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Amoebozoans Are Secretly but Ancestrally Sexual: Evidence for Sex Genes and Potential Novel Crossover Pathways in Diverse Groups of Amoebae

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Abstract

Sex is beneficial in eukaryotes as it can increase genetic diversity, reshuffle their genomes, and purge deleterious mutations. Yet, its evolution remains a mystery. The eukaryotic clade supergroup Amoebozoa encompasses diverse lineages of polymorphic amoeboid forms, including both free-living and parasitic lineages. The group is generally believed to be asexual, though recent studies show that some of its members are implicated in cryptic forms of sexual cycles. In this study, we conduct a comprehensive inventory and analysis of genes involved in meiosis and related processes, in order to investigate the evolutionary history of sex in the clade. We analyzed genomic and transcriptomic data of 39 amoebozoans representing all major subclades of Amoebozoa. Our results show that Amoebozoa possess most of the genes exclusive to meiosis but lack genes encoding synaptonemal complex (SC). The absence of SC genes is discussed in the context of earlier studies that reported ultrastructural evidence of SC in some amoebae. We also find interclade and intrageneric variation in sex gene distribution, indicating diversity in sexual pathways in the group. Particularly, members of Mycetozoa engage in a novel sexual pathway independent of the universally conserved meiosis initiator gene, *SPO11*. Our findings strongly suggest that not only do amoebozoans possess sex genes in their genomes, but also, based on the transcriptome evidence, the present sex genes are functional. We conclude that Amoebozoa is ancestrally sexual, contrary to the long held belief that most of its members are asexual. Thus, asexuality in Amoebozoa, if confirmed to be present, is a derived-trait that appeared later in their evolution.

Key words: sexual reproduction, genome, transcriptome, gene inventory, meiosis, life cycle.

Introduction

Understanding the origin and evolution of sex in eukaryotes has proven a formidable task (Bonner 1944; Wenrich 1954; Goodfellow et al. 1974; Bé and Anderson 1976; Schuster 1976; Raikov 1982; Goldstein 1997; Kondrashov 1997; Goldstein 1999b; Parfrey et al. 2008; Garg and Martin 2016). This challenge is exacerbated as microbial eukaryotes have long been excluded from the discussion due to the false assumption that they are primitive (Haeckel 1866) and asexual (Maynard Smith 1978). Despite this assumption, several microbial eukaryotes including some amoebozoans, the focus of this study, are believed to engage in sexual acts (Martin and Alexopoulos 1969; Arnold 1972; Erdos et al. 1973a; Goodfellow et al. 1974; Erdos et al. 1975; Schuster 1976; Dacks and Roger 1999a; Goldstein 1999a; Ramesh et al. 2005; Stanley 2005). However, the exact mechanism of sexual developments in most of these lineages is obscure or deviant from those observed in animals, fungi and plants (Lahr et al. 2011c).

The Amoebozoa encompasses diverse groups of amoebae characterized by complex and diverse life cycles (Erdos et al. 1973b; Goodfellow et al. 1974; Schuster 1976; Raikov 1995; Adl et al. 2012; Tekle et al. 2014), most of which are traditionally considered asexual. The group includes lobose naked (e.g., *Amoeba proteus*) and testate (e.g., *Arcella*) amoebae, pelobionts (e.g., *E. histolytica*), lobose flat-shaped amoebae (e.g., *Acanthamoeba*), cellular (dictyostelid), and acellular (e.g., myxogastrid) slime molds as well as some less known

diverse forms (reticulate, filose and amoeboflagellate). The observed life cycles in the group range from simple binary fission or alternating a/sexual morphotypes to formation of cysts or spores involving fruiting bodies. Asexuality for some members of this group is either used as a defining character (Hurst et al. 1992) or implied due to absence of reports of sexuality (Cavalier-Smith 2002).

The hallmark of sex is meiosis, a well-defined process that allows reduction of parental ploidy by half and genetic exchange through crossing over between homologous chromosomes. The physical and molecular components of meiosis in multicellular eukaryotes are well characterized and have been used as a blueprint to explore sexuality in other microbes (Olive 1963; Raikov 1982; Mignot and Raikov 1992; Ramesh et al. 2005; Poxleitner et al. 2008; Chi, Mahe et al. 2014; Chi, Parrow et al. 2014). Attempts on the observation of meiosis in most microbes have been very challenging, due to a combination of challenges in cultivation combined with complex life cycles. Moreover, canonical meiosis as observed in animals, fungi and plants is rarely observed in amoebae and most other eukaryotic microbes (Erdos et al. 1973b; Raikov 1982; Mihake 1996; Tekle et al. 2014). Thus, lack of physical evidence of sexual stages including meiosis has led some to assume that some microbes are exclusively asexual (Maynard Smith 1978; Tibayrenc et al. 1990). Contrary to this belief, some recent cytological studies using advanced techniques have shown some aspects of sexual stages in a few eukaryotic microbes, including members of Amoebozoa (Poxleitner et al. 2008; Tekle et al. 2014). For example, our recent study shows that *Cochliopodium*, previously considered asexual, engage in multiple cell fusion followed by karyogamy (nuclear fusion) to form a large polyploid plasmodium, which eventually fragments into uninucleate amoebae (Tekle et al. 2014). This process likely allows the amoeba to undergo genetic exchange through random mixing of chromosomes from multiple individuals.

