Meiosis genes inventory in alveolates and other protist provide evidence for cryptic sex and the prevalence of a synaptonemal complex-independent crossover pathway

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Declaration

I, Jingyun Chi, hereby declare that this dissertation is the presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.
Furthermore, I declare that this present dissertation has not been submitted complete or in parts to any other institution or university with the intent to obtain an academic degree. Also, I submitted this present dissertation not previously to another governmental or scientific examination board.

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Introduction

1.1 Bioinformatics

1.1.1 Definition and History

Just as its name implies, Bioinformatics is an interdisciplinary of biology and computer sciences, about the collection, interpretation, annotation and storage of biological data (Abdurakhmonov 2016; Bayat 2002). Specifically, it uses DNA sequences as a source to predict the coding protein sequences, which, on the one hand is used to predict and simulate the secondary structure of protein molecules and then according to the putative function of the protein to design drug, on the other hand is used to analyze the evolutionary relationship between different species by constructing phylogenetic tree (Eisenhaber 2000-2013; Kono and Sarai 1999; Morozov, et al. 2005; Yu, et al. 2004). With the development of NGS, it is also possible to sequence the transcriptom and whole genome sequence, which provide more reliable evidence for the analysis of molecular mechanism pathway and evolution (Behjati and Tarpey 2013; Serratì, et al. 2016). As a conclusion, bioinformatics is defined as the discipline of applying informatics technology to understand and organize the information associate with organisms and molecular processes(Le Gallo, et al. 2017).

As a modern interdisciplinary, biology and medicine, mathematics and computer sciences are three components. Biologist and medical scientist think bioinformatics aims to clarify biological or medical sense, mathematicians think that algorithms and mathematical model is its core; while, computer scientists think database and software development are the bases of bioinformatics. Thus it can be seen, bioinformatics covers a wide range of fields and its function and value are fully demonstrated in the human genome project.

Bioinformatics emerged with the development of the substitutions matrix by Dayhoff and the Needleman-Wunsch algorithm (Needleman and Wunsch 1970; Pertsemlidis and Fondon 2001). In the 80s, the establishment of biological database opened the door for people who are interested in using the data to verify their hypothesis. The corporation of three international biological databases enables the data sharing around all biology scientists. In the meantime, to manage and analyze the all the data efficiently, program tools which were represented by BLAST and FASTA raises(Altschul, et al. 1990; Lipman and Pearson 1985). In this period, bioinformatics had established as an independent discipline. Since 90s, with the springing up of various software tools, genome sequencing and sequence analysis plays more and more important roles in bioinformatics. Genome project, especially human genome project, is producing thousand millions of data, which fueled a computational evolution in biology. Genomic data analysis is now the most developing field in bioinformatics (Gauthier 2018; Hagen 2000)

Before the invention of next-generation sequencing, Sanger Sequencing method (dideoxy method) which was invented by Frederick Sanger and his collaborators in 1975 and Shotgun sequencing method were widely used(Sanger and Coulson 1975). The latter one was even applied in the Human Genome project organized by Celera Genomics cooperation. The increasing demand of low-cost sequencing pushed the development of Next Generation sequencing (NGS) technology forward. Consist of Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent (Proton / PGM sequencing) and SOLiD sequencing, next generation sequencing revolutionized our research on the genomics and genetics biology and take our understanding of genome to a deeper level.

1.1.2 Data and Tool

Since 20 century, especially the end of 20 century, the biological technology develops with full speed. The explosion of data enriches the

abundance of biological resources in a large extent and meanwhile, urges people to find an extremely strong tool to manage and store them. In 1972, the first protein database was founded by Dayoff by initiating the collection of protein sequences in her book Atlas of protein sequence structure (Dayhoff 1965). At present, many Bioinformatic centers were established to collect, organize, manage and publish biological data as well as provide search and analysis tools to researches.

Current various bioinformatic databases have become bridges to connect all the labs and promote their research. Most nucleotide sequences are stored in the currently three largest bioinformatic databases, which are DDBJ from Japan, EMBL from Europe and GenBank from the USA. Since 1982, those three databases cooperate with each other by updating and exchanging data every day.

Because of the convergent evolution, classification by morphological characters has its own limitations, e.g. organisms with a long distance relationship might have similarity in some aspects (Liu, et al. 2014). Meanwhile, observation of morphological characters is difficult in organisms with small size and numerous quantities, such as all kinds of microbial. Moreover, the common character between organisms is too scanty to be studied. Currently, the development of molecular biology and the maturity of amino acid/nuclear acid sequencing technologies make the construction of phylogeny in molecular level possible. With the rapid development and reducing costs of sequencing technology, massive amount of data are stored in databases such as GenBank, EMBL and DDBJ, which promoting the construction of phylogeny in wider rang. Phylogeny in molecular level has imponderable advantage than the traditional approaches: the exact period and rate of evolution could be evaluated by the diversity of protein or gene sequences; the evolutionary relationship between distant related organisms could be determined; the relationship among microbial could be studied.

Since bioinformatics is playing increasingly important roles in discovering

the evolution of life, computational algorithms become indispensable in processing phylogenetic data. Various algorithm and software package appear constantly. The workflow of constructing a phylogenetic tree using computational approach could be divided into four steps: selecting homolog sequences, sequence alignment, inferring phylogeny and evaluation.

As the first step, selecting homolog sequences including collecting sequences data and then confirm their homology.

Sequence alignment provide a method for measuring the relevance among amino acid or nucleotide sequences by identify regions of similarity (Altschul, et al. 1990; Apostolico and Giancarlo 1998; Edgar and Batzoglou 2006). Currently, there are more than 50 multiple sequence alignment softwares and new ones are still emerging. Common multiple sequence alignment softwares include BLAST(Altschul, et al. 1990), FASTA (Lipman and Pearson 1985), ClastalW (Larkin, et al. 2007), MAFFT (Katoh, et al. 2002), MUSCLE (Edgar 2004), T-coffee (Notredame, et al. 2000) and etc. By statistically evaluation of those softwares in dealing with high-diverse sequences (Wong, et al. 2008), MAFFT is considered as the most accurate one comparing to MUSCLE, ClustalW and T-coffee. Despite the accuracy is lower than MAFFT (although still higher than ClustalW and T-coffee), MUSCLE is also impressive by its highest operation speed. In our study, we align our query protein sequences for homolog search by MUSCLE and for constructing phylogeney by MAFFT.

Since 80s 20centry, various scoring matrix have been developed to improve the accuracy of alignment and algorithms to improve the sensibility of searching for homolog sequences. Although none of those algorithms could guarantee the optimal alignment, some of them present much better performance than others. Currently, two methods are widely used in sequence comparison-BLAST (Altschul, et al. 1990) and HMMer (Eddy 2011).

In bioinformatics, BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) is one of the most popular tools for database search. As a package, BLAST can not only

search against protein and nucleotide database, but also translate the query sequence into protein sequence or translate the database into proteins to improve the sensitivity of search.

As another database search tool, HMMER (Durbin, et al. 1998) uses the implements methods using probabilistic models called profile hidden Markov models (profile HMMs). A position-specific scoring system for substitutions, insertions, and deletions is used for the (Eddy and Wheeler 2015). Sequences in database would then be compared with profile-HMM and those which scoring greater than profile-HMM would considered to be homolog candidates (Jones, et al. 2014). Relying on the strength of its mathematic model, HMMer has more advantages than BLAST in detecting remote homologs.

1.1.3 Phylogeny

Molecular phylogeny inferring methods could be divided into two categories: based on discrete character data and based on distance. Methods based on discrete character data construct phylogeny by searching for all possible trees among which the one fitting best with evolution relationship would be selected. These methods define a statistically optimal standard, with which each generated tree is evaluated. Methods based on discrete character data include Maximal parsimony methods (Farris 1970; Fitch 1971), Maximal likelihood methods (Myung 2003; Pagel 1999), and Bayesian methods (Holder and Lewis 2003). Distance-based methods, however, follows the Minimum evolution (ME) principle (Rzhetsky and Nei 1992). In these methods, a distance matrix presenting the evolution distances among all evolved species is defined and applied in classification of those species by clustering algorithm. To obtain a final phylogenetic tree, two nodes with shortest distances are merged iteratively and new distance matrix is generated. Distance-based methods include unweighted pair-group method with arithmetic mean (UPGMA) (Szostak, et al. 1983), Neighbor-Joining (NJ), transformed distance

method and neighbor-related methods, etc. Although there are various kinds of phylogeny constructing methods, none of them is absolutely fit for all data in all conditions. With less diverse and long enough sequences, Neighbor-Joining, Maximum likelihood and Maximum parsimony methods would obtain similar topology (Saitou and Imanishi 1989). When the sequences are highly diverse, transferring DNA into distance matrix would cause the loss of information (Penny 1982). The performance of Distance methods relies on the quality of distance matrix, thus accurate result could only be obtained when sequences meet some conditions. Maximum parsimony does not rely on distance matrix, however, without allowing for convergence along long branches as an explanation of similarity, this method is susceptible to long-branch attraction, in which two long branches that are not adjacent on the true tree are inferred to be the closest relatives of each other by parsimony (Holder and Lewis 2003). So with the highly diverse sequences, Maximum parsimony could lead to incorrect tree topology. Moreover, with large dataset, Maximum parsimony is quit time-consuming as being a NP-problem (Foulds and Graham 1982). Maximum likelihood is a statistic method based on evolutionary models, with statistical consistency and robustness (Bryant, et al. 2005). Nevertheless, like Neighbor-Joining, it also relies on models. With different models, the tree topology may vary. The algorithm of Maximum likelihood is relatively complicate, thus only suitable for small datasets. As the posterior probability not only referring to all the trees, and listing for each tree every possibilities of branch length and substitution models, the result of Bayesian could not be inferred with regular analysis approaches. Inferring with Markov Chain Monte Carlo Methods (MCMC), Bayesian could rapidly handle large dataset and evaluate the confidence of tree.

In our study, we constructed the phylogeny with Maximum likelihood method. Being introduced for phylogeny in 1964 by L. L. Cavalli-Sforza and A. W. F. Edwards, this method require a special substitution model for analyzing the given sequence data to maximum the likelihood value of each tree

topology. The tree with highest likelihood value is considered as best tree. Considering tree topology and branch lengths as two parameter, maximum likelihood methods seek the maximum value of likelihood ratio to estimate the branch length. Assuming that the rate of evolution is variable, the result of distance methods is better than Maximum Parsimony, and Maximum likelihood (Zhong, et al. 2001). In other words, maximum likelihood method allows different rate of evolution in every branch. For this reason, when we have sequences from various species, we choose Maximal likelihood method for construct phylogeny tree.

The core of this method is the substitution models, commonly used protein evolution models include empirically-based models like Whelan And Goldman (WAG) model (Whelan and Goldman 2001), single-doublet-triplet (SDT) model (Whelan and Goldman 2004), Dayhoff model (Schwarz and Dayhoff 1979), JTT model (Jones, et al. 1992) and secondary structure hidden Markov model (HMM) approach (Goldman, et al. 1996); Codon-based models like REV0 (Yang, et al. 1998) and HKY model (Yang and Nielsen 2000). In our study, we selected WAG model which is an empirical model of globular protein evolution. It combines the best contributes of Dayhoff and JTT models and is implemented to estimate a new model of amino acid replacement from a database of globular protein sequences comprising 3,905 amino acid sequences split into 182 protein families. Respecting to maximum-likelihood values for a great amount of the protein families in the database, WAG model outperformed the Dayoff and JTT model by providing a better overall fit to the evolutionary process in globular proteins and may leading to more accurate phylogenetic tree inferring (Whelan and Goldman 2001).

Phylogenetic trees could be constructed and evaluated with hundreds of softwares, such as Phylip (Felsenstein 2005), MEGA (Tamura, et al. 2013), MrBayes (Ronquist and Huelsenbeck 2003), PAUP (Swofford 2011), RaxML (Stamatakis 2014). In this study, we constructed our trees using RaxML, simply because it has a fast maximum likelihood tree search algorithm that

returns trees with good likelihood scores.

1.1.4 Programming

Currently, several program languages have developed their own program packages, in another words, many languages have their own Bio* project, e.g. bioperl, bioSQL, bioruby, biopython, biojava. All those packages have integrated the most commonly used bioinformatic tools to reduce code duplication for biologists.

Among all those program languages, why we prefer python? There are several advantages that attract us most. First of all, it is easy to learn for the total beginner in programming and even easier for man with some programming experiences. The code of python is easy to read. Secondly, the data structure of python is suitable for bioinformatics. As an object-orientated language, python is able to convert the biological data into its own object. More importantly, python is supported by a large amount of program packages. Lastly, python also have regular expression function, which make it equally strong as Perl in text processing part.

Biopython is an non-commercial Python tools for computational molecular biology, as well as bioinformatics using python. One of its many strengths is that it is very easy to learn, with a a very clear syntax. Biopython features include parsers for various Bioinformatics formats (BLAST, Clustalw, FASTA, Genbank,...), access to online services (NCBI, Expasy,...), interfaces to common and not-so-common programs (Clustalw, DSSP, MSMS...), a standard sequence class, various clustering modules, a KD tree data structure etc. and even documentation (Jeff Chang, et al. 2013). The general function of Biopython includes parsing sequences, multiple sequence alignment with various programs, BLAST and other sequence searching tool, accessing databases (e.g. NCBI, Swiss-prot, ExPaSy), protein 3D-structure analysis using PDB module, population genetic analysis, phylogenetic analysis, cluster analysis, etc. In our research, we use a designed pipeline using biopython.

The detailed workflow would be introduced in other chapter.

To summarize, as an interdisciplinary of biology and computer sciences, bioinformatics has become a significant tool in almost all field of biology. As a young research field, it still has a tremendous space to be improved, from both theoretical side and practical application side. Its theoretical support need the strive of many disciplinarians including molecular and genetic evolution, popular genetics, biostatistics, genome biology, computer sciences and mathematics. From practical application part, firstly, the integration of current program and the development of new programs are extremely urgent. Although more and more programs spring up in the past decades, many of them lack corresponding description, which makes it difficult for newly developed programs to utilize current program resource. Programmers are forced to develop new software from very beginning. The consequence is that the input and output format of many programs are not compatible and the functions of them are overlapped. Meanwhile, scientists, who facing all these programs, have problems choosing the most suitable one. To solve above problems, it is necessary to compare the features and technical parameters of programs with similar functions. Secondly, the large-scaled databases such as GenBank, EMBL, Swiss-Prot, PDB etc need to be improved and prepared to deal with the explosion of biological sequence data. More importantly, the sequence technology improved with each passing day, the amount and quality of data changes with it. Bioinformaticians should always ready to those changes.

1.2 Meiotic recombination and Sex

1.2.1 General information about meiotic recombination and sex

Meiosis is the special type of cell division, in which haploid cells are generated from a diploid cell. Meiosis is a key event in the life of all sexually reproductive organisms (Ricardo Benavente and Volff 2008). Ordinarily, a cell has two complete sets of chromosomes, giving rise to haploid cells (gametes)

each having one set of chromosomes. Two such gametes arising from different individual organisms merge by the process of syngamy to generate a new diploid cell, thus completing the sexual cycle. The origin of meiosis is closely associated with the origin of sex itself, which is so far still a debatable topic in the research field of evolution.

It is almost certain that meiosis is evolved from mitosis (Wilkins and Holliday 2009); however, how and when the mitosis gene divide into meiosis genes is still unknown.

