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Genetic Evidence for Sexuality in *Cochliopodium* (Amoebozoa)

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Abstract

Microbial eukaryotes, including amoeboids, display diverse and complex life cycles that may or may not involve sexual reproduction. A recent comprehensive gene inventory study concluded that the Amoebozoa are ancestrally sexual. However, the detection of sex genes in some lineages known for their potentially sexual life cycle was very low. Particularly, the genus *Cochliopodium*, known to undergo a process of cell fusion, karyogamy, and subsequent fission previously described as parasexual, had no meiosis genes detected. This is likely due to low data representation, given the extensive nuclear fusion observed in the genus. In this study, we generate large amounts of transcriptome data for 2 species of *Cochliopodium*, known for their high frequency of cellular and nuclear fusion, in order to study the genetic basis of the complex life cycle observed in the genus. We inventory 60 sex-related genes, including 11 meiosis-specific genes, and 31 genes involved in fusion and karyogamy. We find a much higher detection of sex-related genes, including 5 meiosis-specific genes not previously detected in *Cochliopodium*, in this large transcriptome data. The expressed genes form a near-complete recombination machinery, indicating that *Cochliopodium* is an actively recombining sexual lineage. We also find 9 fusion-related genes in *Cochliopodium*, although no conserved fusion-specific genes were detected in the transcriptomes. *Cochliopodium* thus likely uses lineage specific genes for the fusion and depolyploidization processes. Our results demonstrate that *Cochliopodium* possess the genetic toolkit for recombination, while the mechanism involving fusion and genome reduction remains to be elucidated.

Subject area: Reproductive strategies and kinship analysis
Key words: fusion, gene inventory, karyogamy, meiosis, sexual reproduction, transcriptome

Sexual reproduction, commonly defined as a process of ploidy reduction and restoration via meiosis and nuclear fusion (karyogamy) of gametes, respectively, is one of the major features of eukaryotes. Although well-documented in multicellular eukaryotes (i.e., plants and animals), sexual processes are generally poorly understood in most microbial eukaryotes including Amoebozoa (Wenrich 1954; Raikov 1982; Kondrashov 1997; Parfrey et al. 2008; Lahr et al. 2011; Tekle et al. 2014; Garg and Martin 2016). This is mainly due to diverse and complex life cycles and difficulties in cultivating and observing sexual reproduction in microorganisms. Moreover, most eukaryotic microbes were previously assumed be to asexual (Haeckel 1866; Maynard Smith 1978). More recently, however, sex or recombination has been observed in a number of microbial lineages across the eukaryotic tree of life (Erdos et al. 1973, 1975; Blanc et al. 1989; Mihake 1996; Chepurnov et al. 2004; Singh et al. 2013). In others, processes similar to sex but apparently missing one or more parts of the full sexual cycle, including meiosis, have been documented (Poxleitner et al. 2008; Carpenter et al. 2012; Tekle et al.

2014). These sex-like processes, where parts of the conventional sexual stages as seen in plants and animals are not observed, are collectively called "parasexual." Amoebozoans display diverse parasexual life cycles, involving presumed sexual stages during the cyst (Erdos et al. 1973; Mignot and Raikov 1992; Singh et al. 2013) or vegetative stage (Tekle et al. 2014), or alternating sexual and asexual stages (Schaudinn 1899). The genetic basis of these various parasexual pathways and their evolutionary origin is poorly understood. However, genetic evidence, including gene inventories documenting meiosis and karyogamy, has been reported in various microbial eukaryotes including amoebozoans, indicating that sex is ancestral in eukaryotes (Ramesh et al. 2005; Malik et al. 2008; Chi et al. 2014a, 2014b; Patil et al. 2015; Speijer et al. 2015; Tekle et al. 2017). Genetic and cytological studies elucidating the diverse parasexual pathways observed in microbial eukaryotes will greatly enhance our understanding of the evolution and origin of sex in eukaryotes.

Cochliopodium, the focus of this study, is a genus of discoid or globose amoebae within the supergroup Amoebozoa (order Himatismenida), characterized by a dorsal covering, or tectum, composed of surface microscales. The genus consistently forms a strongly-supported monophyletic group (Kudryavtsev et al. 2005, 2011; Lahr et al. 2011; Tekle et al. 2008, 2016). The genus includes around 25 described species (Kudryavtsev et al. 2005, 2011; Anderson and Tekle 2013; Tekle et al. 2013, 2015), several of which are known to undergo a unique and unusual process of cellular fusion, karyogamy, and fission (Tekle et al. 2014). During this process, multiple cells from the same culture undergo cellular fusion resulting in giant, multinucleated cells, which then undergo karyogamy to form polyploid nuclei. These cells and their nuclei eventually fragment back into single, uninucleate cells, apparently without undergoing visible meiosis. This lack of observation of conventional meiosis, has led to the description of this process as parasexual (Tekle et al. 2014). The genetic basis of fusion and karyogamy in *Cochliopodium*, as well as the mechanism of the fragmentation process, is currently unknown.