In general, we can identify four categories of sexual stages where meiosis or nuclear fusion is assumed to occur in Amoebozoa. These include sexual cysts (Erdos et al. 1973b; Goodfellow et al. 1974; Seravin and Goodkov 1987; Mignot and Raikov 1992; Smirnov and Goodkov 1999; Ehrenkaufer et al. 2007), vegetative cellular and/or nuclear fusion (Seravin and Goodkov 1987; Michel and Smirnov 1999; Tekle et al. 2014), a distinct sexual morphotype (Schuster 1976) and putative amoeboid or flagellate gametes (Wrigley de Basanta et al. 2012). The complete stages of canonical meiosis have never been observed in any amoebae studied; meiosis is simply assumed to occur during these various putative sexual stages (Lahr et al. 2011c). It is likely that amoebozoans have several ways of achieving the products of sex, as evidenced by the varied life cycles reported for them (Erdos et al. 1973b; Blanc et al. 1989; Tekle et al. 2014). The evolution of these putative sexual stages within Amoebozoa is poorly understood, as the described life cycles are diverse, and in some instances, amoebae seem to have evolved similar life cycles independently. For example, studies show that amoebozoans such as *Endostelium* (Olive et al. 1984; Kudryavtsev et al. 2014) are capable of producing fruiting bodies, a character mostly attributed to the distantly related protostelid amoebae (slime molds). Similarly, sexual cysts are reported in some distantly related amoebozoan lineages (Goodfellow et al. 1974; Mignot and Raikov 1992). Lahr et al. (2011) provided a detailed account of amoeboid (a)sexuality, showing that seven of the approximately 14 lineages of Amoebozoa reviewed might be implicated in sex.

Whereas these are compelling reports on sexuality in these amoebae, most of the evidence described needs further investigation due to its incomplete or circumstantial nature. For example, in *Cochliopodium*, mechanisms of depolyploidization or meiosis still remain to be investigated (Tekle et al. 2014). In some cases, technical complications make a complete study of meiosis in some amoeba difficult. Ultrastructural studies based on transmission electron microscope (TEM) suffer from a lack of sequential sections and other technical difficulties related to fixation that may result in incomplete or artifactual results. Additionally, some amoebae are assumed to undergo meiosis during a cyst stage (Erdos et al. 1973b; Goodfellow et al. 1974; Mignot and Raikov 1992), which creates a technical hurdle for live observation and experimentations; cysts and spores are covered by thick translucent walls, making live observation difficult. Moreover, some published reports have never been reproduced in the laboratory. For example, the life cycle of *Trichosphaerium*—alternating between two morphs, gamont (sexual) and schizont (asexual)—reported by Schaudinn (1899) has not been observed in more recent laboratory cultures.

Views on the evolution and sexuality of microbial eukaryotes have been changing with the ever-growing molecular genetic data. Particularly, the availability of genomes and RNAseq data for some lineages have allowed us to better understand their evolutionary placement in the tree of life (Hampl et al. 2009; Koonin 2010; Grant and Katz 2014; Tekle et al. 2016). The wealth of genetic data have also opened an opportunity to gain new insights on the molecular basis of sex in microbes. These include discovery of molecular signatures of sex, such as the genes involved in recombination (Ramesh et al. 2005; Malik et al. 2008; Chi et al. 2014a). Several putative asexual eukaryotic microbes such as diplomonads (e.g., *Giardia*), some ciliates and members of Amoebozoa (e.g., *Arcella*, *Entamoeba*) are reported to undergo genetic recombination (Lovlie et al. 1988; Blanc et al. 1989; Deak and Doerder 1998; Caccio and Sprong 2010; Lahr et al. 2011b). Further genome exploration in model organisms (e.g., *Dictyostelium*) and important human pathogens (e.g., *Entamoeba, Giardia*) have revealed discovery of homolog genes exclusively used in meiosis (Malik et al. 2008). The discovery of meiosis specific and other sex related genes have been put forward as support for their capability to engage in

sexual reproduction, because such genes would likely be pseudogenized if they were no longer used. This leads to an alternative approach to documenting meiosis that has been exploited in recent studies: searching the genomes of amoebae for genetic signs of sex. Consequently, several studies have successfully documented full complements of meiotic genes in diverse putative asexual microbes including some ciliates (e.g., *Ichthyophthirius multifiliis*) (Chi et al. 2014a), dinoflagellates (Chi et al. 2014b), fungi, diplomonads, and amoebae (Malik et al. 2008; Schurko and Logsdon 2008).

In this study, we conducted a comprehensive sex gene inventory for 39 amoebozoan lineages representing all major subclades of Amoebozoa. We used both genomic and transcriptomic data to investigate the evolution of sex in the group. Our findings show that all analyzed taxa possess several genes unique to meiosis, suggesting conservation of this ancient mechanisms. We conclude that Amoebozoa is ancestrally sexual; thus, asexuality in this group is likely a derived trait that appeared later in their evolution, if indeed they are entirely asexual.

Materials and Methods

Taxa and Genes Studied

We analyzed data for 39 species in Amoebozoa that represent all major subclades (tables 1 and 2). These include taxa belonging to Eudiscosea (17), Mycetozoa (6), Archamoebae (5), Tubulinea (2), Himatismenida (3), Variosea (3), and the *incertae sedis* taxon *Pessonella* sp. ATCC® PRA-29. Among these, eight taxa representing three major subclades have completed genomes (table 2), whereas the data for the rest come from different published RNAseq projects (supplementary table S1, Supplementary Material online). Transcriptome data coverage for the latter taxa varied by species (see supplementary table S1, Supplementary Material online).

We focused on a total of 44 sex-related genes, including 11 meiosis-specific genes, selected based on literature and availability in the OrthoMCL database (http://www.orthomcl.org/orthomcl/). The majority of the genes and taxa analyzed including non-amoebozoan eukaryotes were obtained from a phylogenomic pipeline developed by Grant and Katz (2014). This pipeline includes 13,104 "orthologous groups" (OGs, clusters of homologs organized in OrthoMCL) that are used to capture homologs from newly added taxa. Additional sex related genes and amoebozoan transcriptomes previously not included in the pipeline were later added from OrthoMCL and NCBI databases, respectively. These databases were last accessed June 2016.

Gene Inventory Analysis

Initially, we used the phylogenomics pipeline, a conservative approach, to determine the presence of the sex genes in Amoebozoa. We performed two runs of the pipeline; the first run used a stricter sequence cutoff parameter in Guidance (Penn et al. 2010) of 0.6 and the second used a more relaxed sequence cutoff of 0.3. In both runs, the column cutoff was 0.4. Guidance generates a reference multiple sequence alignment and assigns a score to every residue, which it calculates by building progressive alignments from bootstrap trees (in this case, the number of bootstraps was set to 10) and counting the proportion of alignments that contain the same residue. The average residue score per column and row in the reference alignment can be used to set a column and sequence cutoff, respectively, and any column or row which on average doesn't meet the cutoff is removed from further analysis.