Meiotic sex, has been explored deeply both theoretically and empirically. The genes enable meiotic sex are just begin to be explored through the inventory and analysis of meiotic genes by a method called “meiosis detection toolkit” (Schurko and Logsdon 2008). Observation has its powerlessness in study the sex in microbial sexual for two reasons. First, the morphological features for sex such as microbial are not obvious. Meanwhile, for facultative sexual organisms, the laboratory conditions may inappropriate for the occurring of sex; for example, the occurrence of sex for *Tetrahymena* is induced by stressful conditions such as starvation and the condition of sexual reproduction for most other ciliate remains unclear.

1.2.2 Cost and benefit of sex

Sexual reproduction has no much benefit for the individual organism. For males, they have to spent a lot of time and energy, sometimes even risk their life, to find and ingratiate themselves with mate. For Female, sexual reproduction passed on only fifty percent of their genes to offspring, while by parthenogenesis they can pass on all of their genes. Nowadays, thousands animal and plants still undergo asexual or parthenogenesis, thus avoid the "fifty percent" of cost. However, the other millions of animal and plants are sexual, or facultative reproductive. For what evolutionary benefit would their female pays the cost? This is one of the most famous enigma in the evolutionary biology. Various answers have been proposed, among which the

most prevailing opinion is that, sexual reproduction lead to genetic recombination, causing plentiful mutations, thus the competitive ability of surviving the challenge of existence. Like the William's lottery model explained: asexual is like buying a lot of ticket with the same number, which would not increase the chance of winning, while sexual reproduction is like buying tickets with various numbers, which are more likely to win.

By accumulate mutations in each generation, sexual organisms would better elude the chase of enemies (parasites and predation). For surviving, organisms must keep genetically updating (correspondingly, parasites and predation must also keep updating). Asexual organisms, which parent and offspring are genetically identical, would be caught up by the enemies, thus leading to extinct. This explanation is called "red queen hypothesis". It derived from the statement of Red queen in Lewis Carroll's Through the looking-Glass that "Now, here, you see, it takes all the running you can do, to keep in the same place".

1.2.3 Meiotic recombination and meiotic genes

Meiosis is a division process that create haploid cells by reduce the chromosome number by half. During meiosis, the cell divides twice to generate four daughter cells. According to the cytological features, the first round of cell division (meiosis I) comprises five phases: Interphase, Prophase I, Metaphase I, Anaphase I and Telophase I. In prophase I, the homologous chromosomes pair and form synaptonemal complex to facilitate the meiotic recombination (also called crossover). Meiotic crossover result in the mosaic gametic chromosomes that contain the genetic material from its homologous parental chromosome. This process is deliberately controlled by a set of meiosis-specific genes and other gene shared with mitosis. In eukaryotes, crossover could be roughly divided into four stages: double-strand break (DSB) initiate, DSB end processing, DNA strand exchange, DSB repair.

Meiotic crossover is initiated by programmed DSB, which is catalyzed by

widely-conserved protein called Spo11. Two Spo11, which contains a tyrosine to ligates and dissociates phosphodiester backbone of the DNA, acts as a homodimer to generate a transient, covalent protein-DNA intermediate (Cao, et al. 1990; Keeney 2008). In *Saccharomyces cerevisiae*, at least nine other genes, including MNR complex (Mre11, Nbs1 and Rad50), are involved in the formation of DSB with Spo11 (Keeney 2001). Those genes are, however proved to be dispensable in the DSB formation in Arabidopsis (Puizina, et al. 2004).

After the DSB formation, Spo11 is covalently linked to the 5′ ends of DNA, leaving 3′ hydroxyls and nicks offset by 2 base pairs (bp) (Cole, et al. 2010). Spo11 is then removed from DNA by cleavage of single strands 3' by Mre11 nuclease with other cofactors, such as Sea2 (Com1), leaving a resected 5' end and a protruding 3' overhang (Borde 2007; Lee, et al. 2012). The protruding 3' overhang is then further extended in the opposite direction by the 5' to 3' exonuclease Exo1 (Tsubouchi and Ogawa 2000).

The DNA strand exchange is performed in most eukaryotes by two RecA homolog: the ubiquitous Rad51 and meiosis-specific Dmc1. These two proteins form a overlapping but slightly offset “co-foci”, which load differentially on opposite ends of the meiotic DSB (MacQueen 2015). By binding on the 3' overhanging ssDNA tails, the nucleoprotein filaments have the remarkable capacity to search surrounding double-stranded DNA (dsDNA) and melt homologous duplex DNA through strand invasion and exchange events. Research on Tetrahymena shows that in the absent of Dmc1, efficient Rad51-dependent repair takes place, but only between sister chromatid, which indicate that Dmc1 functions in search similar but non-identical DNA (Howard-Till, et al. 2011). Although Rad51 provide support for the action of Dmc1, in the absent of Rad51, the Dmc1 nucleoprotein filament can form and no significant effect of the strand-exchange activity in Arabidopsis(Singh, et al. 2017). However, in the rad51Δ meiosis, metaphase I chromosomes keep broken, indicating that Rad51 plays an crucial role in the DSB repair. The

Rad51-Dmc1-ssDNA nucleoprotein filament is stabilized by complex, which is consist of meiosis-specific protein Hop2 and Mnd1. Meanwhile, a bunch of other proteins, such as Mei5, Sae3, RPA, Hed1, XRCC3, BRCA2, etc., are also involved in the DNA strand exchange.

The product of DSB strand exchange is a D-loop intermediate, when the 3' overhanging ssDNA tails invades the homologous duplex DNA. Heterodimer of Msh4-Msh5 are involved to stabilize the D-loop. If the D-loop is not stabilized, it would then disrupted and the extended 3′ end anneals to the ssDNA tract on the opposite end, forming a interhomolog non-crossover. This mechanism is also called synthesis-dependent strand-annealing (SDSA). If the D-loop is stabilized, it would then be repaired, using the invading 3' end as a primer and the homolog as a template, resulting a crossover.

1.2.4 Two pathways involved in crossover

There are two product of early stage strand invasion and exchange: crossover and non-crossover. In most sexual species, the formation of one crossover would interfere the others, which is called interference crossover (crossover pathway I). Correspondingly, the other pathway, in which one crossover does not interfere the formation of another one, is call non-interference crossover (crossover pathway II). In *Saccharomyces cerevisiae*, the formation of crossover pathway I requires the ZMM proteins (ZIP1, ZIP2, ZIP3, ZIP4, MSH4, MSH5, MER3). Some members of the ZMM proteins are the component of Synaptonemal complex (SC) and the others plays crucial role in synapsis (Zickler 2006).

Synaptonemal complex (SC) is an ultrastructurally detectable protein structure which is once used as the indicator of occurring of meiosis (Heywood and Magee 1976). Considered as the marker of parallel chromosome paring in meiotic crossover pathway I, the SC functions to "zippers" the pair of homologous chromsomes together during the prophase I of meiosis(Heyting 1996; Moens 2001). Although the tripartite structure of SC, including axial

element, transverse filament and central element, is relatively ubiquitous among eukaryotic organisms, their protein components various and with low, if any, sequence similarity among animal, plant and fungi (Anuradha and Muniyappa 2005; Grishaeva and Bogdanov 2014). The distribution and protein component of SC in the trees of eukaryotes is still obscure.

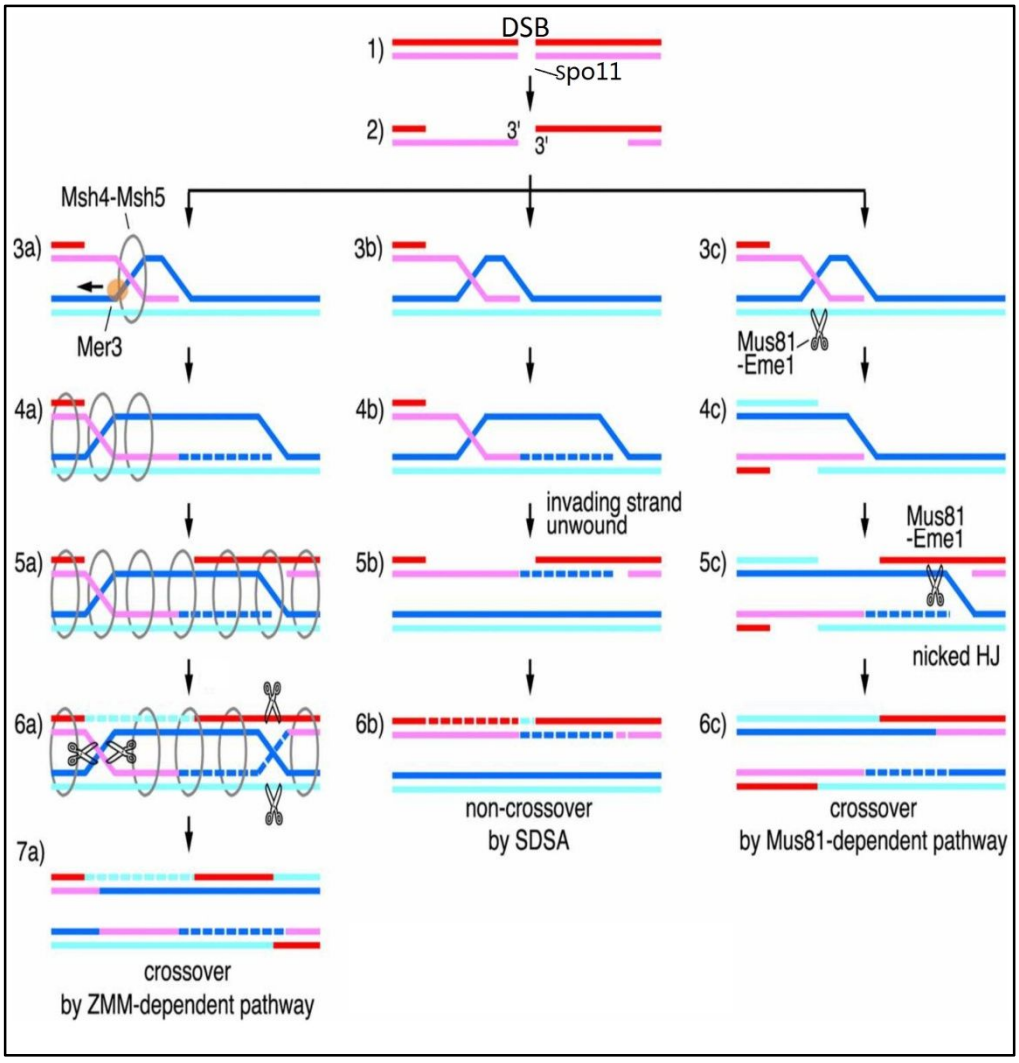

Fig.1. The formation of crossover by both ZMM-dependent pathway (pathway I) and Mus81-dependent pathway (pathway II) and non-crossover as proposed by (Szostak, et al. 1983). Figure was slightly modified from (Whitby 2006).

Meiotic crossover pathway I undergoes with the facility of SC and Msh4-Msh5, Mlh1-Mlh3, and Mer3 proteins and exhibits crossover

interference. While pathway II is MUS81-Mms4/Eme1 dependent and exhibits no interference. In crossover pathway I, the canonical double Holliday Junction (dHJ) structures are generated after strand invasion and then resolved by Sgs1 helicase, Exo1, and a putative endonuclease activity encoded by the DNA mismatch repair factor Mlh1-Mlh3 (Rogacheva, et al. 2014). The exact mechanism of Mus81 action remains obscure. It may interact with Mms4/Eme1 to form a DNA structure-specific endonuclease to cleave intact single HJs in *S. pombe* (Holloway, et al. 2008). Although crossover pathway I is prevalent in most eukaryotes, pathway II may cohabit in some species (Mercier, et al. 2005), and in species such as S.pomb, crossover formation relies only on pathway II and not subject to interference.

1.2.5 Meiosis detection toolkit

By searching directly genomes for meiotic genes using a set of meiotic gene that present the best marker for the present of meiosis, this method could be used in the observation of potential capability for sex in some putative obligate asexual organism, especially for microorganisms, whose sexual reproduction are deeply influenced by conditions, and the investigation of their evolution. Using the “meiosis detection toolkit”, meiotic genes in several organisms have been observed (Ramesh, et al. 2005). including mircoralge such as *Nannochloropsis gaditana* (Corteggiani Carpinelli, et al. 2013), alveolates such as Dinoflagellata (Chi, et al. 2014b), Ciliates(Chi, et al. 2014a), fungi such as *Magnaporthe oryzae* (Saleh, et al. 2014) and various parasitic protists(Weedall and Hall 2014). The sexually of most of those microorganisms are controversial, because of the influence of the genetic, physiological and environment

Since the sexual reproduction in many taxa is not observed in lab, a bioinformatics approach called “Meiosis detection toolkit” emerged as the time required. This toolkit is consist of a set of meiotic genes that represent the best marker for the present of meiosis in eukaryotes (Schurko and Logsdon 2008).

All, or at least most of those genes being presented in a genome would suggest a genetic capability of having sexual reproduction of this species; while the absence of most gene suggest the loss of meiosis and asexuality. This approach is aimed to understand the evolution of meiosis in eukaryotes and the detection of cryptic sexuality in putative obligate asexual.

The meiosis detection toolkit consists of eight meiosis-specific genes (SPO11, HOP1, HOP2, MND1, REC8, DMC1, MSH4 and MSH5) and four meiotic genes which also involved in mitosis (RAD21, RAD51, MSH2 and MSH6). The exchange of genetic information of meiotic recombination initiated with the programmed double-strand breaks (DSB) created by SPO11 as well as other members in the complexes including MRE11, RAD50 and NBS1. Those double-strand breaks in the DNA could be repaired homologous recombination to bypass lesion that could cause

There are three aims of inventory meiotic genes in whole genome sequences: 1) to detect cryptic sex in putative asexual lineages; 2) to infer which meiotic crossover pathways are using in particular species and 3) to fill the gaps between sexual and asexual lineages, thereby to broaden the perspective of the evolution of asexuality to sexuality. As mentioned before, observation is powerless in detecting sexuality in microbials, because the size of microbials and their extreme environment conditions of having sex.

1.3 Alveolates

1.3.1 General information of Alveolates

Alveolates are a group of protists under the classification of chromalveolata, which all share a system of sacs underneath their cell membranes. These closely packed sacs are called alveoli, which forms continuous layer of membrane vesicles which resemble with muscle sarcoplasmic reticulum and with unknown function (Stelly, et al. 1991). Besides alveoli, alveolates are with the feature of micropores throughout the cell surface involved in pinocytosis; closed mitosis; large

mitochondria with tubular cristae (Leander 2008). It is inferred that the last common ancestor of dinoflagellates and apicomplexa has two heterodynamic flagella, micropores, trichocysts, rhoptries, micronemes, a polar ring, and a coiled open-sided conoid (Kuvardina Olga, et al. 2002). However, whether these characters are also present in the LACA remains unclear, given that ciliates ingest by a different machanism. It is reported that a large amount of alveolate-exclusive proteins, including a family of protein called Alveolins, are associated with the alveolate pellicle formed by in ciliates, apicomplexa and dinoflagellates (Gould, et al. 2011; Gould, et al. 2008). Although all kinds of trophic behavior, predatory, parasite or photosynthetic, present in alveolates, genetic evidences support that the common ancestor of alveolates is photosynthetic and possesses a plastid (Archibald 2008; Reyes-Prieto, et al. 2008; Sato 2011). The genetic vestiges of plastid, which is retained in chromerida, some apicomplexa and dinoflagellates, are found in the genome of ciliates (Tetrahymena and Paramecium tetraurelia), suggesting that ciliates share a photosynthetic ancestor with other alveolates species.