Cellular fusion is commonly observed in most eukaryotic clades including several members of Amoebozoa (Page 1971; Seravin and Goodkov 1984; Michel and Smirnov 1999; Lahr et al. 2011; Tekle et al. 2014). However, the genetic basis of cellular fusion and karyogamy in eukaryotes has been difficult to elucidate. This is mainly due to the fact that many of the involved genes are lineage-specific (Chen et al. 2007; Aguilar et al. 2013). However, a few genes involved in fusion seem to be conserved across eukaryotic species. For example, the cell fusion protein HAP2 (GCS1), an ancient derivative of the viral class II fusogens (Fedry et al. 2017; Pinello et al. 2017) has been detected across major eukaryotic clades (Wong and Johnson 2010; Speijer et al. 2015). The functional conservation of HAP2 has been confirmed in deletion and rescue assays in diverse lineages such as *Chlamydomonas* and *Plasmodium* (Hirai et al. 2008; Liu et al. 2008), *Tetrahymena* (Cole et al. 2014), and *Dictyostelium* (Okamoto et al. 2016). Even in some organisms where HAP2 does not seem to be present, such as nematodes and vertebrates (Wong and Johnson 2010; Speijer et al. 2015), other viral class II fusogens have been described which function in gamete or somatic cell fusion (Avinoam and Podbilewicz 2011; Perez-Vargas et al. 2014). Another conserved protein is GEX1 (fungal ortholog: KAR5; Ning et al. 2013), a karyogamy gene, which is even more widespread in eukaryotes than HAP2 (Speijer et al. 2015). GEX2 is known to be functionally conserved in fungi (Beh et al. 1997; Melloy et al. 2009), *Chlamydomonas*, *Plasmodium* (Ning et al. 2013), and vertebrates (Abrams et al. 2012). These genes are among the prime targets for detection in *Cochliopodium*, given the extensive cellular

and nuclear fusion observed in the genus. The detection of these genes in *Cochliopodium* would indicate fusion and karyogamy in *Cochliopodium* are derived from evolutionarily conserved eukaryotic gamete fusion factors, supporting sexual nature of the behavior observed in this amoeba.

Meiosis, a signature of sexual reproduction, is a process by which genetic material is exchanged and halved in progeny cells. Unlike fusion, the genetic toolkit of meiosis is well-conserved across eukaryotes, enabling the formation of a core inventory of meiotic machinery, which can be tested in diverse eukaryotes (Ramesh et al. 2005; Malik et al. 2008; Schurko and Logsdon 2008). Meiotic gene inventories take a genomics-level approach to documenting meiosis in lineages where sexual reproduction has not previously been recognized. This approach has successfully been used to detect complements of meiotic genes in diverse eukaryotic microbial lineages such as Diplomonads (Ramesh et al. 2005), Parabasalia (Malik et al. 2008), Ciliates (Chi et al. 2014), Dinoflagellates (Chi et al. 2014), Diatoms (Patil et al. 2015), and Amoebozoa (Tekle et al. 2017). The presence of meiosis genes in a lineage is thought to indicate the presence of meiosis, as these genes would likely decay by genetic drift or other processes if meiosis was lost. Because of its life cycle, *Cochliopodium* is an excellent target for an inventory of meiosis and sex-related genes to determine whether it is (or was recently) capable of true sexual reproduction. In general, these inventories are performed on whole genomes of an organism (e.g., Ramesh et al. 2005; Malik et al. 2008; Chi et al. 2014a, 2014b; Patil et al. 2015) to ensure maximal detection of sex-related genes. However, no *Cochliopodium* genome has been sequenced, and sequencing such a genome is challenging at present due to the size and complexity of Amoebozoa genomes (Glöckner and Noegel 2012). Instead, transcriptome data, which is easier to collect from Amoebozoans, can be de novo assembled and inventoried. This will likely miss some genes that are lowly- or non-expressed, however, it will also provide evidence for the activity and functionality of the genes in the organism.

A previous study found that Amoebozoa is ancestrally sexual, with sex genes well distributed across its phylogeny (Tekle et al. 2017). However, the life cycles found in the supergroup are extremely diverse and can be complex, with varying amounts of a/sexuality (Parfrey et al. 2008; Lahr et al. 2011). The genomes included in the previous study showed an abundance of sex and meiosis-specific genes. However, the wide variation in detection of the sex genes across clades and species (Tekle et al. 2017) resulted in little ability to determine possible genetic bases for the life cycles represented by the transcriptomes. One such transcriptome of a *Cochliopodium* species, *Cochliopodium minutoidum*, had a very low detection of sex genes and no meiosis-specific genes, which was surprising given the parasexual life cycle of the genus. However, the transcriptome used had very low amount of data, which may have led to an artificially low detection of sex genes (Tekle et al. 2017). Additionally, this species fuses much less frequently than some other species within the genus (Tekle et al. 2014), so genes related to its life cycle may not have been highly expressed when the data were collected.