To ensure that no homologs were missed because of taxon removal during Guidance, we then used local BLAST (Altschul et al. 1990) to retrieve potential homologs from each of the analyzed genomes and transcriptomes, as well as from outgroup genomes across the eukaryotic tree of life and genomes of some bacteria and archaea, where available. For the instances where both the taxon and the gene were taken from the phylogenomics pipeline, we used the BLAST results included in the pipeline to find putative homologs for each gene in each species. In cases where either the gene or the taxon was not found in the pipeline, a new BLASTp search was performed using the gene sequences as queries and the taxon sequences as the subject. In both cases, we collected all hits with an *e*-value less than 1e−15. As this sometimes resulted in a very large number of hits, custom Python scripts were used to ensure only one sequence per contig or scaffold was retained, and to remove identical sequences from the subsequent list of hits.

Two alternative methods of homology searching were also tested to ensure that no distant homologs were missed. The first was psi-BLAST (Altschul et al. 1997), which uses hits generated from a first BLASTp run to build a protein profile to BLAST against the chosen protein database. This process can be repeated multiple times, with each repeat adding new hits to the profile to better detect distant homologs. The second homology search program we used was HMMer (hmmer.org, version 3.1b2), which uses an input sequence or alignment to build a hidden Markov model including the protein sequence and predicted secondary structure elements, and searches the given protein database for sequences which match the generated model.

To confirm the presence of the genes for each taxon, as well as differentiate paralogous genes from each other, the remaining hits were aligned using MUSCLE (Edgar 2004) via Seaview (Gouy et al. 2010). Columns with more than 75% missing data were masked, and phylogenetic trees were built using RAxML BlackBox (Stamatakis et al. 2008) through the CIPRES portal (Miller et al. 2010). RAxML analyses were run with default parameters, with the exceptions of the Protein Substitution Matrix (RTREV instead of JTT) and the use of empirical base frequencies. For the trees, outgroups from across

Table 1
Phylogenetic Distribution of Sex Genes in Major Subclades of Amoebozoa

Gene	OG	Archamoeba	Eudiscosea	Himatismenida	Mycetozoa	Tubulinea	Varipodida	*Incertae sedis* (*Pessonella*)
Bouquet Formation								
SAD1	129586	+	+	+	+			+
Crossover Regulation								
DMC1	126834	+	+		+			
HOP1	128667	–	–		+			
HOP2	128568	+	+		+		+	+
MER3	129931	–	+		–		+	
MND1	127882	+	+	+	+		+	+
MSH4	130077	+	+		+			
MSH5	129379	+	+		+			
RED1	180525	–	–		–			
ZIP1	171209	–	–		–			
DNA Damage Sensing/Response								
MEC1/ATR	128386	+	+	+	+	+	+	+
MRE11	127969	+	+		+		+	
RAD17	127538	–	+		+		+	
RAD23	130351	+	+	+	+		+	+
RAD24	126706	+	+	+	+	+	+	+
RAD50	127792	+	+	+	+		+	+
TEL1/ATM	128955	+	+		+	+	+	+
Double-Strand Break Formation								
SPO11	127274	+	+		–			
Double-Strand Break Repair (Nonhomologous End Joining)								
KU70	129086	+	+	+	+	+	+	
KU80	129372	–	+		+		+	
LIG4/DNL1	130132	+	+	+	+		+	+
XRCC4/LIF1	135131	–	+		+			
Double-Strand Break Repair and Meiotic Divisions								
REC8	150817	–	–		–			
Recombinational Repair								
BRCA2	131863	+	+		+			
DNA2	129631	+	+	+	+		+	+
EXO1	127511	+	+	+	+		+	
FEN1	127472	+	+	+	+		+	+
MLH1	127201	+	+		+	+		
MLH3	130552	+	+		+	+		
MMS4/EME1(s)	135664	–	–		+			
MPH1/FANCM(a,m)	128649	–	+	+	+		+	
MSH2	127538	+	+		+	+		
MSH3	130351	+	+		+		+	
MSH6	126895	+	+		+		+	
MUS81	129162	–	+		+		+	
PMS1	128001	+	+	+	+	+	+	
RAD51	126834	+	+	+	+		+	+
RAD52	130806	+	+		+		+	
RAD54	127098	+	+		+	+	+	+
RTEL1	127294	+	+		+		+	+
SGS1	126644	+	+	+	+		+	+
SLX1	128732	+	+		–			
SMC5	128615	+	+	+	+		+	+
SMC6	127751	+	+	+	+		+	+
Total Detected		33	39	17	38	9	28	17

Table 2

Meiosis Genes Inventoried in Amoebozoa Genomes

Gene	*A. castellanii*	*D. discoideum*	*D. purpureum*	*E. dispar*	*E. histolytica*	*E. invadens*	*E. nuttalli*	*P. pallidum*
Bouquet Formation								
SAD1	+	+	+	–	+	+	+	+
Crossover Regulation								
DMC1	+	–	–	+	+	+	+	–
HOP1	–	–	–	–	–	–	–	–
HOP2	+	+	+	+	+	+	+	+
MER3	–	–	–	–	–	–	–	–
MND1	+	+	+	+	+	+	–	+
MSH4	+	+	+	+	+	+	+	+
MSH5	+	+	+	+	+	+	+	+
RED1	–	–	–	–	–	–	–	–
ZIP1	–	–	–	–	–	–	–	–
DNA Damage Sensing/Response								
MEC1/ATR	+	+	+	+	+	+	+	+
MRE11	+	+	+	+	+	+	+	+
RAD17	+	+	+	–	–	–	–	+
RAD23	+	+	+	+	+	+	+	+
RAD24	+	+	+	+	+	+	+	+
RAD50	+	+	–	+	+	+	+	+
TEL1/ATM	+	+	+	+	+	+	+	+
Double-Strand Break Formation								
SPO11	+	–	–	+	+	+	+	–
Double-Strand Break Repair (Nonhomologous End-Joining)								
KU70	+	+	+	–	–	–	–	+
KU80	+	+	+	–	–	–	–	+
LIG4/DNL1	+	+	+	+	+	+	+	+
XRCC4/LIF1	–	+	+	–	–	–	–	+
Double-Strand Break Repair and Meiotic Divisions								
REC8	–	–	–	–	–	–	–	–
Recombinational Repair								
BRCA2	+	+	+	–	–	+	–	–
DNA2	+	+	–	+	+	+	+	+
EXO1	+	+	+	+	+	+	+	+
FEN1	+	+	+	+	+	+	+	+
MLH1	+	+	+	+	+	+	+	+
MLH3	+	+	+	+	+	+	+	+
MMS4/EME1(s)	–	+	+	–	–	–	–	+
MPH1/FANCM(a,m)	+	+	+	–	–	–	–	+
MSH2	+	+	+	+	+	+	+	+
MSH3	+	+	+	–	–	–	–	+
MSH6	+	+	+	+	+	+	+	+
MUS81	+	+	+	–	–	–	–	+
PMS1	+	+	+	+	+	+	+	+
RAD51	+	+	+	+	+	+	+	+
RAD52	+	+	+	+	+	+	+	+
RAD54	+	+	+	+	+	+	+	+
RTEL1	+	+	+	+	+	+	+	+
SGS1	+	+	+	+	+	+	+	+
SLX1	+	–	–	+	+	+	+	–
SMC5	+	+	+	+	+	+	+	+
SMC6	+	+	+	+	+	+	+	+