Alveolates include three main subgroups of ciliates, dinoflagellates, apicomplexa and lineages such as *Chromerida*, *Perkinsus Colpodella* , *Chromera*, *Colponema*, Ellobiopsids, *Oxyrrhis*, *Rastrimonas*, and *Parvilucifera*, that do not fit neatly into those three groups (citation).

Both sexual and asexual reproduction has been found in three major subgroups of alveolates. In Apicomplexa, sporozoite, an invasive form of organism, enters a host cell and then undergoes asexual replications to form numerous merozoites. Some of the merozoites transform into sexually reproductive cells, or gamonts, which join together in pairs and form a gamontocyst. Within the gamontocyst, the gamonts divide to form numerous gametes. Pairs of gametes then fuse to form zygotes, which give rise by meiosis to new sporozoites, and the cycle begins again (Levine 1985).

1.3.1 Ciliates

Ciliates are with high specificity in cytological and genetic characters comparing with other eukaryotes: They have two sets of nucleus, macronuclear and micronuclear, with distinct functions; DNA are deleted and rearrange during the development of macronuclear; gene-sized nanochromosomes in some species such as *Oxytricha* and *Stylonychia*. Those characters make ciliates the model organism for molecular and cellular biology.

The most noticeable feature of ciliates is their dualism. Unlike other eukaryotes, whose most genetic information is stored in one single nuclear, ciliates possess two nucleus with distinct function: macronuclear and micronuclear. The micronucleus serves as the germ line and the macronucleus provides the nuclear RNA for vegetative growth. During the sexual reproduction stage of ciliate, the mating cell exchange the haploid micronuclei, and a new macronucleus generated from the new diploid micronucleus by amplification and editing, meanwhile, the old macronucleus is destroyed (Prescott 1994). Ciliates normally reproduce asexually by fission; however, many of them can also undergo sexual reproduction, which is called conjugation. The conjugation of ciliates is usually induced by lack of food or the change of extracellular environment such as temperature, illumination or salinity, and between two opposite mating types. Unlike human or other higher organisms, which have either male or female, ciliates can exist in several different sexes, e.g. *Tetrahymena thermophila* has 7 mating types.

The widely accepted classification of ciliates was proposed by John Corliss in 1975 (Corliss 1975). According to the morphology of cilium and cytostome, he divided ciliates into three subclasses: Kinetofragminophora, Oligohymenophora and Polyhymenophora.

In our study, we mainly focus on four ciliate genomes: *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Ichthyophthirius multifiliis*, and *Oxytricha trifallax*, whose whole genome assembles could be downloaded from their own websites (Arnaiz, et al. 2007; Stover, et al. 2006; Stover, et al. 2012). The genome structure of those ciliates is special comparing to other eukaryotes in

two aspects. First of all, they all have a macronuclei and a micronuclei, which the two set of chromosomes are quite distinct. During MAC differentiation of the germline cell, diminution and fragmentation occur. The segments of the MIC genome known as internally eliminated sequences (IESs) are deleted (Rédei 2008). The IES eliminating is demonstrated to be a precise, highly ordered process (Wen, et al. 1996). In *Tetrahymena thermophila*, approximately 6,000 IESs are removed, resulting in the MAC genome being an estimated 10% to 20% smaller than that of the MIC (Eisen, et al. 2006). A key aspect of the process is the preferential removal of repetitive DNA, which results in 90% to 100% of MIC repeats being eliminated.

Been studied for over three centuries, ciliates are proved to be the most successful group in protist kingdom. They lived in all kinds of water body and moist environments. Except some free-living ciliates, most ciliates are symbiosis or parasitic and usually seen in the digestive track of ruminants. Some ciliates, e.g. Balantidium coli, are parasite in human body (Smith 2003). Ciliates take a large portion of the total amount of microeukaryotes and the most structural complicate group of them.

With highly diversity of each organelle and their rational arrangement though millions years of evolution, ciliate has been used as model organism for eukaryotic molecular biology and phylogeny research. The research of phylogeny on ciliate relies on the development of technology. In the past decades, the research on ciliate is almost parallel with the technology of microscope. Nowadays, the development of sequencing technology and the springing of more accurate algorithms of phylogeny enable us to obtain deeper understanding of the evolution of ciliate and its relationship with other species.

Notably, certain ciliate are genetic-code deviant. They share the translation table 6 with Dasycladacean and Hexamita species, that TAG and TAA encode glutamine or glutamic acid in these organisms and TGA is the only stop codon (Harper and Jahn 1989).

1.3.2 Apicomplexa

The apicomplexa is a group of intracellular, obligate parasitical and pathogenic alveolates, including *Toxoplasma gondii* (causes the disease toxoplasmosis), *Cryptosporidium* spp (causes a respiratory and gastrointestinal illness called cryptosporidiosis), *Plasmodium* spp (cause the disease malaria), *Babesia* spp (causes the disease Babesiosis) and *Eimeria* spp (causes Coccidiosis in animals). Currently, there are about 4000 known species in the group of Apicomplexa. The morphological feature of this group is the apical complex of microtubules, which facilitates their attachment to and penetration of the host cell, and in parasite proliferation. Most apicomplexans have an apicoplast (a nonphotosynthetic plastid) except for Cryptosporidium species and gregarines (Lim and McFadden 2010). The lifestyle of Apicomplexa is complicate and variable among species in the group. Both sexual and asexual reproduction has been found in the group. The basic life cycle start from the infective stage, when sporozoite enters a host cell and then divides repeatedly to form large amount of merozoites. Some of the merozoites transform into sexually reproductive cells, or gamonts. Gamonts join together in pairs and form a gamontocyst. Within the gamontocyst, the gamonts divide to form numerous gametes. Pairs of gametes then fuse to form zygotes, which give rise by meiosis to new sporozoites, and the cycle begins again.

1.3.3 Dinoflagellates

Dinoflagellates which are known for their period bloom that causes "red tide" which may kill fish and shellfish, and apicomplexa which is a group of parasitic and pathogenic protists. Among all alveolates, Apicomplexa and its close relative Dinoflagellates both feed from myzocytosis, thus have the common name Myzozoa. Apicomplexa has asymmetrical distributed microtubules, fibrin and vacuoles. Both Apicomplexa and Dinoflagellates have microtubules at the top of cell which form the apical complex to assist invading

into the host cell, and chromatophores. Although there are both autotrophic and heterotrophic organisms in alveolates, research suggests that their ancestor, at least the ancestor of ciliate, maybe photosynthetic (Reichman, et al. 2003).

Dinoflagellates are characterized by its permanently condensed chromosomes that are composed of fibers organized without histones (Costas and Goyanes 2005; Rizzo 1991, 2003). Most Dinoflagellates contain only one nucleus (uninucleate), while a few others, which contain an endosymbiont alga, have two (dinucleate) (Dodge 1971). The nuclei of Dinoflagellates usually contain large amount of DNA (Rizzo 2003). It has been observed that during the chromosome segregation, the newly synthesized DNA is packed inside the original chromosome while the dividing chromosomes keeping condensed. Genetic analysis shows evidences for a usual meiosis in Dinoflagellates (*Crypthecodinium cohnii*) that may result from a centromere linkage or the absent of crossing over in traditional meiosis or an unusual one-division meiosis (Himes and Beam 1975).

1.3.4 Other clade in alveolates

Except the above three main groups of alveolate, 6 minor groups, include Acavomonidia, Colponemidia, Perkinsozoa, Chromerida, Colpodellida and Voromonadida, are also phylogenetically belong to alveolates.

Introduction of author's research and contribution

In the paper *Meiosis Gene Inventory of Four Ciliates Reveals the Prevalence of a Synaptonemal Complex-Independent Crossover Pathway* (Chi, et al. 2014a), by inventory the meiotic genes in four ciliate genome, we inferred that crossover pathway II is predominant in the ciliates. The author contributed to programming, data analysis and writing.

In the paper *Cryptic Sex in Symbiodinium (Alveolata, Dinoflagellata) is Supported by an Inventory of Meiotic Genes* (Chi, et al. 2014b), by inventory the meiotic genes in the genome of *Symbiodidium*, we discovered the evidence of cryptic sex in this putative asexual species. The author contributed to programming, data analysis and writing

In the paper *Meiotic gene inventory in Alveolate genomes* (to be published), by inventory the meiotic genes in all available alveolate genome data to infer the origin and diverse of two meiotic pathways. The author contributed to programming, data analysis and writing.

In the paper *Meiotic Genes in Colpodean Ciliates Support Secretive Sexuality* the author contributed in programming and data analysis.

In the paper *Putatively asexual chrysophytes have meiotic genes: evidence from transcriptomic data (Kraus, et al. 2019)*, the author performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Inference

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Chapter I

Meiosis Gene Inventory of Four Ciliates Reveals the Prevalence of a Synaptonemal Complex-Independent Crossover Pathway

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Summary

Background

Although meiotic sex has been explored deeply both theoretically and empirically. The genes enable meiotic sex are just begin to be explored through the inventory and analysis of meiotic genes by a method called “meiosis detection toolkit” (Schurko and Logsdon 2008). For those organisms which sexual reproduction has never been observed, meiosis gene inventory provide, from one side, an evidence for the capability of sexuality. The present of most meiosis-specific gene would suggest the maintenance of sexual reproduction and the absence would consistent with the loss of meiosis and asexuality. Using this approach, several putative obligate asexual species, including fungi and protists, has been proved to have cryptic sex. In our study, we expended the "meiosis detection toolkit" to a total 51 meiotic genes, among which 11 is meiosis-specific and we inferred the meiotic recombination pathways according to the presence/absence of those genes in four ciliate genomes.

Current model of meiotic recombination are based on the double strand break repair (DSBR) model described by Szostak. Meiotic recombination is initiated by DNA double-strand breaks generated by the meiosis-specific nuclease Spo11. DNA at these double strand breaks is then resected to expose single-stranded ends. Depending on a series of other proteins, these ends undergo strand invasion and form a heteroduplex (Holliday junction) with homologous DNA tracts, leading to strand exchange between homologous chromosomes. While initial double-strand break formation and strand exchange are conserved among eukaryotes, the downstream pathways converting transient joint DNA molecules into crossovers are diverse. One crossover pathway maybe dominate in one organism but missing in another. The prevalent class I pathway uses the meiosis-specific set of ZMM group

genes and is manifested by the formation of a synaptonemal complex (SC). Crossovers in this pathway are interfering; that is, crossovers suppress nearby crossovers. The class II pathway involves only genes that also have mitotic DNA repair functions, and it produces crossovers that are non-interfering. Meiotic genes involved in initial double-strand break formation, as well as downstream processes such as crossing over, are just beginning to be assessed in ciliates.

Methods

In this study, we inventoried meiosis genes in the genomes of *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Ichthyophthirius multifiliis*, and *Oxytricha trifallax*. To search for the homologs in both whole sequence and motif level, we combined two different methods of data mining, BLASTp and HMMER, to uncover all potential paralogs, and phylogenies are inferred to confirm gene identification. To further evaluate whether ciliate genome architecture allows for relatively faster evolving genes, some genes are also analyzed for non-synonymous to synonymous substitution rates.

we searched for SCs in *T. thermophila* and *Stylonychia mytilus* using nuclear spreading and silver staining methods that highlight SCs in light and electron microscopy in a variety of organisms (Albini, et al. 1984; Loidl, et al. 1998).

Results

Of the 11 meiosis-specific genes inventoried here, seven are found in ciliates. The most central and conserved of these is *SPO11*, whose product initiates recombination by forming double-strand breaks in DNA. Five other genes found are: *DMC1*, which is essential for the homolog (non-sister)-bias in meiotic recombination(Bugreev et al. 2011); *HOP2* and *MND1* whose protein products form a complex that stabilizes the association of Dmc1 with DNA (Chen et al. 2004); and *MSH4* and*MSH5*, whose products act as a

heterodimer (Snowden et al. 2004), and are believed to stabilize recombination intermediates (Nishant et al. 2010).*REC8*, the seventh gene found, poses a special situation in that in all other organisms investigated so far it encodes a meiosis-specific component of the sister chromatid cohesin complex, whereas in *T. thermophila* it is important both for mitosis and meiosis(Howard-Till et al. 2013). Forty meiosis-related genes were also inventoried. These genes are involved in double-strand break formation, DNA damage sensing, double-strand break repair, crossover regulation, and other processes that are relevant but not exclusive to meiosis. Of these genes, 29 are found in the ciliates, of which 23are found in all four species. Paralogs are found in 20meiosis-related genes.

The absence of *HOP1*, *RED1* and *ZIP1*, which encode SC proteins, is consistent with the failure to observe these structures in electron microscopy sections of *T. thermophila* meiotic nuclei (Wolfe et al. 1976).

Discussion

SCs are thought to provide a tight physical link between homologous chromosomes, and to regulate crossing over, in a not yet fully understood way. Only a few eukaryotes are able to perform crossing over in their absence. None of these SC-related genes—*HOP1*, *RED1*, and *ZIP1*—were found in a previous gene inventory of *T. thermophila* (Mochizuki, et al. 2008), and they are not found here (table 1). Moreover, SCs were not detected microscropically in *T. thermophila* in an earlier electron microscopy study (Wolfe, et al. 1976), as well as here with silver staining (fig. 4A,B,C,D). Thus, all molecular and microscopic evidence to date do not support the existence of SCs in *T. thermophila*. It has been proposed that in the absence of these structures, the elongated shape of meiotic micronuclei in *T. thermophila* promotes physical contact of homologous chromosomes during recombination(Loidl and Scherthan 2004).

Similarly, here we did not find the SC genes *HOP1*, *RED1*, and *ZIP1* in *P.*

tetraurelia, *I. multifiliis*, and *O. trifallax* (table 1). However, we did observe axial element-like structures in *S. mytilus* (fig.4), which is closely related to *O. trifallax*(Lynn 2008). The absence of some of the genes in our inventory may be due to the limitations of the data mining methods. The released drafts of the genomes we used could have excluded these genes (e.g., Florea, et al. 2011; Mavromatis, et al. 2012; Zhang, et al. 2012). And, not all gene copies are always present in the macronuclei. Alternatively, structures that appear similar in the microscope could be composed of completely different proteins.

Besides detecting which genes are using in meiosis for ciliate, the more profound meaning of inventory of meiotic genes is to inferring which meiotic recombination pathways are undergoing in ciliates. Although most model organisms are capable of both meiotic recombination class I and class II pathway, their presence and prevalence differ. By inventory the meiotic gene in ciliates, we found that all our model organisms lacking the component proteins for synaptonemal complex, a protein structure to facilitate the binding of homologous chromosomes during meiotic recombination pathway I. This finding is consist with the result of microscope observation and immunostaining methods in *Tetrahymena thermophila* (Howard-Till, et al. 2013; Loidl, et al. 2012), proving the reduction of cannon class I recombination pathway in ciliates is likely a derived condition. the gene inventory would be considered as another powerful approach for analysis the prevalence of pathways in organisms.