In this study, we used next-generation sequencing methods to generate more comprehensive transcriptomes from 2 cochliopodiums, *Cochliopodium pentatrifurcatum* and *Cochliopodium minus*, known to undergo extensive cellular and nuclear fusion. Using these data we inventoried 31 genes involved in fusion and karyogamy in various species, including HAP2 and GEX1, as well as a general meiotic inventory of 11 meiosis-specific and 49 sex-related genes in *Cochliopodium*, to determine whether its unusual life cycle could be based on conserved sex genes. This deep sequencing enabled a

much higher detection of the sex-related genes, including 5 meiosis-specific genes, than from the previous *C. minutoidum* study (Tekle et al. 2017). Three fungal plasmogamy and 6 fungal karyogamy genes were also detected. Although neither HAP2 nor GEX1 were detected in *Cochliopodium*, the much greater detection of the sex genes lends strong support to the sexual nature of the complex life cycle observed in *Cochliopodium*.

Materials and Methods

RNA Isolation and cDNA Synthesis

Cochliopodium pentatrifurcatum and *C. minus* (CCAP 1357/1A) were grown in a minimum of 4 plastic petri dishes with bottled natural spring water (Deer Park®; Nestlé Corp. Glendale, CA) and autoclaved grains of rice. We observed cultures to confirm that all life cycle stages were represented in large quantities before collecting RNA. After minimum 1 week incubation at room temperature, cells in culture had begun the fusion process, resulting in a mixture of the life cycle stages. Adherent amoebae were removed from dishes and transferred to 15 mL conical tubes. Total RNA was isolated from collected amoebae using a NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol. RNA was quantified using a Qubit® RNA HS Assay Kit and Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, CA). Quality of isolated RNA was measured by visualizing on an Ambion® NorthernMax® denaturing formaldehyde gel (Thermo Fisher Scientific, Waltham, MA). Small (18S) and large (28S) subunits of rRNA bands were inspected visually for intensity and smearing to assess RNA degradation. cDNA was synthesized from total RNA using a Thermo Scientific™ RevertAid™ Premium Double Stranded cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA quantity was measured using a Qubit® DNA HS Assay Kit on the above Qubit® fluorometer.

To maximize our detection of the inventoried meiosis and fusion genes, data were also included from other RNA-seq experiments in both species. Additional cDNA was generated from *C. pentatrifurcatum* by picking 1–5 single cells from culture into 0.2 mL PCR tubes. RNA was isolated from the cells and cDNA was synthesized using a SMART-Seq® v4 Ultra® Low Input RNA Kit (Takara Bio USA, Mountain View, CA) per manufacturer's instructions, except that one quarter of the recommended reagents were used. Additional cDNA was also generated from *C. minus* by isolating total RNA with the NucleoSpin® kit, then synthesizing cDNA with the SMART-Seq® kit. cDNA quantity was measured using the DNA HS Assay kit with the Qubit® flurometer, as above.

Preparation of Libraries and Sequencing

Libraries were prepared from 1 ng cDNA using a Nextera® XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA) according to manufacturer's instructions, without the normalization step. Libraries were quantified using the DNA HS Kit with the Qubit® fluorometer, as above, and sequenced on a HiSeq 2500 in paired-end, high-output mode with 75 bp reads at Yale Center for Genomic Analysis. 10 cDNA libraries were multiplexed in one sequencing lane—4 from *C. pentatrifurcatum*, 2 from *C. minus*, and 4 from other Amoebozoa (*Pellita catalonica*, *Mayorella cantabrigiensis*, *Flamella aegyptia*, and *Endostelium zonatum*) not used in this study.

Sequence Assembly

Sequencing generated 28.8–72 million raw reads per sample (Supplementary Table S1). FastQC (http://www.bioinformatics. babraham.ac.uk/projects/fastqc/) was used to inspect reads for quality and length. BBDuk (Joint Genome Institute, US Department of Energy, Walnut Creek, CA) was used to remove low quality bases (Phred score below 30) and Illumina adaptor sequences from the reads, discarding short reads (length below 35) after trimming (Supplementary File S1). The remaining reads from each sample were separately assembled de novo using rnaSPAdes (Bankevich et al. 2012) with default parameters (Supplementary File S1). Contigs less than 300 bp in length were removed from each assembly. Ribosomal sequences were sorted into a separate file by blasting the assemblies with blastn (Altschul et al. 1990) against a Refseq database of ribosomal sequences, with an e-value cutoff of 10^{-10} (Supplementary File S1). All hits below the cutoff were removed. The remaining contigs were then enriched for eukaryotic sequences using usearch, a faster alternative to blastx (Edgar 2010). Contigs were blasted with usearch against RefSeq eukaryotic and prokaryotic databases with an e-value cutoff of 10^{-15} (Supplementary File S1). Contigs were kept if the e-value for the best eukaryotic hit was more than 100 times smaller than the e-value for the best bacterial hit, or if there was a eukaryotic hit but no bacterial hit. The resulting eukaryotic and ribosomal contigs from each assembly were then combined into 2 files representing *C. pentatrifurcatum* and *C. minus* and duplicate contigs within each file were removed.