the eukaryotic tree of life, as well as some bacterial and archaeal taxa when available, were included in the analyses. When the resulting phylogenetic tree showed multiple clusters of sequences (corresponding to gene paralogs), sequences from each cluster were reciprocally BLASTed against NCBI's non-redundant protein database to determine gene identity, and clusters outside the gene of interest were eliminated from the analysis. Presence of each gene in each Amoebozoa taxon was assigned based on remaining taxa in each gene tree; taxa not represented in a given tree were assigned "absent" (with genomes) or "no detection" (with transcriptomes).

Results

Gene Inventory Approaches

We inventoried eight genomes and 31 transcriptomes representing all major subgroups from across Amoebozoa using a phylogenomics pipeline and BLASTp for 11 meiosis-specific and 33 sex-related genes (tables 1 and 2, supplementary table S2 and file S1, Supplementary Material online). Of the genes inventoried, all but three were present in at least one lineage of Amoebozoa, and 15 were present in over half of the taxa analyzed (table 2, supplementary table S2, Supplementary Material online). Similarly, at least one sex related gene was detected in every amoeba analyzed, and 17 of the taxa, including all eight genomes, have over half of the sex genes in this inventory (tables 1 and 2).

Both the strict and relaxed runs of the pipeline returned fewer detections of the sex genes than the BLAST approach, even after confirming BLAST homologs with RAxML trees (data not shown). This is not surprising, as the phylogenomic pipeline was designed for large-scale taxonomic analyses, rather than individual gene identification. Additionally, the results of the psi-BLAST and HMMer searches did not differ significantly from the results of the BLASTp searches (data not shown). For these reasons, as well as to maximize detection of sex genes, we chose to base our further analyses on the hits returned from BLAST and confirmed with RAxML phylogenetic analysis.

Amoebozoa Subclades

Meiosis-related genes were found in every major subclade of Amoebozoa (table 1). Eudiscosea had the largest number of meiosis specific and other sex related genes, with 39 out of 44. Mycetozoa and Archamoebae had similarly high detections, with 38 and 33 genes detected, respectively (table 1). Tubulinea had the lowest rate of detection, with only 9 genes detected. Similarly, subclades Himatismenida and an incertae sedis (ATCC® PRA-29), had the next lowest sex gene detections (table 1). These three lineages (Tubulinea, Himatismenida and ATCC® PRA-29) do not have completed genomes and are represented by lower numbers of transcriptomic data (supplementary table S1, Supplementary Material online). Thus, this low detection may be due to lack of data and representation within these clades, rather than truly lacking many of the meiosis genes.

Eight of the 11 meiosis-specific genes were detected in Amoebozoa. *MND1* is detected in every clade except Tubulinea (table 1). *HOP2* is in every clade except Tubulinea and Himatismenida (table 1). *DMC1*, *MSH4*, and *MSH5* are only detected in Archamoebae, Eudiscosea, and Mycetozoa (table 1). *MER3* is only detected in Eudiscosea and Varipodida. *SPO11* is detected in Archamoebae and Eudiscosea. *HOP1* was consistently absent in almost all Amoebozoa analyzed but was oddly found in one member of Mycetozoa, *P. polycephalum*.

Amoebozoa Genomes

As expected, the eight completed genomes of Amoebozoa showed the highest detection of sex-related and meiosis-specific genes (table 2). Six of the 11 meiosis-specific genes were found within these genomes: *SPO11*, which initiates recombination by creating double-stranded breaks in DNA; *DMC1*, which promotes double-stranded break repair using the homologous chromosome; *MND1* and *HOP2*, which form a heterodimer that stabilizes *DMC1's* association with DNA and promotes Holliday Junction formation; and *MSH4* and *MSH5*, which form a heterodimer that stabilizes recombination intermediates.

Although three of the six meiosis specific genes (*HOP2*, *MSH4*, and *MSH5*; table 2, figs. 1 and 2) were found in all genomes, we observed some variation in the number of presences in the remaining three genes by clades and individual species. *MND1* is found in every genome except *Entamoeba nuttalli* (table 2, fig. 1). *DMC1* and *SPO11* are found in all *Entamoeba* and *Acanthamoeba* genomes (table 2, fig. 2) but are noticeably absent in the three mycetozoan genomes. Additionally, five meiosis-specific genes—*HOP1*, *ZIP1*, *RED1*, *MER3*, and *REC8*—are not found in any of the genomes inventoried. The three genes (*HOP1*, *ZIP1*, and *RED1*), making up the components of the synaptonemal complex (SC), were consistently absent in all genomes (table 2).