This inventory and analysis of meiosis genes in four genomes shows that not all eukaryotic meiosis-specific and meiosis-related genes are needed in ciliates. Our data, together with a previous functional study in *T. thermophila*, suggests that ciliates are capable only of a slimmed meiosis, using a set of mitotic repair proteins for meiotic recombination. This reduction is likely a derived condition, and may be the result of the abandonment of the more complex class I pathway within the ciliates.

Chapter I

Meiosis Gene Inventoryof Four Ciliates Reveals the Prevalence of a Synaptonemal Complex-Independent Crossover Pathway

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Abstract

To establish which meiosis genes are present in ciliates, and to look for clues as to which recombination pathways may be treaded by them, four genomes were inventoried for 11 meiosis-specific and 40 meiosis-related genes. We found that the set of meiosis genes shared by *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Ichthyophthirius multifiliis*, and *Oxytricha trifallax* is consistent with the prevalence of a Mus81-dependent class II crossover pathway that is considered secondary in most model eukaryotes. There is little evidence for a canonical class I crossover pathway that requires the formation of a synaptonemal complex (SC). This gene inventorysuggests that meiotic processesin ciliates largely depend on mitotic repair proteins for executing meiotic recombination. We propose that class I crossovers and SCs were reduced sometime during the evolution of ciliates. Consistent with this reduction, we provide microscopic evidence for the presence only of degenerate SCs in *Stylonychia mytilus*.Additionally, lower non-synonymous to synonymous mutation rates of some of the meiosis genes suggest that, in contrast to most other nuclear genes analyzed so far, meiosis genes in ciliates are largely evolving at a slower rate than those genes in fungi and animals.

Key words: Ciliophora, crossover pathway, genomearchitecture,*Ichthyophthirius*,meiosis, *Paramecium*, phylogeny, *Oxytricha*, *Tetrahymena*.

Introduction

Meiotic recombination is initiated by DNAdouble-strand breaksgenerated by the meiosis-specific nuclease Spo11(Keeney 2001; Keeney et al. 1997). DNA at these double-strand breaks is then resected to expose single-stranded ends. These ends can engage in heteroduplex formation with homologous DNA tracts, leading to strand exchange between homologous chromosomes(San Filippo et al. 2008). While initial double-strand break formation and strand exchange are conserved amongeukaryotes, the downstream pathways converting transient joint DNA molecules into crossovers are diverse(Kohl and Sekelsky 2013). The predominant class I pathwayuses the meiosis-specific set of ZMM group genes(Lynn et al. 2007; Schwartz and Heyer 2011; Svendsen and Harper 2010), and is manifested by the formation of a synaptonemal complex (SC). Crossovers in this pathway are interfering; i.e., crossovers suppress nearby crossovers (Berchowitz and Copenhaver 2010). The class II pathway involves only genes that also have mitotic DNA repair functions, and itproduces crossovers that are non-interfering.

Meiotic genes involved in initial double-strand break formation, as well as downstream processes such as crossing over, are just beginning to be assessed in ciliates. Ciliates (=Ciliophora) are a large clade of microbial eukaryotes with “germline” micronuclei and “somatic” macronuclei in each cell (Lynn 2008). Sex in ciliatesentails the mutual exchange of haploidproducts of meiotically-divided micronucleibetween cells of complementary mating types (Cervantes et al. 2013; Phadke and Zufall 2009; Sonneborn 1937). So far, meiosis genes have only been analyzed in *Tetrahymena thermophila.* In an inventory of 29 meiosis genes in the publishedgenome*,* Malik et al. (2008) used BLAST to find six meiosis-specific and 11meiosis-relatedgenes. With the same method, Mochizuki et al. (2008) found four meiosis-specific genes and 33 meiosis-related genes.And using gene knockout/knockdown and

immuno-staining methods, the function of 4 meiosis-specific and 11meiosis-relatedgenes involved in chromosome pairing and recombinationhasbeen investigated (Howard-Till et al. 2011; Howard-Till et al. 2013; Loidl and Mochizuki 2009; Loidl and Scherthan 2004; Lukaszewicz et al. 2013; Lukaszewicz et al. 2010; Mochizuki et al. 2008).

Here we expand the inventory of ciliate meiosis genes in the *T. thermophila*(Eisen et al. 2006)genome by looking for homologs of 11genes that are known to be meiosis-specific in yeast and other eukaryotes, and 40meiosis-related genes. We also broaden the inventory by searchingthe genomes of *Paramecium tetraurelia* (Aury et al. 2006), *Ichthyophthirius multifiliis* (Coyne et al. 2011) and *Oxytricha trifallax*(Swart et al. 2013). Given that gene duplications of nuclear protein-coding loci are rampant in ciliates(e.g., Katz et al. 2004), two different methods of data mining are used to uncover all potential paralogs, andphylogenies are inferred to confirm gene identification.We use the inventory to look for clues as to which recombination pathwaysmay be treaded by ciliates and discuss the evolutionary implications of our findings.To further evaluate if ciliate genome architecture allows for relatively faster evolving genes(e.g., Zufall et al. 2006), some genes are also analyzed for non-synonymous to synonymous substitution rates. Throughout we indicate gene names by capital letters and italics, and proteins in lowercase with a capital first letter, as is common practice in *Saccharomyces cerevisiae* nomenclature.

Results

Gene inventory and phylogeny

Here, we use data from the published genomes of *T. thermophila*, *P. tetraurelia*, *O. trifallax* and *I. multifiliis*. These genomes were queried for homologs of 51meiosis genes. Both the BLASTp (Altschul et al. 1990)and the HMMER (Eddy 2011) methods, with support from RAxML (Stamatakis 2006) phylogenetic analyses, resulted in the same inventory (table 1, fig. 1,fig. 2, supplementary file 1). Twelve un-annotated open reading frames encoding meiosis geneswere found in *I. multifiliis* (supplementary file 2).

Of the 11 meiosis-specific genes inventoried here, seven are found in ciliates. The most central and conserved of these is *SPO11*, whose product initiates recombination by forming double-strand breaks in DNA. Five other genes found are: *DMC1*, which is essential for the homolog (non-sister)-bias in meiotic recombination(Bugreev et al. 2011); *HOP2* and *MND1* whose protein products form a complex that stabilizes the association of Dmc1 with DNA (Chen et al. 2004); and *MSH4* and*MSH5*, whose products act as a heterodimer (Snowden et al. 2004), and are believed to stabilize recombination intermediates (Nishant et al. 2010).*REC8*, the seventh gene found, poses a special situation in that in all other organisms investigated so far it encodes a meiosis-specific component of the sister chromatid cohesin complex, whereas in *T. thermophila* it is important both for mitosis and meiosis(Howard-Till et al. 2013).

Four of these genes are not found in at least one of the ciliates: *DMC1* was not identified in *P. tetraurelia*, nor does *P. tetraurelia* or the other ciliates have *MCM8* (data not shown) which facilitates double-strand break repair in the absence of *DMC1* in *Arabidopsis thaliana* (Crismani et al. 2013); *MSH4* and *MSH5*were not identified from *I. multifiliis*; and *REC8* is only found in *T. thermophila*. Multiple gene copies are found for three meiosis-specific genes: *HOP2* has two paralogs in *T. thermophila*, *I. multifiliis, and O. trifallax*; *MND1*

has two paralogs in *T. thermophila* and *O. trifallax*; and *MSH4* has two paralogs in *P. tetraurelia* and *O. trifallax* (table 1).Four meiosis-specific genes (*HOP1*, *MER3*, *RED1*, and *ZIP1*)are not found in the ciliates; they are all known to be involved in the formation of the SC.

The finding here of *MSH5* contradicts the two earlier gene inventories of *T. thermophila* (Malik et al. 2008; Mochizuki et al. 2008). We feel that the identification of this gene here is supported by several aspects. First, the phylogenetic analysis shows that our *MSH5* candidate from *T. thermophila* (GI 118384428) nests in the same clade with *MSH5* genes from other organisms(fig.1, supplementary file 1). Second, *MSH5* is also found here in *P. tetraurelia* and *O. trifallax*. Third, Msh5's meiotic dimerization partner, Msh4, is also present in *T. thermophila*, *P. tetraurelia*, and *O. trifallax*.

Forty meiosis-related genes were also inventoried. These genes are involved in double-strand break formation, DNA damage sensing, double-strand break repair, crossover regulation, and other processes that are relevant but not exclusive to meiosis. Of these genes, 29 are found in the ciliates, of which 23are found in all four species. Paralogs are found in 20meiosis-related genes.

dN/dS ratios

To estimate the rate of mutation, Data monkey (Delport et al. 2010; Kosakovsky Pond and Frost 2005a; Kosakovsky Pond et al. 2005)was used to measure the ratio of non-synonymous to synonymous substitutions in23meiotic genes that are present in all four ciliates (fig. 3, supplementary table 1, supplementary table 2). Ratios were <1 for all genes measured, suggesting negative selection; that is, there was a purification of deleterious alleles due to changes in protein sequences. Ciliates have the highest dN/dS ratios for ten of these genes (*ATR*, *DNA2*, *FEN1*, *HOP2*, *MLH1*, *MRE11*, *MSH6*, *MUS81*, *PMS1*, and *RAD50*); for most of these genes, fungi have the second highest values. Fungi have the highest dN/dS ratios for eleven genes (*CDC2*,

EXO1, *KU70*, *KU80*, *LIG4*, *MND1*, *MSH2*, *RAD51*, *SAD1*, *SGS1*, *SPO11*), and animals have the highest values for two (*MPH1* and *RAD23*).

Search for SC-related structures

The absence of *HOP1*, *RED1* and *ZIP1*, which encode SC proteins, is consistent with the failure to observe these structures in electron microscopy sections of *T. thermophila* meiotic nuclei (Wolfe et al. 1976). Here, we searched for SCs in *T. thermophila* and *Stylonychia mytilus* using nuclear spreading and silver staining methods that highlight SCs in light and electron microscopy in a variety of organisms (Albini et al. 1984; Loidl et al. 1998). In *T. thermophila*, meiotic pairing occurs in micronuclei at a stage when they are extremely elongated(fig. 4A; Mochizuki et al. 2008). From 3-4 h after induction of meiosis, when this stage is most abundant, SC-like structures were not detected (fig. 4B,C,D), thereby confirming the observation of Wolfe et al.(1976).

By contrast, distinct linear structures are present in *S. mytilus* (figure 4E). They closely resemble axial elements, which, in many organisms, are precursors to SCs. Axial elements form along chromosomes at the leptotene stage and become connected by transversal filaments. Together, these structures normally become a mature SC that intimately links homologous chromosomes at the pachytene stage(Zickler and Kleckner 1999). However, in *S. mytilus*, connected axial elements were not observed. We therefore presume that *S. mytilus*develops only residual SC structures. This is similar to meiosis of *Schizosaccharomyces pombe*, where only so-called linear elements (LinEs) are formed instead of full-fledged SCs(Loidl 2006).

Discussion

A ciliate meiosis gene toolkit

Meiotic sex—the fusion of haploid meiotic products from different individuals—is thought to have originated in the common ancestor of all eukaryotes (Dacks and Roger 1999). The advantages of maintaining sex since this origin have been explored in depth both theoretically (e.g. Bell 1982; Burt 2000; Hamilton 2001; Kondrashov 1993; Maynard Smith 1978; West et al. 1999; Williams 1975), and empirically (e.g., Becks and Agrawal 2012; Misevic et al. 2010; Morran et al. 2011; Tucker et al. 2013). The underlying genes enabling sex in eukaryotes, though, are just beginning to be explored through the inventory and analysis of a toolkit of meiosis genes (Malik et al. 2008; Ramesh et al. 2005; Schurko and Logsdon 2008).

Detection of the underlying genes involved in sex, or detecting the potential capability of having sex, by inventorying and analyzing a toolkit of meiosis genes has occurred in a broad range of taxa: e.g., in the microbial eukaryotes *Trichomonas vaginalis* (Malik et al. 2008) and *Giardia intestinalis* (Ramesh et al. 2005);in the animals *Daphnia pulex*(Schurko et al. 2009), monogonont rotifers(Hanson et al. 2013), and *Nasonia vitripennis* (Schurko et al. 2010); and in the fungi *Glomus* spp.(Halary et al. 2011; Riley and Corradi 2013) and *Penicillium roqueforti* (Ropars et al. 2012). Overall, published studies have found that although meiosis-specific genes are generally conserved across all the major clades of eukaryotes(Schurko and Logsdon 2008), absence of one or several of them is common. For example, *DMC1*, *HOP2*, *MER3*, and *MND1*are missing in *Caenorhabditis elegans* and *Drosophila melanogaster* (Masson and West 2001; Pezza et al. 2007);*HOP1*is missing in *Anopheles gambiae* and *Neurospora crassa* (Malik et al. 2008); and *MSH4* and *MSH5* is absent in *D. melanogaster* (Kohl et al. 2012),*G. intestinalis* (Ramesh et al. 2005), *Plasmodium falciparum*(Gardner et al. 2002),and *S.*

pombe(Schurko and Logsdon 2008).

In ciliates, meiosis genes have so far only been inventoried in *T. thermophila*. Using BLAST searches, Malik et al.(2008) identified six meiosis-specific (*DMC1*, *HOP1*, *HOP2*, *MND1*, *MSH4*, *SPO11*), and 11 meiosis-related genes (*MRE11*, *RAD1*, *RAD50*, *RAD51*, *MSH2*, *MSH6*, *MLH1*, *PMS1*, *SMC2*, *SMC3*, and *SMC4*). Likewise using BLAST, Mochizuki et al. (2008) found four meiosis-specific genes (*DMC1*, *HOP2*, *MND1*, and *SPO11*), and 39 meiosis-related genes.

T. thermophila is also the only ciliate where functional studies of meiotic proteins have been performed by knockout/knockdown approaches and immunolocalization. In brief, most protein products from meiotic genes exert functions similar to their homologs in other model eukaryotes.Spo11 was found to be essential for the formation of double-strand breaks, confirming its universal role in this process. In particular in *T. thermophila*, Spo11-induced double-strand breaks cause the elongation of micronuclei and, thereby, the pre-assortment of chromosomes that is essential for meiotic homologous pairing(Mochizuki et al. 2008). Similarly, the localization and/or the deletion phenotypes of ATR, Com1(Sae2), Dmc1, Hop2, Mre11, and Rad51were largely consistent with their expected functions(Howard-Till et al. 2011; Loidl and Mochizuki 2009; Lukaszewicz et al. 2010; Mochizuki et al. 2008). Notably, *T. thermophilam* possesses, unlike all other eukaryotes studiedso far, a single version of the kleisin component of cohesion, Rec8, which is crucial for normal chromosome segregation in mitosis and meiosis, and for meiotic double-strand break repair(Howard-Till et al. 2013).

To establish a more robust meiosis gene toolkit for ciliates, we inventoried 11meiosis-specific and 40meiosis-related genes not only in *T. thermophila*, but also in *P. tetraurelia*, *I. multifiliis,* and *O. trifallax* (table 1, fig. 1, fig. 2,supplementary file 1). This inventory of four genomes shows that not all meiosis-specific genes are needed in this clade of microbial eukaryotes. Among the meiosis-specific genes inventoried, seven (*DMC1*, *HOP2*, *MND1*,

MSH4,*MSH5*,*REC8*, and*SPO11*) are found in at least one of the ciliates. While these genes are known to be meiosis-specific in yeasts and other eukaryotes, their meiotic specificity among the ciliates has not been investigated; the one exception is *REC8*, which is also involved in mitosis in *T. thermophila*(Howard-Till et al. 2013), but is absent in the other three ciliates. Likewise, this inventory shows that not all meiosis-related genes are required in ciliates.