Gene Inventory

A total of 31 genes involved directly or indirectly in fusion or karyogamy in various organisms were chosen for inventory in *Cochliopodium* based on a literature review searching for factors known to be involved in fusion or karyogamy in other eukaryotic organisms—mostly fungi (Rose 1996; Melloy et al. 2009; Aguilar et al. 2013; Merlini et al. 2013). These genes were added to 11 meiosis-specific and 49 sex-related genes, chosen based on previous studies (Ramesh et al. 2005; Malik et al. 2008; Schurko and Logsdon 2008; Chi et al. 2014a, 2014b; Patil et al. 2015; Tekle et al. 2017). The new transcriptomes were inventoried for these genes following the methods of Tekle et al. (2017). Briefly, sequences for each gene were collected from orthologous groups (OGs) within the OrthoMCL database (http://www.orthomcl.org/orthomcl/) via a phylogenomic pipeline developed by Grant and Katz (2014), or directly from the database when the OG was not present in the pipeline. We used a custom Python script (Supplementary File S2) to blast each OG against each transcriptome using tblastn (Altschul et al. 1990), collecting all nonredundant hits with an e-value less than 1e-15. To ensure that no distant homologs were missed, we also used HMMer (hmmer.org, version 3.1b2) to build profiles from alignments of the OG sequences and search translated versions of the transcriptomes (Supplementary File S1). To remove potential paralogous false positives among the meiosis-specific genes with positive hits in *Cochliopodium*, hits were aligned with sequences previously discovered by Tekle et al. (2017) using MAFFT (Katoh and Standley 2013), columns with more than 75% missing data were removed, and gene trees were built using RAxML (Stamatakis et al. 2008) as implemented on the CIPRES portal (Miller 2010) with PROTGAMMALGF matrix and default parameters (Supplementary File S1). Similarly, to confirm hits and assess broad conservation or lineage specificity in the chosen fusion and karyogamy genes, Blast search was conducted on 6 outgroup taxa from across eukaryotic subgroups. Hits were collected and aligned with the *Cochliopodium* hits, and gene trees were inferred using RAxML as above (Supplementary File S1). Sequences from each cluster on the resulting trees were blasted using blastp against NCBI's nonredundant (nr) protein database to determine cluster

identity and definitively assign presence/nondetection to the new transcriptomes. When the number of returned hits for an OG was too large to infer the sequence tree in a timely manner, the alignments were manually inspected and sequences were blasted against the NCBI nr database to confirm the identity of the hits. We also re-inventoried the transcriptome of *C. minutoidum* as a control to ensure that genes in the new transcriptomes were not over- or under-detected compared to the previous inventory (Tekle et al. 2017).

Results

Transcriptome Data

Extensive sequencing was used to generate transcriptomes for 2 species of *Cochliopodium*, *C. minus* and *C. pentatrifurcatum*. The amount of data for each of these transcriptomes is much larger than the previous transcriptome of *C. minutoidum*; the *C. minus* transcriptome contains 11 078 nonredundant eukaryotic contigs in 17.5 MB of data, while the *C. pentatrifurcatum* transcriptome contains 9430 contigs in 11.8 MB of data (Supplementary Table S1). For comparison, the previous *C. minutoidum* transcriptome (Cavalier-Smith et al. 2015) had 4013 contigs in only 1.4 MB of data.

Meiosis Specific and Sex-Related Genes

A total of 60 sex-related genes, including 11 meiosis-specific genes, were inventoried in the transcriptomes of *Cochliopodium*. Of the 11 meiosis-specific genes inventoried, 5 were detected in *Cochliopodium* (Table 1; Figure 1). Four of these were detected in both *Cochliopodium* species (DMC1, MND1, HOP2, and MSH4), while one, SPO11, was only detected in *C. minus* (Table 1; Figure 1). Of the genes not detected in *Cochliopodium*, 4 (HOP1, RED1, ZIP1, and REC8) are also absent in the rest of Amoebozoa and are thus unlikely to be present in *Cochliopodium*, while the remaining 2 (MER3 and MSH5) are detected to varying degrees in the rest of Amoebozoa (Tekle et al. 2017). None of these genes were detected in *C. minutoidum* (Table 1; Figure 1), congruent with previous study (Tekle et al. 2017). A total of 49 nonmeiosis-specific sex-related genes were also inventoried in *Cochliopodium*. Of these, 35 were detected in both *C. pentatrifurcatum* and *C. minus*, and 7 of these 35 were also detected in *C. minutoidum* (Table 1). An additional 4 were detected in 1 of the 2 species; 3 in *C. minus* and 1 in *C. pentatrifurcatum* (Table 1).