We also inventoried 33 sex-related genes in these eight genomes (table 2, supplementary file S1, Supplementary Material online). All 33 of these genes were found in at least one Amoebozoa genome; 20 were found in every genome, and an additional four were found in every subclade but not every species within those subclades (table 2). Six were not found in *Entamoeba*, one was not found in Mycetozoa, and two were not found in *Entamoeba* or *Acanthamoeba* (table 2).

Amoebozoa Transcriptomes

In addition to the eight genomes analyzed, we inventoried transcriptome data of 31 species of amoebae for the same set of sex genes (supplementary table S2 and file S1,

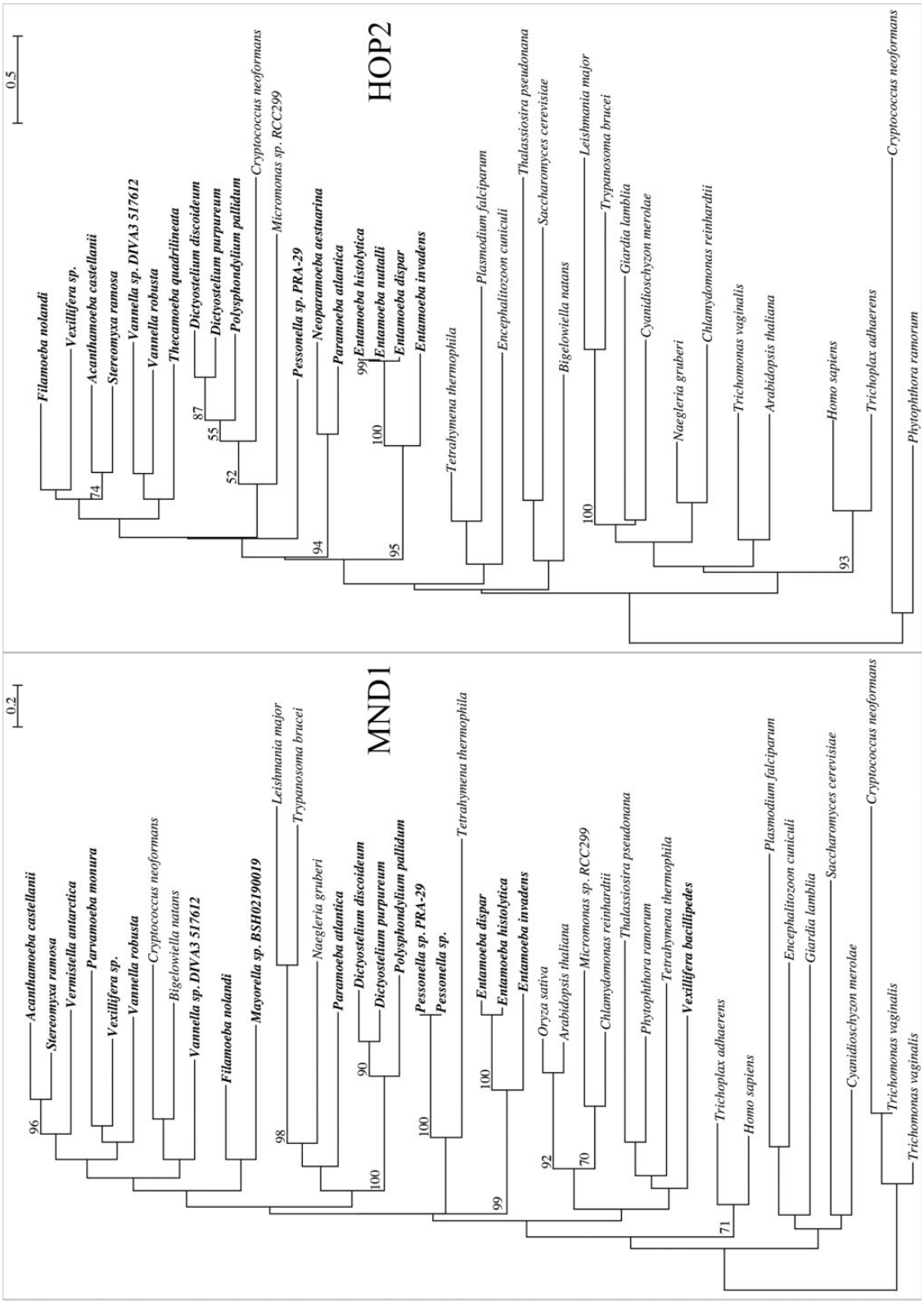

FIG. 1.—Maximum likelihood trees of meiosis-specific genes *MND1* and *HOP2*. Trees rooted at midpoint. Bootstrap support values ≥50% are shown above their bipartitions.