We found multiple gene copies in 3 meiosis-specific, and 20 meiosis-related, genes (table 1, fig. 1, fig. 2, supplementary file 1). This extensive paralogy reflects observations in previous studies that found paralogs in other ciliate nuclear protein coding loci (Israel et al. 2002; Katz et al. 2004; Snoeyenbos-West et al. 2002; Snoke et al. 2006; Yi et al. 2012; Zhan et al. 2013; Zufall and Katz 2007), and is consistent with the multiple whole genome duplications known in *P. tetraurelia* (Aury et al. 2006; Hughes et al. 2007). Because of these paralogs, molecular phylogenetic inferences in ciliates using these meiosis genes could be mislead by a confounding of orthologs and paralogs.

Given that sex, and the genes underlying this process, in microbial eukaryotes is little known (Dunthorn and Katz 2010; O'Malley et al. 2013), these data of four known sexual ciliates can serve as a baseline for inventorying meiosis genes in other sexual ciliates, as well test for the ability to have cryptic sex in putative asexual ciliates; e.g., in the Colpodea (Dunthorn and Katz 2010; Foissner 1993).As discussed below, this gene inventory also allows forevolutionary insights into the slimmed SCs, as well as recombination pathways, in ciliates.

Are functional SCs lacking in ciliates?

Almost all eukaryotes capable of meiosis form SCs (Wettstein et al. 1984). These protein complexes consist of two parallel axial elements that form along the axes of paired chromosomal partners. These axial elements, and the

chromosomes to which they are attached, are connected by transversal filaments. Three meiosis-specific proteins are major components of the canonical SC. Hop1 and Red1 help form axial elements (also known as lateral elements) (Hollingsworth et al. 1990), and Zip1 helps form transversal filaments(Dong and Roeder 2000).

SCs are thought to provide a tight physical link between homologous chromosomes, and to regulate crossing over, in a not yet fully understood way. Only a few eukaryotes are able to perform crossing over in their absence. The best known examples are *S. pombe*(Kohli and Bähler 1994), and *Aspergillus nidulans*(Egel-Mitani et al. 1982). However, these two fungi form rudimentary axial element-like structures (the so-called linear elements or LinEs) that are likely remnants of once existing SCs(Loidl 2006). Accordingly, *S. pombe* has a Hop1, and a protein(Rec10) with some similarity to Red1(Lorenz et al. 2004). LinEs seem to serve in the recruitment or activation of recombination factors(Estreicher et al. 2012).

HOP1 can be easily detected in homology searches due to its conserved HORMA domain, and homologs are known from *A. thaliana*(Caryl et al. 2000; Nonomura et al. 2004), *C. elegans*(Couteau and Zetka 2005; Martinez-Perez and Villeneuve 2005), *Mus musculus*(Shin et al. 2010),*S. cerevisiae*(Hollingsworth and Byers 1989; Hollingsworth et al. 1990), and *S. pombe*(Lorenz et al. 2004). On the other hand, *ZIP1* is not a convenient indicator of the presence of the SC, since its sequence is highly variable and there are similarities only at the structural level(Heyting 2005; Higgins et al. 2005).

None of these SC-related genes—*HOP1*, *RED1*, and *ZIP1*—were found in a previous gene inventory of *T. thermophila* (Mochizuki et al. 2008), and they are not found here (table 1). Moreover, SCs were not detected microscropically in *T. thermophila* in an earlier electron microscopy study(Wolfe et al. 1976), as well as here with silver staining (fig. 4A,B,C,D). Thus, all molecular and microscopic evidence to date do not support the existence of SCs in *T.*

thermophila. It has been proposed that in the absence of these structures, the elongated shape of meiotic micronuclei in *T. thermophila* promotes physical contact of homologous chromosomes during recombination(Loidl and Scherthan 2004).

Similarly, here we did not find the SC genes *HOP1*, *RED1*, and *ZIP1* in *P. tetraurelia*, *I. multifiliis*, and *O. trifallax* (table 1). However, we did observe axial element-like structures in *S. mytilus* (fig.4), which is closely related to *O. trifallax*(Lynn 2008). Moreover, in a transmission electron microscopic study it was reported that *Paramecium primaurelia* (stock 540 of syngen 1 of the *Paramecium aurelia* species complex) has "ill-defined synaptonemal complex-like material"(Stevenson 1972). Residual SC structures have also been microscopically detected in other ciliates. In *Dileptus anser*, there are fragmentary SCs(Vinnikova 1976), as well as massive polycomplexes similar to those that often accompany abnormal or incomplete SC formation(Goldstein 1987). In *Tracheloraphis totevi*, only the less condensed chromosomes or chromosome regions at the periphery of pachytene nuclei show SCs(Kovaleva and Raikov 1992). Altogether, mature, canonical SCs have not yet been found in ciliates, although it seems that there is some variability as to the extent of residual SC formation. It will be a worthwhile task for the future to determine whether there exist any ciliates with canonical SCs.

There is an inconsistency between the apparent presence of fragmentary SCs, or axial element-like structures, in some of the ciliates and the failure to detect homologs of genes encoding axial element proteins, such as *S. cerevisiae HOP1* or *RED1*, or *S. pombe REC10*. The absence of some of the genes in our inventory may be due to the limitations of the data mining methods. The released drafts of the genomes we used could have excluded these genes (e.g., Florea et al. 2011; Mavromatis et al. 2012; Zhang et al. 2012). And, not all gene copies are always present in the macronuclei. Alternatively, structures that appear similar in the microscope could be composed of completely different proteins.

Ciliate recombination pathways

Zalevsky, et al.(1999) proposed that there may be at least two different pathways for meiotic crossover formation in *S. cerevisiae*. Similarly, Copenhaver et al.(2002)suggested that *A. thaliana*has two crossover pathways, only one of which is subject to interference. The existence of two pathways in *S. cerevisiae* was later confirmed by de los Santos et al.(2003), who found that class I crossovers are dependent upon Msh4-Msh5 and exhibit interference, while class II crossovers are dependent upon Mus81-Mms4 and exhibit no interference. Finally, it was established that class I crossovers are dependent upon the so-called ZMM group of proteins (encoded by *ZIP1*, *ZIP2*, *ZIP3*, *MSH4*, *MSH5*, and *MER3*) whose presence is linked to the formation of a SC(Bishop and Zickler 2004; Börner et al. 2004; Hollingsworth and Brill 2004).

This two-pathway classification was found applicable to most crossovers in a range of model organisms. While most model eukaryotes are capable of both recombination pathways, their presence and prevalence differ(Kohl and Sekelsky 2013; Schwartz and Heyer 2011; Svendsen and Harper 2010). Notably, most crossovers in *C. elegans* and *D. melanogaster* are through the class I pathway (Kelly, et al. 2000; Kohl, et al. 2012; Meneely, et al. 2002; Zalevsky, et al. 1999). *S. pombe* is the only organism found so far to exclusively rely on the class II pathway(Boddy et al. 2001; Smith et al. 2003; Villeneuve and Hillers 2001). It should be noted, however, that the two-pathway classification is becoming somewhat blurred in *C. elegans*, where Mus81may also be involved in the generation of interfering SC-dependent crossovers (Bellendir and Sekelsky 2013).

In ciliates, recombination pathways have only been studied in *T. thermophila*. Most, if not all, crossovers in this ciliate are generated by the class II; i.e., the SC-independent Mus81-dependent pathway(Lukaszewicz et al. 2013). *P. tetraurelia*,*O. trifallax*, and *I. multifiliis* presumably are also

capable of class II crossovers given that they have *MUS81*(table 1). The presence of *MSH4* and *MSH5* homologs(table 1) is surprising because ZMM proteins Msh4 and Msh5 are considered to play a role in the stabilization of class I crossover-designated recombination intermediates in the presence of a SC(Börner et al. 2004; Nishant et al. 2010). It is not known if *T. thermophila*is capable of class I crossovers, but given the presence of *MSH4* and *MSH5* there is a possibility that class I crossovers can occur in the absence of a canonical SC. Similarly, given the presence of *MSH4* and *MSH5*in *P. tetraurelia* and *O. trifallax*, a slimmed class I crossover pathway may occur in other ciliates. Gene knockout experiments are therefore needed to study the function of *MSH4*- and *MSH5*-encoded proteins in *T. thermophila*, and to determine if a subset of crossovers in *T. thermophila*are subject to interference. It will also be interesting to know which of these pathways are treaded during autogamy in ciliates, which occurs in ciliates by the fusion of haploid nuclei within a single cell(Lynn 2008; Miyake 1996).

Since the class II pathway is similar to mitotic recombination double-strand break repair, Kohl and Sekelsky (2013) posit that meiotic recombination originally occurred via the class II pathway, and that later the class I pathway evolved for more precise crossover control. Given both class I and class II pathways are found in animals, fungi, and plants, we add to this hypothesis in positing that both crossover pathways would have already been in place within the ancestor of all extant eukaryotes. Similarly, the presence of SC-like structures in several of the ciliates indicates that its absence in *T. thermophila*, and *T. thermophila´*s dependence on the class II recombination pathway, is a derived condition. We hypothesize that a reduction of the SC- and ZMM-dependent class I crossover pathway had occurred at the separation of the ciliates from the other Alveolata, or sometime afterwards. Support for the gradual loss of the class I pathway comes from the observation that *MSH4* and *MSH5* homologs are missing in *I. multifiliis* (table 1).

Rates of evolution in ciliate meiosis genes

Genome architecture is thought to be one of the driving forces of the rates of gene evolution (Lynch 2007; Lynch and Conery 2003). For example, it is hypothesized that the peculiar ciliate genome architecture, in which protein synthesis occurs in the macronuclei, allows deleterious mutations in micronuclear genes to evade selective pressures during long periods of cell replication(Katz et al. 2004; Katz et al. 2006; Zufall et al. 2006). From the micronuclei's point of view, the generation time is greatly extended. This evasion allows for enough time for compensatory mutations to arise, such that non-synonymous mutation rates are high in ciliates. Consistent with this hypothesis, using dN/dS ratio comparisons, Zufall et al. (2006) found that ciliates have the highest rates of mutation for six genes (actin, β-tubulin, EF1α, Histone H4, and HSP90) when compared to animals, fungi, and plants; fungi only have a higher dN/dS ratio for α-tubulin.

For the same group of organisms, here we assessed dN/dS ratios for 23meiotic genes (figure 3, supplementary table 1, supplementary table 2). Ciliates have the highest dN/dS ratios for only 10 of these genes. Fungi, or animals, have the highest values for the 13other genes. The genome architecture of ciliates thus does not always lead to higher dN/dS ratios, as seen with α-tubulin in Zufall et al. (2006), and as seen here in more than half of the meioticgenes examined.

Conclusion

This inventory and analysis of meiosis genes in four genomes shows that not all eukaryotic meiosis-specific and meiosis-related genes are needed in ciliates. Our data, together with a previous functional study in *T. thermophila*, suggests that ciliates are capable only of a slimmed meiosis, using a set of mitotic repair proteins for meiotic recombination. This reduction is likely a derived condition, and may be the result of the abandonment of the more complex class I pathway within the ciliates.

Materials and Methods

Data mining

A query database of 11 meiosis-specific genes, and 40 meiosis-related genes from non-ciliate eukaryotes was established using literature and keyword searches of the NCBI protein database (http://www.ncbi.nlm.nih.gov) and the Uniprot Knowledgebase (http://www.uniprot.org) from September to November 2012. For some genes that belong to a certain protein family or consist of certain conserved domains, full sequences from Pfam database (Punta et al. 2012) and NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) were also used as complementary queries (supplementary table 3). For *REC8*, we used both the typical eukaryotic sequences, as well as the sequence for the *REC8* uncovered in *T. thermophila* by Howard-Till et al. (2013).

Four ciliate macronuclear genomes were retrieved from their online databases: *T. thermophila* (http://ciliate.org), *P. tetraurelia* (http://paramecium.cgm.cnrs-gif.fr), *I. multifiliis* (http://ich.ciliate.org), and *O. trifallax* (http://oxy.ciliate.org). Although the genomes of the four organisms have been sequenced and assembled to scaffolds (Aury et al. 2006; Coyne et al. 2011; Doak et al. 2003; Eisen et al. 2006; Swart et al. 2013), annotations of many genes are incomplete. Artemis (Rutherford et al. 2000) was used to extract open reading frames (ORFs) from each genome.

The query database of meiotic genes was used to search the subject database constructed with ORFs from the four ciliate genomes using two complementary approaches: BLASTp (Altschul et al. 1990), which returns the most similar protein sequences from the user-specified protein database, and HMMER v3.0. (Eddy 2011)using HMMERsearch, which uses a profile hidden Markov model to detect remote homologs. For both methods, only hits with E-values $<10^{-4}$for the full sequence were retained. All candidate homologs were then verified by reciprocal BLASTp search against the non-redundant

protein sequence database of NCBI.

Sequence analyses

Phylogenetic inferences of each meiotic gene were used to confirm gene identification, and provide insight into the evolution of the genes in the four ciliates. Taxon sampling in animals, fungi, and plants followed Zufall et al. (2006); the microbial eukaryotes *Dictyostelium*, *Entamoeba*, *Giardia*, *Micromonas*, *Plasmodium*, *Trichomonas*, *Trypanosoma*were also sampled as outgroups. Multiple amino acid sequence alignments were constructed using MAFFT v6.850 (Katoh et al. 2002). The WAG-I-Γ model of evolution (Whelan and Goldman 2001) was used in all analyses. Maximum Likelihood analyses were run in RAxML v7.3.0 (Stamatakis 2006), with bipartition support from 1,000 bootstrap replicates. Trees were visualized with FigTree v1.3.1 (Rambaut 2006).

For selection analyses, amino acid sequences and cDNA sequences of 23 meiotic genes from animals, ciliates, fungi, and plants were retrieved from NCBI database by keyword search (supplementary table 1). These genes were chosen if they are present in all four ciliates. Taxon sampling in animals, fungi, and plants followed Zufall et al. (2006). Alignments of the amino acid sequences were constructed using MAFFT with default settings. Alignments of cDNA were performed using PAL2NAL v14 (Suyama et al. 2006). Using these alignments, dN/dS ratios were measured with Data monkey (Delport et al. 2010; Kosakovsky Pond and Frost 2005a; Kosakovsky Pond et al. 2005). Comparisons were made with the likelihood SLAC method (Kosakovsky Pond and Frost 2005b), with the best fitted substitution model automatically chosen by the program.

Cell preparation and staining for microscopy

*S. mytilus*was cultured at 20-23°C in Pringsheim solution and fed with *Chlorogonium*(Ammermann et al. 1974). Strains of different mating types were

mixed in the morning and fed in a manner that all food was depleted in the afternoon. Under these conditions cells conjugated during the following 16-24 h (Dieter Ammermann, pers. commun.). Samples were taken from 16-23.5 h at 1.5 h intervals. 20 µl concentrated suspension of conjugating cells were dropped onto a clean slide and 80 µl 10% Lipsol detergent were added to lyse the cells. Lysis was stopped after 5 sec by the addition of 120 µl fixative (4% paraformaldehyde + 3.4% sucrose). Liquids were mixed by tilting the slide. Slides were air-dried and stained with $AgNO_3$ solution (1 g $AgNO_3$ in 2 ml H_2O). Staining and preparation for light and electron microscopy followed the protocol for yeast SCs in(Loidl et al. 1998).

