al. 2003; Higgins et al. 2008). The resolution of crossovers produced by the action of the meiotic recombination machinery in amoebozoans may be performed by the main meiotic pathway I or the secondary pathway II as both are conserved in the group. Such a result is noteworthy, considering a group long held to be "asexual." Thus, is the mere existence of all of meiotic genes, with some specific losses, enough information to presume sexual cycles in any group? Similar positive results with *Giardia*, *Trichomonas*, and others led to the conclusion that they are secretively sexual. One could suppose that some genes considered to be meiosis-specific may undergo neofunctionalization in some groups and, thus, would not work upon meiosis anymore; however such a hypothesis needs to be demonstrated.

In the case of Amoebozoa, as the ancestor of all lineages was clearly sexual, our positive results support the assumption that the whole lineage is sexual, many of these taxa with unknown sexual or meiotic processes. This permits us to make overarching conclusions that will need to be further investigated: 1) meiotic sex is cryptic, 2) current laboratory conditions are not suitable for sexual cycles and, 3) perhaps in some cases meiotic events are mistakenly reported as mitosis. The latter might well be the case, as meiotic divisions could be interpreted as two sequential mitotic divisions. In many cases we hypothesize that haploid and diploid forms may have roughly the same morphological appearance. But the assumption that most or all amoebozoan would perform meiosis and sex in the same manner seems to be rather simplistic given the high diversity of forms in the group. Alternative processes may exist in some lineages, hypothetically among polyploid forms such as *Acanthamoeba*, *Polychaos*, and *Entamoeba*. Maciver (2016) proposed that polyploid amoebae and other organisms presenting high numbers of genome copies may have the possibility to recombine their chromosomes rather frequently and revert deleterious mutations through the process of recombination and gene conversion. For this process they would employ their conserved meiotic machinery. This would allow for genome stability without the necessity of spending energy undergoing meiosis or fusing with other individuals. In this framework, a parasexual process would maintain genome stability in polyploids. Although an interesting idea, this hypothesis is not supported with evidence.

One can entertain the idea that a given protein involved in meiosis could be coopted for another function (pending functional demonstration). Even if it were the case, it is unlikely that several of them would assume new functions in the same lineage. Traditionally seen as an assemblage of asexual mitotically reproducing organisms, amoebozoans (especially Tubulinea and Discosea) should be understood as putative sexual organisms, with direct implications to different fields. Regarding public health, the results presented here change the way we approach pathogenic species, their response to drugs used in their control, as well as dynamics and evolution of drug resistance. Some instances can be observed in pathogenic fungi and apicomplexan parasites: crossings between different plant pathogens *Tapesia yallundae* strains yielded progeny with higher level of fungicide resistance (Dyer et al. 2000); sex and recombination were also associated to spread of drug resistance and virulence in human pathogens (Heitman et al. 2014); a highly virulent *Toxoplasma gondii* strain was demonstrated to be produced by out-crossing and that clonal population structure and expansion of an epidemic clone was maintained by selfing (Wendte et al. 2010). Thus, clonal population structures are not an evidence of asexuality, but rather a consequence of repeated unisexual reproduction in a self-compatible strain (Feretzaki and Heitman 2013). Multidrug resistance in *E. histolytica* (Orozco et al. 2002) may be explained by sexual recombination among different strains. Regarding taxonomy, some corrections may issue from molecular data in the same way it happened with some fungi where some species may be synonymized because haploid and diploid forms vary morphologically. *Aspergillus fumigatus*, an ascomycete implicated in human disease was thought to be asexual and recently discovered to have a fully functional sexual cycle (O'Gorman et al. 2009). The sexual part of the cycle (teleomorph) known as *Neosartorya* is now synonymized with *Aspergillus*.

Similarly to other groups of protists, there is a bias of fully annotated amoebozoan genomes currently available toward parasitic organisms, that is, *Entamoeba* species. This is a problem because those groups lack typical mitochondria, have reduced genomes, may perform parasexual processes, may lack mating systems, and are not representative of the biology of Amoebozoa. Thus, they are not reliable for more general studies aiming at deepening our knowledge of evolutionary processes in amoebozoans. Our results indicate that the rich diversity of life cycles, ecological strategies and wide-ranging evolutionary strategies present in the Amoebozoa has, in fact, evolved from sexual populations.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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