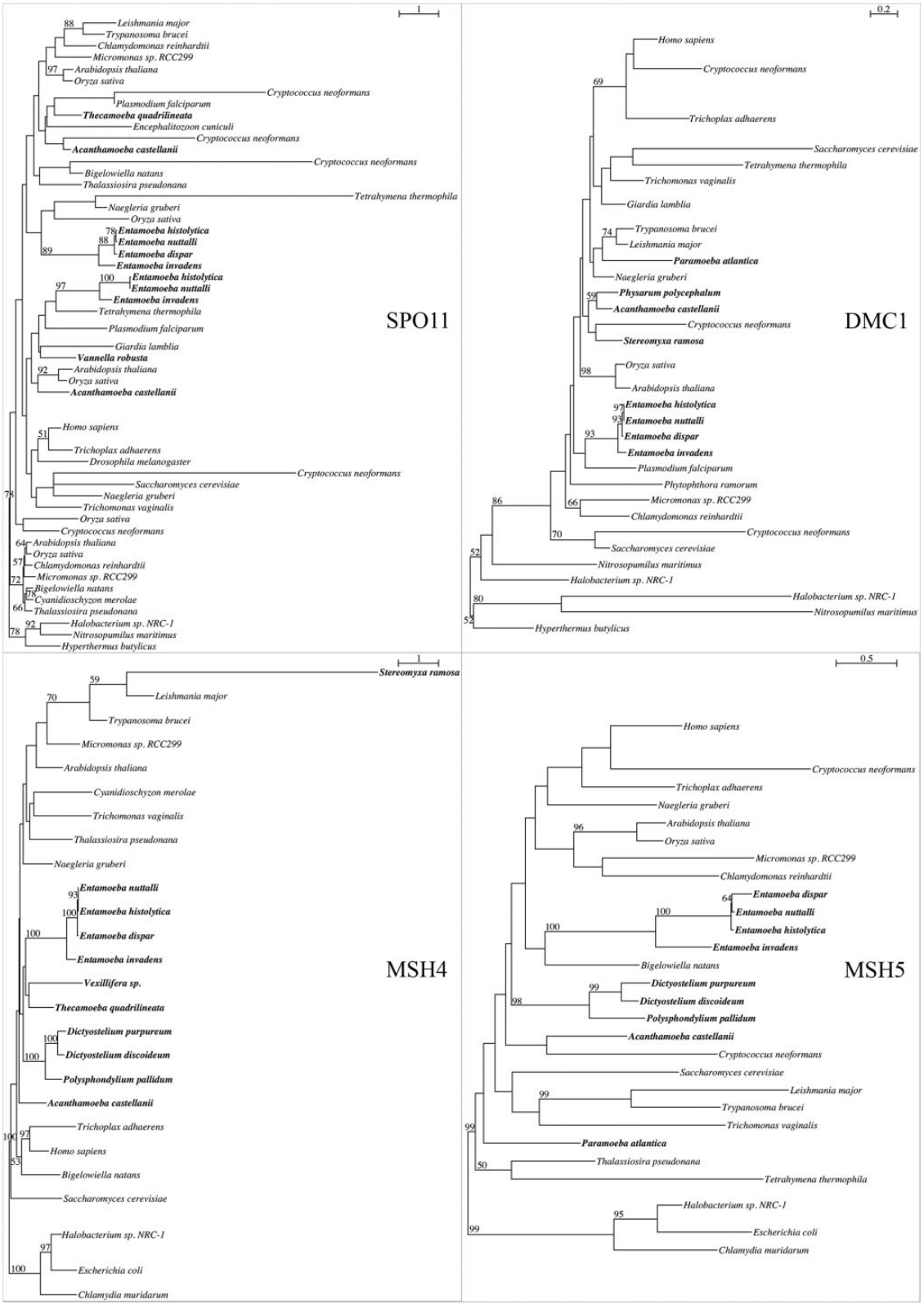

Fig. 2.—Maximum likelihood trees of meiosis-specific genes *SPO11*, *DMC1*, *MSH4*, and *MSH5*. Trees rooted based on prokaryotic outgroup position. Bootstrap support values ≥50% shown above or beside their bipartitions.

Supplementary Material online). Detection of sex genes in these amoebae transcriptomes correlated with the size of data analyzed (supplementary fig. S1, Supplementary Material online) and was much lower than in the genomes. Lineages with smaller transcriptome data generally rendered fewer detections (supplementary fig. S1, Supplementary Material online).

Meiosis-specific genes occur in 14 of the transcriptomes analyzed (supplementary table S2, Supplementary Material online). The most common meiosis-specific gene is *MND1*, occurring in 13 transcriptomes, including three transcriptomes (*Vexillifera bacillipedes*, *Vermistella antarctica*, and *Parvamoeba monura*) for which *MND1* is the only detected meiosis-specific gene (fig. 1, supplementary table S2, Supplementary Material online).

The other relatively common meiosis-specific gene is *HOP2*, occurring in 10 transcriptomes (fig. 2, supplementary table S2 and file S1, Supplementary Material online). *MSH4*, *DMC1*, *MSH5*, *SPO11*, and *MND3* are found in few amoebozoan transcriptomes (figs. 1 and 2, supplementary fig. S2; see supplementary table S2 and file S1 Supplementary Material online). The three genes associated with SC (*HOP1*, *RED1*, and *ZIP1*) and *REC8*, involved in holding sister chromatids together during meiosis, were not detected in any transcriptome analyzed, except for a single detection of *HOP1* in the transcriptome of *P. polycephalum* (fig. S2 and table S2, Supplementary Material online).

Of the 33 sex-related genes, all 33 were detected in at least one transcriptome, and 12 were detected in half or more of the transcriptomes analyzed (supplementary table S2, Supplementary Material online). The transcriptome with the highest number of detected genes was *Vannella sp.* DIVA3 517612, with 29 sex-related genes (figs. 1 and 2; supplementary fig. S2 and table S2, Supplementary Material online). Conversely, the transcriptomes with the lowest number of detections were *Acanthamoeba healyi*, *Ovalopodium desertum*, and *Nolandella abertawensis*, each of which only contained *RAD24* (supplementary table S2 and fig. S2, Supplementary Material online). The low detection rates in these taxa are likely due to low number of available transcriptome data that might have been caused due to the physiological states of the amoebae during RNA collection or methods of sequencing (supplementary table S1 and fig. S1, Supplementary Material online).

Discussion

Amoebozoa Is Ancestrally Sexual

Evidence for the ancestral origin of sex in eukaryotes is accumulating (Dacks and Roger 1999b; Malik et al. 2008; Lahr et al. 2011b). Our findings reinforce this conclusion by providing comprehensive analyses of sex genes in a mostly putative asexual eukaryotic supergroup, Amoebozoa. The current study provides evidence that amoebozoans possess most of the known molecular genetic toolkit important for sex. This finding lends support to the sexual nature of previously unconfirmed life cycles or sex-like (parasexual) behaviors reported in various groups of amoebozoans [reviewed in Lahr et al. (2011b)]. Amoebozoa not only possess sex genes in their genomes, but these genes are also functional and actively expressed, as confirmed by their detection in our transcriptome data. The presence of these genes in the genomes and most of the transcriptome data representing the major subclades of Amoebozoa demonstrates that amoebozoans are ancestrally sexual. Therefore, our study debunks the long held view that the majority of amoebozoans are purely asexual microbes.