*T. thermophila*strains B2086 and CU428 were cultivated at 30°C according to standard methods(Orias et al. 2000), and they were made competent for sexual reproduction by starvation in 10mM Tris–Cl (pH 7.4) for 14-16 h. Conjugation and meiosis were induced by mixing equal amounts of starved strains. 20 µl of a concentrated suspension of conjugating cells from 3 h and 4 h after induction of meiosis were put on a slide, and 40 µl of 10% Lipsol detergent were added. Cell lysis was monitored under phase contrast. When about 2/3 of cells were lysed, the process was stopped by the addition of 120 µl fixative (as above). Further processing of the slides was done as for *S. mytilus*.

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Table 1. Meiosis genes inventoried in four ciliate genomes. Genes are grouped according to functions. Meiosis-specific genes are bolded. Letters in parentheses after some gene names designates that name is used in specific organisms: a in *Arabidopsisthaliana*, c in *Caenorhabditis elegans*, d in *Drosophilamelanogaster*, m in *Mus musculus*, and s in *Schizosaccharomyces pombe*. Superscripts designate the papers in which those homologs were identified previously, and their roles in meiosis experimentally established, in *T. thermophila*:[1]Mochizuki et al. (2008), [2]Howard-Till et al. (2011),[3]Howard-Till et al. (2013), [4]Loidl and Mochizuki (2009), [5]Lukaszewicz et al. (2010), [6]Lukaszewicz et al. (2013). Numbers are the NCBI database GI accession numbers; except for *DNA2* of *O. trifallax*, which is a contig number that does not have a GI number. Unannotated = genes without GI accession numbers; their amino acid sequences are in supplementary file 1. "-" = genes not identified in the genomes.

Gene	*Tetrahymena thermophila*	*Paramecium tetraurelia*	*Ichthyophthirius multifiliis*	*Oxytricha trifallax*
DOUBLE STRAND BREAK FORMATION				
REC114/*REC7*(s)	-	-	-	-
***SPO11*/*REC12*(s)**	146174859 [1]	145508327	unannotated	403366622
CROSSOVER REGULATION				
DMC1	118382143 [2]	-	340503751	403376234
HOP1	-	-	-	-
HOP2	146164587 146180477 [1]	145484139	340506041 unannotated	403340394 403356227
MER3	-	-	-	-
MND1	118364583 118382874 [1]	145503596	340502971	403366359 403374287
MSH4	118377506	145511321 145501156	- -	403343032 403340570
MSH5	118384428	145543484	-	403355869
***RED1*/*ASY3*(a)/*REC10*(s)**	-	-	-	-
ZIP1	-	-	-	-
DOUBLE STRAND BREAK REPAIR AND MEIOTIC DIVISIONS				
REC8	118381268 [3]	-	-	-
BOUQUET FORMATION				
MPS3/*SUN*-1(c)/*SAD*1(m)	146144065	145486212 145490578 145542514 145480857	unannotated	403362248
DNA DAMAGE SENSING/ RESPONSE				
MEC1/*ATR*	118387169 [4]	145524936	unannotated	403344435
TEL1/*ATM*	-	-	-	-
MRE11	146185466 [5]	145544352 145536832	unannotated	403367531 403369501
RAD17	146168062	145483611 145552798	unannotated	-
RAD23	146161955	145499454	340505565	403370790
RAD24	146162627 118374567	145553485 145510192 145530239	471226163 471223876 471231354 471221868	403363735 403361798
RAD50	118398608 [2]	145512287	340500394 340500395	403349168
XRS2/*NBS1*(m)	-	-	-	-
DOUBLE STRAND BREAK REPAIR (non-homology end join)				
KU70	146162847 146182742	145509781 145494698	340504921 340509175	403345721
KU80	118379947	145481675 145495442 145493174	340505600	403353375
LIG4/*DNL1*	118372223	74830325 145530083 145541123	471226273	403348596
XRCC4/*LIF1*	-	-	-	-
RECOMBINATIONAL REPAIR				
BRCA1	146144639	-	-	-
BRCA2	118369034	145510666 145550052	unannotated	403362253
DNA2	118372980	145551458 145482823	unannotated	403363527 21922_0_g18
MMS4/*EME1*(s)	118367913 [6]	145508321	-	-
EXO1	118395354	145531403 145532383	340503093	403375999 403361276 403373775
FEN1	146145019 146171182	145514642 145514173	471231312	403347702 403358237 403341860
MLH1	117556979	145510949	unannotated	403339826 403351590
MLH3	118396563	none	unannotated	-
MPH1/*FANCM*(a,m)	118380585	145547248	471224730	403331000
MSH2	146164189	124088618	340503635	403372692
MSH3	-	-	-	-
MSH6	118376906 118361141 118355604 118368049	145479585 145480193 145532012 145539700 145547527 145503005	340506212 340500328 340502546	403351686 403351687 403350104 403351679 403365718 403332180 403332270
MUS81	118378880 [6]	145483941	unannotated	403359221
PMS1	229594773	145551871	340500161	403370380 403375263 403361297 403360310
RAD51	118355624 [2]	145492218 145499122	340502670	403356083 403345476 403374245
RAD52	-	-	-	-
RAD54	118383249	145482121	340502773	-
RTEL1	118383994 118387755	145517083 145525952 145512032 145525869	unannotated 340503511	403351116 403335726 403341333 403369956 403361814
SAE2/*COM1*(c,a)/*CTIP*(m)	118371089 [5]	145537105 145553397	-	-
SGS1	146165477 [6]	145496268 145478455	340502580	403364021 403369477 403340774
SLX1	-	-	-	-
SLX4/*HIM-18*(c)/*MUS312*(d)	-	-	-	-
SMC5	-	-	-	-
SMC6	-	-	-	-
YEN1/*GEN1*(m)	-	-	-	-
MEIOTIC ENTRY				
CDC2	118389274 146180395	145539344 145534283 145479295	340505212	403347840 403331849

FIG. 1. Maximum likelihood analyses of *MSH4*, *MSH5*, and *SPO11*. Bipartitions with ≥70% bootstrap support are bolded. Individual trees for all genes inventoried in the four ciliate genomes, as well as all bipartition support values, are in supplementary file 1.

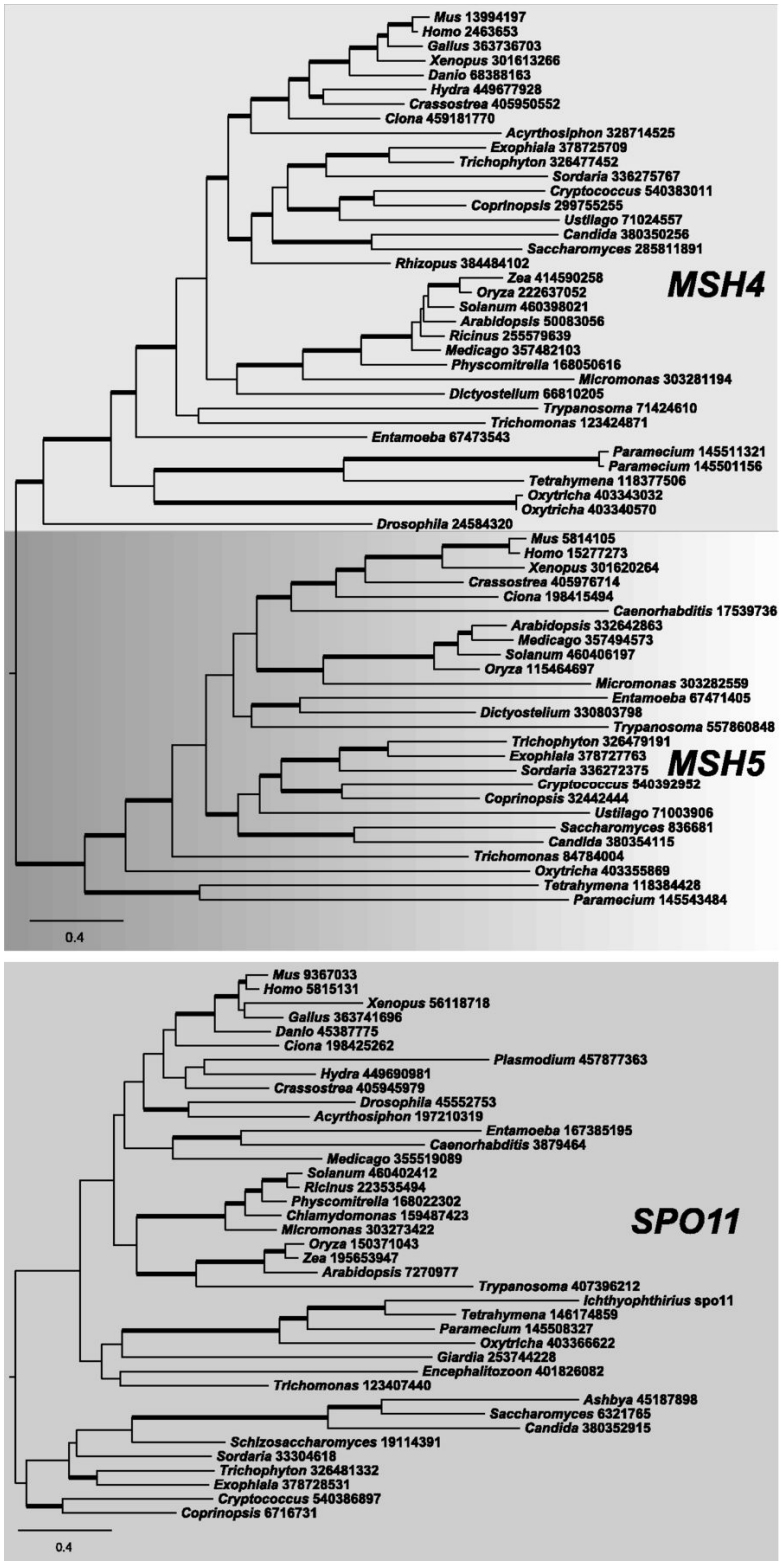


FIG. 2. Maximum likelihood analyses of *HOP2*, *MND1*, *RAD51*, and *DMC1*. Bipartitions with ≥70% bootstrap support are bolded. Individual trees for all genes inventoried in the four ciliate genomes, as well as all bipartition support values, are in supplementary file 1.

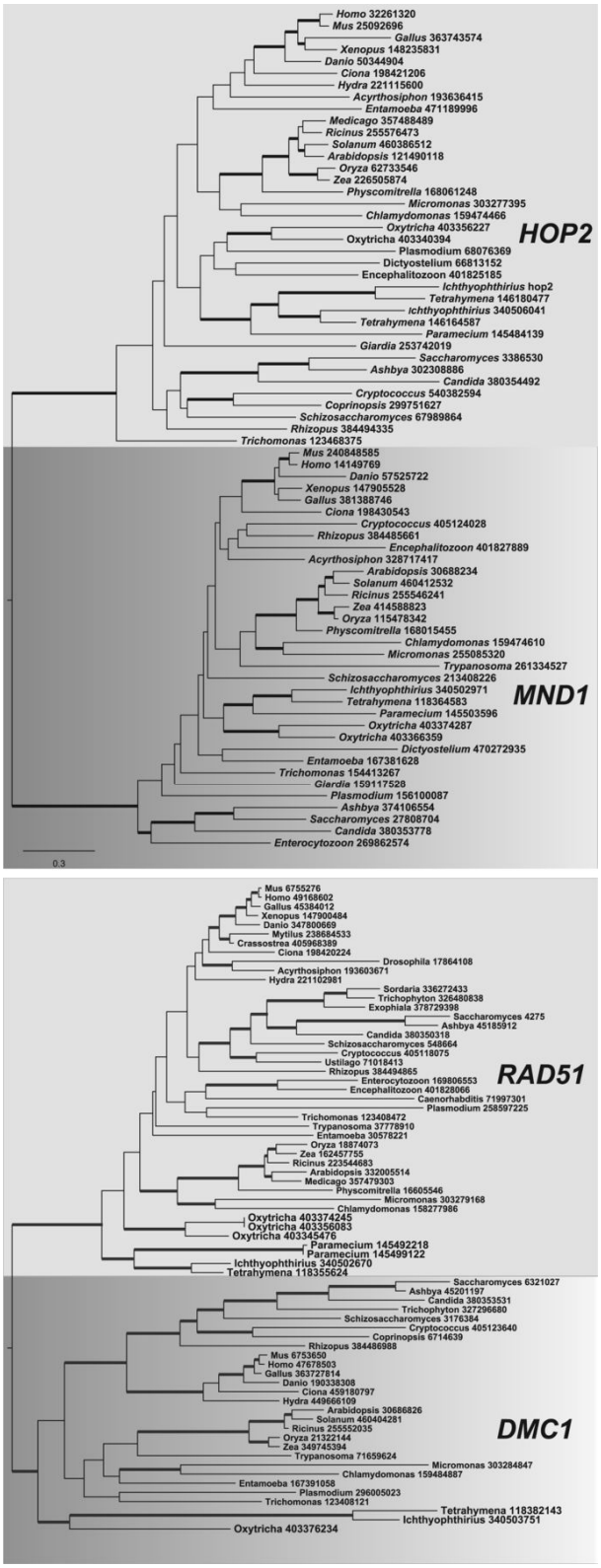


FIG. 3. Estimates of dN/dS ratios for 23 meiotic genes from four eukaryotic clades. Individual values are in supplementary table 2.