Fusion- and Karyogamy-Related Gene Inventory

Of the 31 fusion- and karyogamy-related proteins inventoried in this study, 9 were detected in *Cochliopodium* (Table 2; Figure 1). Two of these genes—BNI1 and MYO2—are actin-associated proteins involved in plasmogamy in fungi; one, KEX2, is a protein processing enzyme acting upstream of plasmogamy factors; and the remaining 6—CDC28, KEM1, KAR3, CIN4, CDC4, and KAR2—are fungal karyogamy factors. Notably, neither HAP2 nor GEX1 were detected in *Cochliopodium* (Table 2). Homologs of all of the above proteins except CDC4, as well as 4 other fusion and karyogamy genes (CIN2, KAR4, JEM1, and SEC63), were found across eukaryotic supergroups (Table 2; Figure 1). CDC4 and 2 other genes (RVS161 and CDC34) were detected only in Amorphea (Opisthokonts + Amoebozoa) (Table 2). The remaining genes were specific to Opisthokonts (Table 2), many of which were only found in the group they were originally described in, either fungi (*Saccharomyces*) or metazoa (*Homo*) (data not shown).

Discussion

Extensive Transcriptome Sequencing Reveals Meiosis Genes in Cochliopodium

A recent study of sex gene inventory in Amoebozoa based on genome and transcriptome data showed that most amoebae possess and express several sex-related and meiosis-specific genes (Tekle et al. 2017). This study concluded that Amoebozoa is ancestrally sexual. However, the detection of sex genes in Amoebozoans was not uniform. Many of the transcriptomes showed a lower detection of sex genes than the genomes, and this detection varied with the amount of data; that is, amoebae represented by large transcriptomes had more sex-related/meiosis genes detected than those represented by smaller amounts of transcriptome data (Tekle et al. 2017). This, combined with the diversity and complexity of Amoebozoan parasexual cycles, limited the ability to determine the genetic basis for any particular Amoebozoan life cycle. In *C. minutoidum*, none of the meiosis exclusive genes were detected (Tekle et al. 2017). This result was surprising given the reported parasexual life cycle of *Cochliopodium* involving extensive cellular and nuclear fusion (Tekle et al. 2014). *Cochliopodium minutoidum* is among species that is represented by a very low amount of transcriptome data, therefore, it is likely that the lower detection of sex genes might be due to the amount of data analyzed. It is also possible that available data reflected the physiological state of the amoeba at the time the transcriptome data were collected, or might correspond to the lower rate of fusion observed in this amoeba (Tekle et al. 2014). In this study, we collected much larger amounts of transcriptome data, representing the different phases of the life cycle, of 2 cochliopodiums that are known for their high frequency of cellular and nuclear fusion (Tekle et al. 2014). The newly sequenced transcriptomes of *C. pentatrifurcatum* and *C. minus* both have a much higher representation of data than the previously sequenced transcriptome of *C. minutoidum*. These newly generated data not only show a much higher detection of sex-related genes in general but also detection of meiosis specific genes in both cochliopodiums (Table 1; Figure 1)—in fact, the detection of sex genes in this study is comparable to that of sequenced genomes in the previous study (Tekle et al. 2017). This finding lends support to the sexual nature of the life cycle of *Cochliopodium* involving extensive cellular and nuclear fusion followed by fission.

The 5 meiosis-specific genes detected in *Cochliopodium* (Table 1; Figure 1) are all directly involved in the recombination process. SPO11 initiates recombination by causing double-stranded breaks (Keeney et al. 1997). HOP2 and MND1 form a heterodimer, which interacts with DMC1 and its nonmeiosis-specific homolog, RAD51, to promote homologous recombination (Chen et al. 2004; Bugreev et al. 2011). Finally, MSH5 acts to resolve crossover intermediates (Hollingsworth et al. 1995), usually as a heterodimer with MSH4 (Novak et al. 2001; Nishant et al. 2010). Thus, genes for the full primary pathway of recombination are present in *Cochliopodium*. Furthermore, the functions performed by the meiotic genes not detected in *Cochliopodium* could likely be replaced using nonmeiosis-specific genes. For example, REC8, which is involved in establishing the cohesin complex that holds sister chromatids together during meiosis (Watanabe and Nurse 1999), has a nonmeiosis-specific homolog, RAD21, which performs the same function in mitosis (Michaelis et al. 1997). RAD21 is present in both cochliopodiums, and the functions of RAD21 and REC8 appear to be interchangeable when only one is present in an organism (Xu et al. 2004; Howard-Till et al. 2013; Patil et al. 2015). As