Life Cycle and Mechanism of Sex in Amoebozoa

The members of Amoebozoa are extremely diverse in their life cycles, both asexual and sexual. However, the exact mechanisms of sexual development in Amoebozoa are mostly unknown. Even in those model amoebozoan lineages proposed to undergo meiosis during the cyst stage (Mignot and Raikov 1992) or alternating between haploid and diploid stages (Martin and Alexopoulos 1969; Erdos et al. 1973b). The genetic and ultrastructural basis of meiosis is poorly understood. This is mainly due to lack of observation caused by experimental challenges. Our current study shows that observed variations in life cycle and sexual behavior in Amoebozoa reflect a similar variability at the genetic level.

SC Independent Sex in Amoebozoa?

One of the common genetic features observed among all amoebozoan genomes examined is the consistent absence of three meiosis exclusive genes, *HOP1*, *ZIP1*, and *RED1*, involved in SC formation (Dong and Roeder 2000; Muniyappa et al. 2000). The SC is an ultrastructurally detectable protein structure that forms between two pairs of sister chromatids during meiosis (Heyting 1996, 2005). It is believed to facilitate chromosome pairing, synapsis, and recombination. The SC has been used as one of the reliable indicators for the occurrence of meiosis (Aldrich 1967; Heywood and Magee 1976; Raikov 1995) and is commonly found among eukaryotes that undergo conventional sex (von Wettstein et al. 1984; Heyting 1996) including members of Opisthokonta and Archaeplastida. Interestingly, despite the absence of detectable SC genes, some ultrastructural studies report the physical detection of SC in some members of Amoebozoa, including those inventoried in this study. These include SC observation in cysts of *Arcella vulgaris* (Mignot and Raikov 1992) and reproductive cysts or spores of several mycetozoans (Carroll and Dykstra 1966; Aldrich 1967; Erdos et al. 1972). Whereas SC independent recombination pathways are known (Lukaszewicz et al. 2013; Chi et al. 2014a) and amoebae are reported to undergo genetic recombination (Lahr et al.

2011a; Singh, et al. 2013), we find the discrepancies between genetic and ultrastructural evidence for SC quite intriguing.

A similar phenomenon is found in a distant eukaryotic lineage, ciliates. There is ample evidence supporting sexuality in ciliates, including some ultrastructural studies that report SC like structures in some species of ciliates (Raikov 1982; Skarlato 1982; Bobyleva 1984). However, similar to amoebae, ciliates lack clear homologs of genes known to encode SC proteins in their genomes (Chi et al. 2014a). Microscopic observations of SC in ciliates are sporadic. For instance, the model organism, *Tetrahymena thermophila*, is reported to lack SC in meiotic nuclei (Wolfe, et al. 1976). Other ciliates either lack fully mature SC structures or contain only a residual SC structure, resembling those found in fission yeast, *Schizosaccharomyces pombe* (Chi et al. 2014a). Residual SC, also known as linear elements (LinEs), are simple filamentous structures found in *S. pombe* (Loidl 2006). The main structural component of LinE is *REC10*, a distant homolog of *RED1* in budding yeast (Lorenz et al. 2004). Both *RED1* and *REC10* are not detected in amoebae (table 2) or ciliates (Chi et al. 2014a) genomes. The structural and genetic homology of residual SC in ciliates and *S. pombe* remain to be elucidated.

Studies show that there is morphological and genetic variation in the eukaryotic SC (Bogdanov et al. 2007). Whereas SCs show overall similarity in morphology, SCs in plants and animals display size variation based on genome sizes (Bogdanov et al. 2007). Similarly, members of fungi display species-specific banding patterns of the lateral SC elements (Zickler 1973; von Wettstein et al. 1984). The genetic makeup of SC also varies. The genes encoding the central-space protein of the SC in budding yeast (*ZIP1*) and mammals (*SCP1*) share no sequence similarity, but have similar physico-chemical properties (Heyting 1996; Penkina et al. 2002). Therefore, it likely that the microscopically observed SC reported in both amoebae and ciliates might have a different origin or coded by different sets of genes. This might also result due to either rapid evolution of genes or replacement with other gene product. Our current findings necessitate further investigation on the morphologic and genetic origin of SC in both amoebae and ciliates.

A minor crossover (CO) pathway independent of SC is known in plants, vertebrates, and budding yeast (Higgins et al. 2008; Holloway et al. 2008; Lukaszewicz et al. 2013). This type of crossover involves MUS81, a non-meiosis exclusive DNA endonuclease with overlapping function in chromosomal CO pathway. This pathway has been proposed as predominant mechanism of meiotic recombination in lineages that lack SC such as ciliates and budding yeast (Lukaszewicz et al. 2013). Interestingly, amoebae possess a MUS81 homolog (table 2). Given some members of Amoebozoa are reported to engage in meiotic like recombination, amoebae may have independently evolved a mechanism of SC independent meiotic recombination similar to ciliates and budding yeast.

Interaclade and Intrageneric Sexual Pathways Variations

Genome wide exploration of sex genes revealed that variation in sexual pathways might exist in amoebozoans. Previous gene inventory studies that included two amoebozoan genomes (*Dictyostelium* and *Entamoeba*) with limited gene sampling show similar results to ours (Malik et al. 2008). However, our study is more thorough, including greater gene inventory sampling from eight completed genomes representing three major subclades of Amoebozoa as well as additional transcriptome data of various amoebae. This comprehensive sampling enabled us to gain some insights into the evolution of sexual pathways in Amoebozoa.

Comparison of gene inventories in the genomes of the three amoebozoan subclades shows that Eudiscosea (*Acanthamoeba*) and Mycetozoa uniquely share six sex-related genes, which are not detected in Archamoebae (table 2). The functions of these six shared genes include DNA damage sensing/response (*RAD17*), double-strand break repair (non-homologous end-joining, *KU70* and *KU80*) and recombinational repair (*MPH1/FANCM*, *MSH3* and *MUS81*, table 2). Archamoebae and Mycetozoa do not share any of the sex genes inventoried that are not also present in *Acanthamoeba*, whereas Archamoebae shares three sex genes with *Acanthamoeba* which are not present in Mycetozoa (table 2). It is interesting to note that among the sex genes present in Archamoebae and *Acanthamoeba* and absent in Mycetozoa are two of the key meiosis exclusive genes, *SPO11* and *DMC1*. *SPO11* is one of the central and universally conserved meiosis genes that plays a role in initiation of recombination by forming double-strand breaks in DNA (Keeney et al. 1997). *DMC1* encodes the main enzyme in meiosis that promotes recombination between homologous chromosomes by repairing programmed DNA double strand breaks (DSBs) (Bugreev et al. 2011). These differences clearly indicate that there is variation in recombination pathways in Amoebozoa.