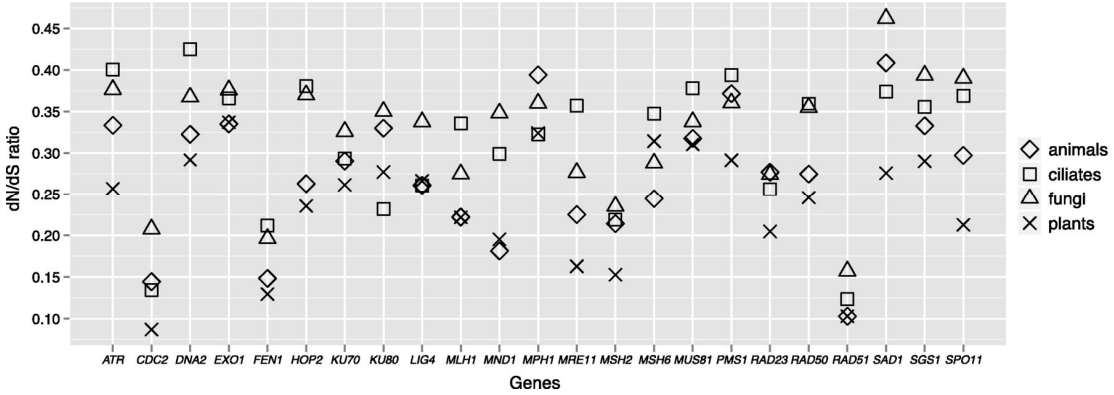
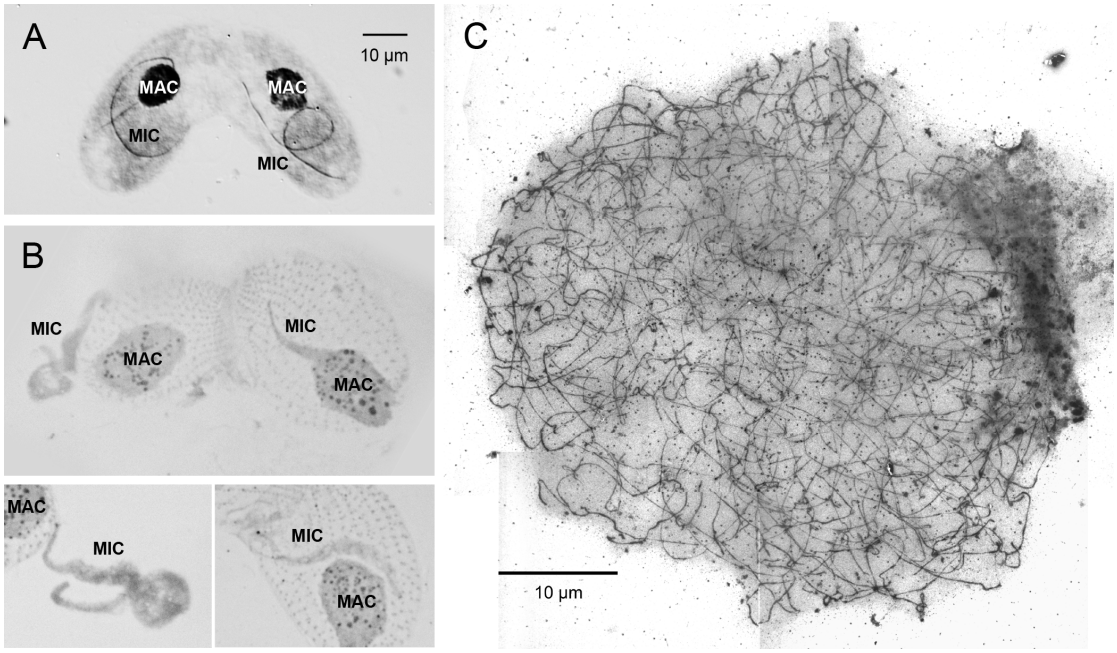


FIG. 4. Spread meiotic nuclei. *(A)* A pair of conjugating *T. thermophila* cells, each with a macronucleus (MAC) and an elongated meiotic micronucleus (MIC). This is the stage when chromosome pairing occurs. Nuclei are stained with Giemsa. *(B-D)*Several examples of detergent-spread, silver stained micronuclei of *T. thermophila*. Light microscopy images. No internal structure is seen. Scale bars for *(B-D)* are the same as in *(A)*. *(E)* Electron microscopic image of a detergent-spread, silver stained meiotic nucleus of *S. mytilus*. Long thin threads, resembling axial element SC subunits, are present. Bars represents 10 μm.


A
10 µm
MAC
MAC
MIC
MIC
B
MIC
MAC
MIC
MAC
MAC
MIC
MIC
MAC
C
10 µm

Chapter II

Cryptic sex in Symbiodinium (Alveolata, Dinoflagellata) is supported by an inventory of meiosis genes

Jingyun Chi , Matthew W. Parrow and Micah Dunthorn (2014), Cryptic Sex in *Symbiodinium* (Alveolata, Dinoflagellata) is Supported by an Inventory of Meiotic Genes. J. Eukaryot. Microbiol., 61: 322-327. doi:10.1111/jeu.12110

Summary

Background

Sexual reproduction is widely distributed, but not universal, in animal, plant and fungi, and the genes involved in meiosis, one significant process of sexual organisms, are well detected in molecular level. However, the present or absents of meiotic genes in protists is remain unclear. Meiotic genes involved in initial double-strand break formation, as well as downstream processes such as crossing over, are just beginning to be assessed in protists. In our previous study, we inventoried 51 meiotic genes including 11 meiosis-specific in the genome of four ciliates: *Tetrahymena thermophila*, *Paramecium tetraurelia, Ichthyophthirius multifiliis* and *Oxytricha trifallax*.

In this study, we inventory the same bunch of genes in the genome of symbiodinium, one of the most prevalent genera of endosymbiotic dinoflagellates in various coral and marine organisms including representatives of ciliates, cnidarians, flatworms, foraminifera, mollusks and radiolarians. The genome of dinoflagellates are among the largest known genomes and the nucleus contains a DNA-binding protein instead of histones. The chromosomes are condensed throughout the cell cycle and are segregated through an unusual mechanism of closed mitosis with an extranuclear spindle that does not pass through the nuclear envelope (Triemer and Fritz 1984). Because of the above features of dinoflagellates, no whole genome sequencing has been applied in this group except for one putative asexual clade, *Symbiodinium minutum*. Meanwhile, EST (Expressed sequence tag) data from *S.minutum* and *S. microadriaticum* are also available.

In most time of their life cycle, *Symbiodinium* undergo asexual reproduction by division. Hypotheses about ecology, observed diversity, and applications of species concepts in *Symbiodinium* have been complicated by a long-standing uncertainty of whether or not sexual recombination occurs in

these dinoflagellates, since neither cytological evidence for sexual recombination nor observation of meiosis has been provided.

Basically three approaches could be applied for detecting cryptic sex: observation, popular genetics to reveal the diversity of complex genetic systems and meiotic gene inventory in the whole genome. However, none of the three approaches are perfect and could demonstrate cryptic sex independently.

As the most direct approach, observation is nevertheless powerless for facultative sexual organisms which undergo only vegetative reproduction in inappropriate laboratory conditions. Meanwhile, for small organisms like most protists, there is no obvious morphological feature for sex. In symbiotic organisms such as *Symbiodinium*, sex might only occur in its rare free-living stage. Popular genetics has its limits considering the Meselson's effect that alleles at a single locus in an asexual population evolve independently of each other and may look likes paralogs in phylogeny after the long-term of evolution. Meiotic gene inventory approach is limited by the uncertainty of expression of those genes, i.e. gene found in the genome may not be used anymore. In summary, none of the three approach for detecting cryptic sex is perfect, which is the reason why we need to integrate the results of at least two of them to confirm our inference.

Application of popular genetics to *Symbiodinium* has revealed high genetic variability in allozymes, randomly amplified polymorphic DNA, and other molecular fingerprints. This variation has been interpreted as evidence of intraclade shuffling of alleles within population gene pools through sexual recombination. Genetic variation in observed markers has even appeared higher in some *Symbiodinium* clades than in known sexual dinoflagellates. Additionally, ribosomal DNA genes are relatively homogeneous within clades of *Symbiodinium*, suggesting concerted evolution at putatively tandem-array rDNA loci through homologous recombination.

Although not all genes known to be involved in meiosis are found in all

sexual microbial eukaryotes, presumably many, if not most, of these meiotic genes would be lost from the genome of derived, obligatory asexuals. As an evidence of cryptic sex, meiotic genes have been found in several putative asexual organisms: the arbuscular mycorrhizal fungus *Glomus spp*., the blue cheese fungi *Penicillium roqueforti*, the parasites *Giardia intestinalis* and *Trichomonas vaginalis*.

Methods

In our study, we inventoried 11 meiosis-specific genes and 40 meiosis-related genes in available genomes of Symbiodinium minutum (Bayer, et al. 2012a; Shoguchi, et al. 2013b) and Symbiodinium microadriaticum (Bayer, et al. 2012a), to unveil the genetic capacity for canonical eukaryotic sex. Symbiodinium minutum (= isolate Mf1.05b; ITS2 type B1) was originally collected from polyps of Orbicella faveolata (= Montastraea, scleractinian coral) in the Florida Keys, while S. microadriaticum (= isolate CassKB8; ITS2 type A1) was originally collected from tentacles of Cassiopea sp. (upside-down jellyfish) in Kaneohe Bay, Hawaii (Bayer et al. 2012). These two species represent different clades within the Symbiodinium phylogeny.

Results

Of the 51 meiotic genes inventoried, 32 were identified inShoguchi et al.'s(2013a) S. minutum'smostly complete genome (Table 1). A subset of those genes was found in the ESTs ofBayer et al.'s(2012b) S. minutumand S.microadriaticum(Table 1). Missing genes are expected since the ESTs are short, fragmented nucleotide sequences derived from genetranscripts collected at a singletime point in a single cell-cycle state (Adams, et al. 1991). Phylogenetic inferences from each meiotic gene confirm identification (Supplementary File 1).

Discussion

The result of our gene inventory consist with the deduction of population genetics, that the present of six (of eleven) meiosis-specific genes suggests the sexual ability of *Symbiodinium*. Presumably, the protein products of these genes are being used to construct meiotic machinery during cryptic sex, otherwise they would have been lost or inactivated.

Sex may have yet to be directly observed in Symbiodinium because, like other symbionts (sensu Margulis 1993; i.e., including parasites), suppression of out crossing when in the symbiotic state may be adaptive in that clonality is promoted(Heitman 2010). Definitive proof of sex in Symbiodinium still awaits cytological observation and/or evidence of meiotic chromosome or trait segregation. The dinoflagellate meiosis genes reported here for the first time can provide a new foundation for future hypotheses on the evolution, occurrence, and molecular mechanisms of sex in Symbiodinium in particular, and dinoflagellates in general.

Chapter II

Cryptic sex in Symbiodinium (Alveolata, Dinoflagellata) is supported by an inventory of meiosis genes

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J. Eukaryot. Microbiol., 61: 322-327. doi:10.1111/jeu.12110

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Abstract

Symbiodinium encompasses a diverse clade of dinoflagellates that are ecologically important as symbionts of corals and other marine organisms. Despite decades of study, cytological evidence of sex (karyogamy and meiosis) has not been demonstrated in *Symbiodinium*, although molecular population genetic patterns support the occurrence of sexual recombination. Here we provide additional support for sex in *Symbiodinium* by uncovering six meiosis-specific and 25 meiosis-related genes in three published genomes. Cryptic sex may be occurring in *Symbiodinium*'s seldom-seen free-living state while being inactive in the symbiotic state.

Key Words. Asexuality, coral reefs, dinoflagellates, symbiosis, zooxanthellae.

Introduction

Dinoflagellate algae in *Symbiodinium* Freudenthal (Taylor 1974) form a large clade of multiple ribosomal DNA phylotypes or “species”, most of which await formal description(Baillie, et al. 2000; Coffroth and Santos 2005; LaJeunesse 2001; LaJeunesse, et al. 2012; Pochon, et al. 2012; Thornhill, et al. 2013). *Symbiodinium*(= zooxanthellae) are common symbionts of marine organisms such as ciliates, cnidarians, flatworms, foraminifera, mollusks, and radiolarians(Coffroth and Santos 2005; Miao, et al. 2005; Rowan 1998; Trench 1993). These symbioses are central in coral reef ecosystems, where these dinoflagellates provide photosynthetically-fixed carbon to reef-building cnidarians and mollusks (Burriesci, et al. 2012; Davy, et al. 2012; Muscatine 1990). As most of these animals have not incorporated *Symbiodinium* into their vertical germ line transmission, this symbiosis must be re-introduced through horizontal transmission from poorly-understood free-living populations of *Symbiodinium* upon each new generation(Coffroth and Santos 2005).

Hypotheses about ecology, observed diversity, and applications of species concepts in *Symbiodinium* have been complicated by a long-standing uncertainty of whether or not sexual recombination occurs in these dinoflagellates(Coffroth and Santos 2005; LaJeunesse 2001). Most dinoflagellates appear to exhibit a haplontic life cycle, with a haploid vegetative stage and a transient sexual diploid stage(Parrow and Burkholder 2004; Tillmann and Hoppenrath 2013). Examination of polymorphic microsatellite loci from symbiont-derived and cultured isolates suggests that *Symbiodinium* is likewise haploid in the vegetative (presumably mitotically-reproducing) stage(Santos and Coffroth 2003). While it has been hypothesized that *Symbiodinium* may exhibit sex with diploid formation (Fitt and Trench 1983), definitive evidence for canonical eukaryotic sex (e.g.,karyogamy and meiosis) has yet to be shown (Freudenthal 1962; LaJeunesse 2001; Taylor 1974; Trench 1997).

One approach to detect cryptic sex is to evaluate genetic patterns within and among populations(Hurst, et al. 1992; Normark, et al. 2003). Application of this approach to *Symbiodinium* has revealed high genetic variability in all ozymes, randomly-amplified-polymorphic DNA, and other molecular fingerprints. This variation has been interpreted as evidence of intra-clade shuffling of alleles within population gene pools through sexual recombination (Baillie, et al. 2000; LaJeunesse 2001; Pettay, et al. 2011; Reichman, et al. 2003; Santos and Coffroth 2003; Thornhill, et al. 2013). Genetic variation in observed markers has even appeared higher in some *Symbiodinium* clades than in known sexual dinoflagellates(Baillie, et al. 2000; Bolch, et al. 1999). Additionally, ribosomal DNA genes are relatively homogeneous within clades of *Symbiodinium*(Sampayo, et al. 2009), suggesting concerted evolution at putatively tandem-array rDNA loci through homologous recombination (Reichman, et al. 2003).

Another approach to detect cryptic sex is to uncover the presence of meiosis genes (Normark, et al. 2003; Schurko and Logsdon 2008). Although not all genes known to be involved in meiosis are found in all sexual microbial eukaryotes (e.g., Chi, et al. 2013; Malik, et al. 2008), presumably many, if not most, of these meiosis genes would be lost from the genome of derived, obligatory asexuals(Collin and Miglietta 2008; Marshall, et al. 1994; Normark, et al. 2003; Schurko and Logsdon 2008). For example, genes have been found, and considered evidence of cryptic sex, in a number of microbial putative asexuals: the arbuscular mycorrhizal fungus *Glomus* spp. (Halary, et al. 2011; Riley and Corradi 2013), *Giardia intestinalis*(Ramesh, et al. 2005),the blue cheese mold *Penicillium roqueforti*(Ropars, et al. 2012), and *Trichomonas vaginalis* (Malik, et al. 2008). Here we use this meiosis gene inventory approach in the available genomes of *Symbiodinium minutum* from Shoguchi et al.(2013a) and Bayer et al. (2012b), and *Symbiodinium microadriaticum* from Bayer et al. (2012b), to unveil the genetic capacity for canonical eukaryotic sex. *Symbiodinium minutum* (= isolate Mf1.05b; ITS2 type B1) was

originally collected from polyps of Orbicella faveolata (= Montastraea, scleractinian coral) in the Florida Keys, while S. microadriaticum (= isolate CassKB8; ITS2 type A1) was originally collected from tentacles of *Cassiopea* sp.(upside-down jellyfish) in Kaneohe Bay, Hawaii(Bayer, et al. 2012b).These two species represent different clades within the *Symbiodinium* phylogeny (Supplementary Fig. 1, Bayer, et al. 2012b; Coffroth and Santos 2005).

Material and Methods

A query database of 51 meiosis-specific and meiosis-related genes (table 1) was established using literature and keyword searches of the NCBI protein database (http://www.ncbi.nlm.nih.gov) and the Uniprot Knowledgebase (http://www.uniprot.org) from September to November 2012

Classification of *Symbiodinium* isolates follows LaJeunesse et al. (2012). A subject database was made by downloading the genome of *S. minutum* and *S.microadriaticum* from their online databases (http://marinegenomics.oist.jp/genomes/viewer?project_id=21&current_assembly_version=symb_aug_v1.120123, and http://medinalab.org/zoox). Shoguchi et al.'s(2013a) *S.minutum*'s Roche-454- and Illumina-sequencing-produced annotated protein database was searched using BLASTp, and nucleotide sequences were searched with tBLASTn (Altschul, et al. 1990). Bayer et al.'s (2012b)*S. minutum*'s and *S.microadriaticum*'s annotated expressed sequence tags (ESTs) were searched with tBLASTn; identified sequences were translated into proteins using Transeq v6.6.0(Goujon, et al. 2010; Rice, et al. 2000), which also allowed for identification of stop codons. For both BLASTp and tBLASTn, hits with E-values<10^{-4} were retained. Gene identity was verified by reciprocal BLASTp searches against the non-redundant NCBI protein sequence database.