Table 1. Meiosis gene inventory in 3 *Cochliopodium* species

Gene	OG	*Cochliopodium minus*	*Cochliopodium pentatrifurcatum*	*Cochliopodium minutoidum*
Bouquet formation				
SAD1	OG5_129586	+	+	nd
Cell cycle regulation				
CDC20	OG5_126765	+	+	nd
Crossover regulation				
DMC1	**OG5_126834**	**+**	**+**	**nd**
HOP1	**OG5_128667**	**nd**	**nd**	**nd**
HOP2	**OG5_128568**	**+**	**+**	**nd**
MER3	**OG5_129931**	**nd**	**nd**	**nd**
MND1	**OG5_127882**	**+**	**+**	**nd**
MSH4	**OG5_130077**	**+**	**+**	**nd**
MSH5	**OG5_129379**	**nd**	**nd**	**nd**
RED1	**OG5_180525**	**nd**	**nd**	**nd**
ZIP1	**OG5_171209**	**nd**	**nd**	**nd**
DNA damage sensing/response				
MEC1/ATR	OG5_128386	+	+	+
MRE11	OG5_127969	+	+	nd
RAD1/MEI9	OG5_130438	+	+	nd
RAD17	OG5_127538	nd	+	nd
RAD23	OG5_130351	+	+	nd
RAD24	OG5_126706	+	+	+
RAD50	OG5_127792	+	+	+
TEL1/ATM	OG5_128955	+	+	nd
XRS2	OG5_180328	nd	nd	nd
Double-strand break formation				
SPO11	**OG5_127274**	**+**	**nd**	**nd**
REC114	OG5_142109	nd	nd	nd
Double-strand break repair (nonhomologous end joining)				
KU70	OG5_129086	+	+	nd
KU80	OG5_129372	+	+	nd
LIG4/DNL1	OG5_130132	+	+	nd
XRCC4/LIF1	OG5_135131	nd	nd	nd
Recombinational repair				
BRCA1	OG5_159932	+	+	nd
BRCA2	OG5_131863	+	nd	nd
DNA2	OG5_129631	+	+	nd
EXO1	OG5_127511	+	+	nd
FEN1	OG5_127472	+	+	nd
MLH1	OG5_127201	+	+	nd
MLH2	OG5_202562	nd	nd	nd
MLH3	OG5_130552	+	+	nd
MMS4/EME1	OG5_135664	nd	nd	nd
MPH1/FANCM	OG5_128649	+	+	nd
MSH2	OG5_127538	+	+	nd
MSH3	OG5_130351	+	+	nd
MSH6	OG5_126895	+	+	nd
MUS81	OG5_129162	+	nd	nd
PMS1	OG5_128001	+	+	nd
RAD51	OG5_126834	+	+	nd
RAD52	OG5_130806	nd	nd	nd
RAD54	OG5_127098	+	+	nd
RTEL1	OG5_127294	+	+	nd
SAE2	OG5_138817	nd	nd	nd
SGS1	OG5_126644	+	+	nd
SLX1	OG5_128732	nd	nd	nd
SLX4	OG5_136855	nd	nd	nd
SMC5	OG5_128615	+	+	nd
SMC6	OG5_127751	+	+	+
YEN1	OG5_132593	nd	nd	nd
Sister chromatid cohesion				
PDS5	OG5_128901	+	nd	nd
RAD21	OG5_129513	+	+	nd

Table 1. Continued

Gene	OG	*Cochliopodium minus*	*Cochliopodium pentatrifurcatum*	*Cochliopodium minutoidum*
REC8	**OG5_150817**	**nd**	**nd**	**nd**
SCC3	OG5_127983	+	+	nd
SMC1	OG5_127449	+	+	+
SMC2	OG5_127360	+	+	+
SMC3	OG5_127789	+	+	nd
SMC4	OG5_127440	+	+	+
Total detected		42	39	7

Meiosis-specific genes are highlighted in bold. "+" = detected, "nd" = not detected.

in the rest of Amoebozoa, the elements forming the synaptonemal complex (SC) are also not detected in *Cochliopodium* (Table 1; Tekle et al. 2017). However, alternate pathways to meiosis not involving the synaptonemal complex do exist and likely replace the conventional pathway in *Cochliopodium* (Lukaszewicz et al. 2013; Chi et al. 2014; Shodhan et al. 2014). This is also congruent with the lack of observation of structures such as meiotic spindle fibers and SC during the *Cochliopodium* life cycle (Tekle et al. 2014). Taken together, this evidence indicates that a functional recombination pathway exists in *Cochliopodium*, most likely facilitating genetic exchange in the giant, polyploid nuclei during karyogamy (Tekle et al. 2014).