The evolutionary relationship of the three subclades (Eudiscosea, Archamoebae, and Mycetozoa) is not well resolved (Tekle et al. 2016). Archamoebae and Mycetozoa are traditionally placed under the more inclusive subclade Conosa (Cavalier-Smith 1998). However, the support for Conosa in molecular studies varies (Tekle et al. 2008; Lahr et al. 2011a; Cavalier-Smith et al. 2014), and it usually is not supported in large-scale analysis (Tekle et al. 2016). The lack of shared sex genes in these subclades is interesting and worth further investigation, though with the current data it is premature to make any evolutionary inferences based on the observed difference of sex genes in these lineages. Besides, sex genes are notorious for convergent evolution, unusual paralogy and relatively accelerated rates of evolution (Malik et al. 2008).

It should be noted that it is common to see independent loss of one or a suite of meiosis-specific genes in sexual eukaryotes. *DMC1*, along with a suite of other genes (*HOP1*,

HOP2, and *MND1*), is missing in *Drosophila*, *Anopheles*, and *Neurospora* (Ramesh et al. 2005; Schurko and Logsdon 2008). Similarly, *Caenorhabditis* lacks *HOP2*, *MND1*, and *DMC1*, while retaining *HOP1* (Malik et al. 2008). All these lineages are sexual and the reported variations show a plasticity that exists in the recombination pathways after the first steps of meiosis. However, given the universal role of *SPO11*, its absence in Mycetozoa is very unusual and poses new evolutionary questions about this subclade. Eukaryotic *SPO11* shows interdomain sequence and structure conservation with one of the Archaeal DNA topoisomerase (Topo VI) subunits (Nichols et al. 1999). *SPO11* is expressed exclusively during meiosis and plays a critical role in the initiation of sex by catalyzing the formation of DSBs prior to synapsis in prophase I of meiosis (Keeney et al. 1997). *SPO11* is ubiquitous; its homologues are found across the tree of life in truly or cryptically sexual eukaryotes (Malik et al. 2008). Whereas independent genes losses and different crossover pathways are known past the first step of meiosis, the initiation of meiosis mediated by *SPO11* is well conserved across eukaryotes (Chi et al. 2014a).

The absence of *SPO11* in Mycetozoa, as evidenced by its absence in all Mycetozoa genomes as well as transcriptomes, adds a further layer of complexity to the evolutionary conundrum and mechanisms of sex in eukaryotes. Members of Mycetozoa are extensively studied for their sexuality and other cellular processes, and their life cycle involving diploid and haploid stages is well documented (Erdos et al. 1973b, 1975). However, the transition between these stages remains obscure due to the technical challenges described earlier. Members of the Mycetozoa are also known to undergo genetic recombination in a consistent manner as in meiosis (Erdos et al. 1975; Francis 1998). These findings posit a novel mechanism of cryptic sexual processes in this lineage. It further questions the universal role of *SPO11* as initiator of meiosis in eukaryotes. A detailed account on the evolutionary role of *SPO11* in general and *SPO11* independent ploidy reduction in *Dictyostelium* in particular is described in a recent review article (Bloomfield 2016).

A few *SPO11*-independent crossover induction pathways have been identified. For example, in *SPO11* null mutants of *Saccharomyces pombe*, knockout of *FEN1*, an exonuclease critical in Okazaki fragment processing in yeast, substantially increased crossover frequency and viability of spores (Farah et al. 2005). Similarly, expression of vertebrate DNA Deaminases in *S. pombe* and *Caenorhabditis elegans SPO11* null mutants also restored crossing over in both organisms (Pauklin et al. 2009). It is possible that members of Mycetozoa induce crossovers by a similar alternative pathway. Alternatively, crossovers in Mycetozoa may be environmentally induced. *Dictyostelium* is known to be highly resistant to irradiation (Deering 1968) and has a very low rate of mutation compared with other eukaryotes (Saxer et al. 2012), suggesting a very efficient DNA repair mechanism that may have evolved under increased mutagenesis pressure. If *Dictyostelium* and other mycetozoans are subjected to increased amounts of DNA damage in their natural environment, this may render additional induction of damage for crossing over redundant and unnecessary (Bloomfield 2016).

In addition to the interclade genomic variation, we also noticed intrageneric genome variations of sex genes in Amoebozoa genomes (table 2). All of observed variations in Mycetozoa are non-meiosis-specific genes, whereas in *Entamoeba* one of the three intrageneric variations was one of the meiosis exclusive genes, *MND1* (table 2); all *Entamoeba* species have this gene except *Entamoeba nuttalli* (table 2). This gene is also found in the remaining whole genome lineages and in most of the amoeba transcriptomes examined (tables 1 and 2). *MND1* works in close conjunction with *HOP2* by forming a heterodimeric complex that interacts with *DMC1* to promote meiotic homolog juxtaposition and strand assimilation (Chen et al. 2004). *MND1* is one of the indispensable genes for meiotic recombination. It is not clear if its absence is due to genome sequence incompleteness or indicative of another deviant pathway employed in this particular species. As described above, loss and gain of sex genes, particularly those not exclusive for meiosis, are common due to redundancy and function overlaps. However, if intrageneric variation like the above example is authentic and of common occurrence, its investigation will further our understanding of the evolution and mechanisms of such genes in cryptic sexual life cycles.

Our study demonstrates that amoebozoans employ diverse sexual pathway strategies to achieve the products of sex (recombination). It further demonstrates that the mechanism of sexuality is as diverse as the reported life cycles in this major clade of eukaryotes. Given this diversity, further genome wide investigations in Amoebozoa will likely unravel yet more unknown mysteries of sexual like processes and contribute substantially to our understanding of the origin and evolution of sex in general, and evolution and the roles of specific sex genes such as *SPO11* in particular.

Supplementary Material

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

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