Despite the larger transcriptome sampling, some notable non-detections among the meiosis-specific genes may indicate that the new transcriptomes still do not capture all of the genes present in *Cochliopodium*. For example, MSH4 is not detected in either of the new transcriptomes, while its interaction partner, MSH5, is detected in both transcriptomes (Table 1). The 2 genes are almost always either both present or both absent in other inventoried genomes (e.g., Malik et al. 2008; Schurko and Logsdon 2008), including other amoebozoans (Tekle et al. 2017), due to their shared function during meiosis (Nishant et al. 2010). Thus, MSH4 is likely present in *Cochliopodium*, but not detected in our transcriptomes, probably due to low or no expression when the transcriptomes were collected. Additionally, SPO11, the near-universally conserved initiator of meiosis, was detected in *C. minus* but not in *C. pentatrifurcatum* (Table 1; Figure 1). It is likely that, similar to MSH4, our *C. pentatrifurcatum* samples were not at the right life stage to express SPO11. Alternatively, SPO11 may have been so lowly expressed as to be missed in our transcriptome data, even with our extensive sequencing of the transcriptome. While transcriptome data are easy to generate and can ascertain the functionality of detected genes in the life cycle of an organism, lack of detection has no conclusive biological meaning. To fully understand the exact mechanism and sexual nature of *Cochliopodium*, data from the whole genome of a *Cochliopodium* is required.

Exploring the Genetic Basis of Cellular and Nuclear Fusion in Cochliopodium

The genetic basis of the life cycle of *Cochliopodium*, involving plasmogamy, karyogamy, and fission, is not well understood. The lack of detection of the conserved gamete fusion factors HAP2 and GEX1 in *Cochliopodium* deepens this mystery. It is possible that, as with the meiosis genes, these fusion factors are present in the *Cochliopodium* genome, but were not found in the transcriptome due to low or no expression in the cells when our samples were collected. Alternately, our homology methods may not be sensitive enough to detect

Cochliopodium HAP2 and GEX1 orthologs. Unlike the well-conserved meiosis genes, HAP2, GEX1 and other fusion genes are highly divergent in different species (as low as 20% protein sequence identity in GEX1 orthologs, for example). This is evident given their spread across multiple OGs in the OrthoMCL database (Table 2). BLAST and HMMer based search tools were unable to detect even known orthologs in the wider eukaryote inventory if the full complement of OGs for each gene were not included (data not shown). If *Cochliopodium* sequences are similarly divergent from other eukaryote sequences, an alternate, more sensitive method of detection will be needed to detect them.

Some other fusion and karyogamy factors are detected in *Cochliopodium* (Table 2; Figure 1). However, despite these genes being required for fusion and karyogamy in fungi, they may not be similarly required for *Cochliopodium* fusion. None of the detected fusion factors are specific to fusion. Some are involved in more general cellular processes related to fusion, such as regulation of the cytoskeleton (Stearns et al. 1990; Sagot et al. 2002) or the cell cycle (Mendenhall and Hodge 1998; Goh and Surana 1999). Others act upstream of fusion as gene regulators (Fuller et al. 1989; Rose et al. 1989; Mendenhall and Hodge 1998; Kim et al. 2004) or as transporters (Johnston et al. 1991; Endow et al. 1994). The inventoried factors directly involved in the fusion process were mostly lineage-specific in our wider eukaryote inventory (Table 2), and it is likely that the same is true for direct fusion factors in *Cochliopodium*. Finding these lineage-specific genes will require an alternate approach, such as whole genome data coupled with differential gene expression analyses and cytological studies across the different stages of the *Cochliopodium* life cycle.

Cochliopodium Is an Unusual Sexual Lineage

The detection of many of the sex-related genes, including several meiosis-specific genes, in *Cochliopodium* transcriptomes (Table 1; Figure 1) lends evidence to the hypothesis that the genus is actively sexual. It is likely that recombination takes place in the large, polyploid nuclei produced after karyogamy. Despite the presence and expression of the meiosis genes, conventional meiosis has never been observed during the polyploid stage of the life cycle (Tekle et al. 2014). Instead, nuclear fission (karyotomy) appears to involve stretching and deforming of the nucleus, a likely process of depolyplodizion in a cell simultaneously undergoing cellular fission. Cells undergoing fission and nuclear fragmentation have no apparent chromosomal condensation, mitotic/meiotic spindle fibers, or conventional signatures of chromosome segregation (Tekle et al. 2014). Adaption of the meiosis toolkit for recombination without classical or observed accompanying meiosis also occurs in several other microbial lineages such as *Giardia* (Poxleitner et al. 2008; Carpenter

Figure 1. Maximum-likelihood phylogenetic trees for the meiosis-specific genes MND1, HOP2, and SPO11 and the fusion-related gene KEM1. Trees inferred with RAxML on the Cipres Science Gateway using GAMMALGF model of evolution. Bootstrap support values above 50% are shown above or below their respective branches. MND1, HOP2, and KEM1 trees rooted at midpoint; SPO11 tree rooted based on prokaryotic outgroup position.

et al. 2012), *Candida* (Forche et al. 2008), and *Entamoeba* (Singh et al. 2013). It is possible that the meiosis genes detected here are also primarily used for recombination in *Cochliopodium*, while another unconventional process performs the actual segregation of the chromosomes. Such a process may be similar to or derived from unusual forms of mitosis documented in several eukaryotic lineages including other Amoebozoa (Raikov 1994). In particular, genome reduction by a process resembling closed intranuclear pleuromitosis (Raikov

1994) could account for the lack of observation of mitotic- or meiotic-like structures in dividing polyploid nuclei. This also occurs in endopolyploid mammalian cells to produce diploid or near-diploid proliferative cells, possibly contributing to genomic instability associated with cancer (Walen 2010). Interestingly, meiosis-specific genes such as SPO11, DMC1, and REC8 are activated in polyploid tumor cells undergoing meiotic-like reductional divisions, which may be a contributing factor to treatment resistance (Erenpreisa et al. 2009;

Table 2. Inventory of genes involved in plasmogamy (including cellular congression) and karyogamy (including nuclear congression) in *Cochliopodium* and in genomes from across the major eukaryotic supergroups

Gene	OG(s)	*Cochliopodium*	Other Amoebozoa	Opisthokonts	Excavates	Plants	SAR
Cellular congression							
BNI1	OG5_127406	+	+	+	n/d	+	+
TPM1	OG5_127228	n/d	n/d	+	n/d	n/d	n/d
Plasmogamy							
CD9	OG5_136376	n/d	n/d	+	n/d	n/d	n/d
FIG1[a]	OG5_138630	n/d	n/d	+	n/d	n/d	n/d
FUS1[a]	OG5_156961	n/d	n/d	+	n/d	n/d	n/d
FUS2[a]	OG5_171667, OG5_216847	n/d	n/d	+	n/d	n/d	n/d
HAP2 (GCS1)	OG5_135474, OG5_143221	n/d	+	n/d	n/d	+	+
IZUMO1[a]	OG5_151190	n/d	n/d	+	n/d	n/d	n/d
KEX2	OG5_127788	+	+	+	+	n/d	+
MYO2	OG5_126577	+	+	+	+	+	+
PRM1[a]	OG5_136560	n/d	n/d	+	n/d	n/d	n/d
RVS161	OG5_129890	n/d	+	+	n/d	n/d	n/d
Nuclear congression							
BIK1[a]	OG5_139423	n/d	n/d	+	n/d	n/d	n/d
CDC4	OG5_129129	+	+	+	n/d	n/d	n/d
CDC28	OG5_126712	+	+	+	+	+	+
CDC34	OG5_129084	n/d	+	+	n/d	n/d	n/d
CDC37[a]	OG5_133705, OG5_135666	n/d	n/d	+	n/d	n/d	n/d
CIK1[a]	OG5_147436	n/d	n/d	+	n/d	n/d	n/d
CIN1[a]	OG5_156139	n/d	n/d	+	n/d	n/d	n/d
CIN2[a]	OG5_132672, OG5_180432	n/d	+	+	+	+	+
CIN4	OG5_128319	+	+	+	+	+	+
KAR3	OG5_126975, OG5_136029	+	+	+	+	+	+
KAR4	OG5_130096	n/d	n/d	+	n/d	+	+
KAR9[a]	OG5_156957	n/d	n/d	+	n/d	n/d	n/d
KEM1	OG5_126774	+	+	+	+	+	+
Karyogamy							
JEM1[a]	OG5_163504	n/d	+	+	+	n/d	n/d
KAR2	OG5_126588	+	+	+	+	+	+
GEX1/KAR5	OG5_142622, OG5_144651, OG5_156781, OG5_168665	n/d	+	+	+	+	+
SEC63	OG5_128413	n/d	+	+	n/d	+	+
SEC66[a]	OG5_135424	n/d	n/d	+	n/d	n/d	n/d
SEC72[a]	OG5_136566	n/d	n/d	+	n/d	n/d	n/d

　　Taxa included in Amoebozoa were *Acanthamoeba castellanii*, *Dictyostelium discoideum*, and *Entamoeba histolytica*; in Opisthokonts, *Homo sapiens* and *Saccharomyces cerevisiae*; In Excavates, *Trichomonas vaginalis*; in Plants, *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*; and in SAR, *Plasmodium berghei* and *Tetrahymena thermophila*. "+" means gene was detected in the group; "n/d" means the gene was not detected but may still be present in other members of the group not included here.

　　[a]Based on initial blastp results only; not confirmed by alignment inspection or tree inference.

Ianzini et al. 2009). Because of the similarity between these processes, *Cochliopodium* may provide a good model for studying these ploidy cycling processes.

　　Several other microbial eukaryotes including amoebae are assumed to undergo meiosis during dormant cyst stages, which creates problems for live experimentation (Erdos et al. 1973; Goodfellow et al. 1974; Mignot and Raikov 1992). By contrast, *Cochliopodium* genome fragmentation occurs during the vegetative stages of its life cycle (Tekle et al. 2014). *Cochliopodium* is thus an ideal organism for the study of genome reduction or depolyploidization processes. Further genome-wide exploration coupled with live experimentation in *Cochliopodium* will unravel the mysteries surrounding the mechanism of genome reduction in eukaryotes with similar life cycles.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

Data Availability

Transcriptomes used in this study have been deposited at the National Center for Biotechnology Information (NCBI) under BioProject 394890.

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