Phylogenetic inferences used multiple amino acid sequence alignments were constructed using MAFFT v6.850(Katoh, et al. 2002).The WAG-I-Γ model of evolution was used in all analyses. Maximum Likelihood analyses were run with RAxML v7.3.0(Stamatakis 2006), with bipartition support from 1,000 bootstrap replicates. Trees were visualized with FigTreev1.3.1(Rambaut 2006).

Results and Discussion

Inventory of meiosis genes.

Of the 51 meiotic genes inventoried, 32 were identified inShoguchi et al.'s(2013a)*S. minutum*'smostly complete genome (Table 1). A subset of those genes was found in the ESTs of Bayer et al.'s(2012b) *S. minutum*and *S.microadriaticum* (Table 1). Missing genes are expected since the ESTs are short, fragmented nucleotide sequences derived from genetranscripts collected at a singletime point in a single cell-cycle state (Adams, et al. 1991). Phylogenetic inferences from each meiotic gene confirm identification (Supplementary File 1).

Six of the 11 meiosis-specific genes were identified in *Symbiodinium*. Those genes included: *SPO11*, a type II topoisomerase that initiates meiotic recombination by creating DNA double-strand-breaks (Keeney, et al. 1997); *MSH4* and *MSH5*, which stabilize crossovers between homologous chromosomes(Borts, et al. 2000; Kohl and Sekelsky 2013); as well as*DMC1*, *HOP2*, *MND1*. Four meiosis-specific genes (*HOP1*, *MER3*, *ZIP1* and *REC8*) were not uncovered in *Symbiodinium*; three of these genes (*HOP1*, *MER3*, *ZIP1*) are also missing in the relatively closely-related Alveolate, *Tetrahymena thermophila* that is a known sexual(Malik, et al. 2008; Mochizuki, et al. 2008).Twenty-five of the 40 meiosis-related genes inventoriedwere also identified in *Symbiodinium*.

Stop codons within the protein-coding regions of the meiosis genes were largely not found in*S. minutum*and S. *microadriaticum*. Only one stop codon was found in the sequence of *RTEL1* of Bayer et al.'s (2012b)*S. minutum*, which is probably due to an error in the EST's one-pass sequencing methodology. This absence of stop codons within the coding regions (except for the one EST of*RTEL1*) is consistent with the genes being able to be transcribed and translated into functional meiotic proteins.

Is *Symbiodinium* sexual?

There are now two sources of evidencesuggesting *Symbiodinium* is cryptically sexual. First,there is population genetic data that is consistent with recombination from environmental and cultured isolates collected from a variety of marine habitats(Baillie, et al. 2000; LaJeunesse 2001; Lajeunesse, et al. 2010; Pettay, et al. 2011; Sampayo, et al. 2009; Santos, et al. 2004; Thornhill, et al. 2013). Second, we present here new data in this geneinventory that uncovered six meiosis-specific and 26 meiosis-related genes in three available*Symbiodinium* genomes (Table 1).Presumably, the protein products of these genes are being used to construct meiotic machinery during cryptic sex, otherwise they would have been lost or inactivated(Collin and Miglietta 2008; Marshall, et al. 1994; Normark, et al. 2003; Schurko and Logsdon 2008).

Meiosis genes uncovered in *Symbiodinium* could be used for another cellular processes besides crossing over (Normark, et al. 2003; Riley and Corradi 2013); e.g., the paralogs of *HOP2* and *MND1* in *T. thermophila*(Mochizuki, et al. 2008). Some of these genes could also be used for a parasexual cycle (non-sexual genetic exchange); e.g., *Candida albicans*, where diploid cells undergo syngamy and karyogamy followed by a stochastic reduction back to diploidy, with no haploid stage (Bennett and Johnson 2003; Forche, et al. 2008; Heitman 2010); and *Giardia intestinalis*, where nuclei within a cyst undergo karyogamy followed by meiotic reduction (Heitman 2010; Poxleitner, et al. 2008). However, the genomic inventory data presented here supports the view that *Symbiodinium*, like most other microbial eukaryotes (Dunthorn and Katz 2010; Heitman 2010; Hurst, et al. 1992; O'Malley, et al. 2013), is a sexual taxon with meiotic recombination functioning to promote genetic diversity and adaptive evolution.

Sex may have yet to bedirectly observed in *Symbiodinium* because, like other symbionts(*sensu* Margulis 1993; i.e., including parasites), suppression of outcrossing when in the symbiotic state may be adaptive in that clonality is promoted(Heitman 2010). When in the symbiotic state, sex may also be

inactive if the creation of new allelic combinationswould affect the relationship between *Symbiodinium*and its partner, or if only one *Symbiodinium*mating type is present because of strain selection by the partner. If sex is occurring, it is most likely during the free-living state (Trench 1997), and it mightrequire specialized as-yet unknownconditions for inducement in the laboratory; for example, a sexual stage in*Aspergillusfumigatus* was shown after 100 years of trying when more natural conditions were eventually mimicked(Heitman 2010; O'Gorman, et al. 2009).

Definitive proof of sex in *Symbiodinium* still awaits cytological observation and/or evidence of meiotic chromosome or trait segregation. Dinoflagellate meiosis is not well understood but appears unusual at the genetic and cytological levels(Beam and Himes 1975; Parrow and Burkholder 2003; Parrow and Burkholder 2004; Tillmann and Hoppenrath 2013). The dinoflagellatemeiosis genes reported here for the first time can provide a new foundation for future hypotheses on the evolution, occurrence, and molecular mechanisms of sex in *Symbiodinium* in particular, and dinoflagellates in general.

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Table**1.** Meiosis genes inventoried in three *Symbiodinium* genomes. Shoguchi et al.'s(2013)*Symbiodinium minutum* genome is from Roche-454 and Illumina sequencing; Bayer et al.'s (2012) *S. minutum* and *Symbiodinium microadriaticum* genomes are from annotated expressed sequence tags (ESTs).Names of sequences indicate their Gene ID or sequence identifiers from their respective databases. "-" = gene not found.

Gene	*Symbiodinium* **minutum** Shoguchi et al. (2013)	*Symbiodinium* **minutum** Bayer et al. (2012)	*Symbiodinium microadriaticum* Bayer et al. (2012)
MEIOSIS SPECIFIC			
DMC1	symbB.v1.2.008353.t1 symbB.v1.2.000608.t1	-	kb8_rep_c3666 kb8_s61344
HOP1	-	-	-
HOP2	symbB.v1.2.026766.t1	-	-
MER3	-	-	-
MND1	symbB.v1.2.036043.t1	-	
MSH4	symbB.v1.2.013503.t1	mf105_c16703	-
MSH5	symbB.v1.2.033801.t1	mf105_c15157	-
REC8	-	-	
RED1	-	-	
SPO11	symbB.v1.2.038121.t1 symbB1.v1.2.012520.t1	mf105_rep_c6015 mf105_c21838 mf105_s61845	kb8_c47674
ZIP1	-	-	-
MEIOSIS RELATED			
ATM	symbB.v1.2.029032.t	-	-
ATR	symbB.v1.2.040344.t1 symbB.v1.2.040393.t1	-	-
BRCA1	-	-	-
BRCA2	symbB.v1.2.003783.t1	-	-
CDC2	symbB.v1.2.017277.t3 symbB.v1.2.008214.t1 symbB.v1.2.004367.t1	mf105_rep_c10109 mf105_rep_c2341 mf105_rep_c19999	kb8_rep_c1509 kb8_rep_c457 kb8_rep_c2571
DNA2	-	-	-
EXO1	symbB.v1.2.019143.t1	mf105_rep_c654	kb8_rep_c5845
FEN1	symbB.v1.2.017794.t1 symbB.v1.2.012846.t1 symbB.v1.2.005353.t1	mf105_c28658 mf105_rep_c14029	kb8_rep_c543 kb8_c15198
KU70	symbB.v1.2.013334.t1	-	-
KU80	symbB.v1.2.017317.t1	mf105_c23114 mf105_s61872	-
LIG4	symbB.v1.2.033586.t1 symbB.v1.2.007862.t5	mf105_c8799 mf105_rep_c9196	kb8_rep_c308 kb8_c25653 kb8_rep_c20313
MLH1	symbB.v1.2.021543.t1 symbB.v1.2.038425.t1	mf105_rep_c4483	kb8_c35906
MLH3	symbB.v1.2.028804.t1	mf105_c43035	kb8_c31640
MMS4	-	-	-
MPH1	-	-	-
MRE11	symbB.v1.2.022929.t1 symbB.v1.2.022929.t3	mf105_c45501	kb8_c21135
MSH2	symbB.v1.2.002929.t1 symbB.v1.2.002929.t2	-	kb8_rep_c4807
MSH3	-	-	-
MSH6	symbB.v1.2.026874.t1 symbB.v1.2.039566.t1 symbB.v1.2.038785.t1	mf105_c38241	kb8_c9361 kb8_c35175
MUS81	symbB.v1.2.018987.t1 symbB.v1.2.018988.t1	-	-
PMS1	symbB.v1.2.037606.t1	-	kb8_rep_c27825
RAD17	-	-	-
RAD23	symbB.v1.2.023245.t1	mf105_c45316	-
RAD24	-	-	-
RAD50	symbB.v1.2.031416.t2 symbB.v1.2.031416.t1	mf105_c32417	kb8_rep_c5255
RAD51	symbB.v1.2.024814.t1	-	kb8_rep_c1564 kb8_s57946
RAD52	-	-	-
RAD54	symbB.v1.2.019527.t1	mf105_rep_c6986	kb8_rep_c4182
REC114	-	-	-
RTEL1	symbB1.v1.2.007167.t1 symbB1.v1.2.031200.t2 symbB1.v1.2.017460.t1	mf105_c42650	kb8_rep_c18897
SAD1	symbB.v1.2.018178.t1	-	-
SAE2	symbB.v1.2.018901.t1	-	kb8_c11808
SGS1	symbB.v1.2.009075.t4 symbB.v1.2.009075.t3	-	kb8_c23447 kb8_c26428
SLX1	symbB.v1.2.003012.t1 symbB.v1.2.026455.t1	mf105_rep_c7537 mf105_s55008	kb8_rep_c7475 kb8_c19702
SLX4	-	-	-
SMC5	-	-	-
SMC6	-	-	-
XRCC4	-	-	-
XRS2	-	-	-
YEN1	-	-	-

Chapter III

Independent Reduction of Meiotic Crossover Pathway I in the Alveolates

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(Unpublished)

Summary

Background

Most of eukaryotes show signs of having sex or sexual recombination, and the other asexual eukaryotes have evidences of evolving from sexual ancestors (Zimmer, 2009). However, the distribution of sex in specific lineage is debating. With the detection of almost full set of meiotic genes in the genomes of some putative asexual species, they are considered as secretively sexual (Chi et al., 2014b; Malik et al., 2008). Meiotic recombination, or crossover are proved to have two pathways in eukaryotes (Argueso et al., 2004; Gottlieb et al., 1989; Mao-Draayer et al., 1996; Peoples-Holst and Burgess, 2005). In the predominate pathway I, a proteinaceous structure that juxtaposes homologs called synaptonemal complex (SC) is assembled at the interface between paired chromosomes (Moses, 1968). The pathway II involves the Mus81-Mms4 endonuclease complex and other genes that also have mitotic DNA repair functions (Schwartz et al., 2012).

The distribution of both meiotic crossover pathways is well studied in many model eukaryotes. Loss of one pathway is not rare. In our previous study of discovering the two meiotic crossover pathways in ciliate, we hypothesis that ciliates have a slimmed pathway I, since most of proteins involved are missing in the genome of four ciliate genomes (Chi et al., 2014a).

Ciliates are in the larger groups of alveolates, together with Dinoflagellates, Apicomplexa and several other lineages. In our previous study of meiotic genes in *Symbiodium* (Dinoflagellates), we discussed only cryptic sex instead of analyze the pathways (Chi et al., 2014b). In this study, all available genome and EST data of alveolates are collected for meiotic inventory. Our aim is to uncover the distribution of both meiotic pathways in alveolates with the complementary of meiotic gene among species, to find out if independent loss of meiotic pathways occur, and to discover the influence of

chromosome structure to meiotic pathways.

Methods

In this study we used bioinformatic approach to search for homologs of 51 meiotic genes in whole genome of seven Apicomplexa (*Cyclospora cayetanensis*, *Eacervulina Houghton, Eimeria falciformis*, *Hammondia hammondi*, *Neospora caninum*, *Plasmodium falciparum*, *Toxoplasma gondii*), two Chromera (*Chromide velia*, *Vitralla brassicaformis*) and one Perkinsus (*Perkinsus marinu*). We also inventoried EST sequences of three Dinoflagellates (*Amphidinium carterae*, *Oxyrrhis marina*, *Karenia brevis*), whose inventory results are not presented due to the missing of too many genes.

The query database of meiotic genes was used to search the subject database constructed with amino acid sequence files from the three Labyrinthulomycete genomes using two complementary approaches: Blastp (Altschul et al., 1990), and HMMER v3.0. (Eddy, 2011) using HMMER search. We developed a customized Python program to integrate the results from both Blastp and HMMER together. For both methods, only hits with E-values $<10^{-3}$ for the full sequence were retained. All candidate homologs were then verified by reciprocal Blastp search against the nonredundant protein sequence database of NCBI and phylogeny analysis of RAxML (Stamatakis, 2014).

Results

In Apicomplexa , 6 of 11 meiotic-specific genes are present in most species, including Spo11, Dmc1, Hop1, Hop2, Mnd1, Mer3. Those genes are particularly mediate meiotic crossover pathway I. Exceptions are the absence of Dmc1 in *H. hammondi* and *T. gondii*, Hop1 in *C. cayetanensis*, Hop2 in *C. cayetanensis* and *E. Houghton*, Mnd1 in *C. cayetanensis*, *H. hammondi*, *T. gondii*, Mer3 in *C. cayetanensis*, *N. caninum*, *P. falciparum*. The protein of Msh4 and Msh5 form heterodimer in yeast and human to bind and stabilize the

double Holliday Junction and facilitate crossover during meiotic crossover pathway I (Nishant et al., 2010; Rakshambikai et al., 2013). These two genes, nevertheless, are largely absent in Apicomplexa. Three other meiosis-specific genes, Red1, Zip1 and Rec8, which are the participants in the forming of SC are absent in all Apicomplexa. Of the other 40 meiotic genes, 24 are present in most Apicomplexa. Mus81, which is required for meiotic crossover pathway II are presented in most species except *E. Houghton*.

In Chromerida, *V. brassicaformis* posses 8 of the 11 meiosis-specific genes, while Red1, Zip1 and Rec8 are absent. However, the other Chromerida, *C. velia* lacks 7 of the 11 meiosis-specific genes, only Spo11, Dmc1, Msh4 and Msh5 are present. Of the other 40 meiotic genes, 16 are presented in both *V. brassicaformis* and *C. velia*, while 8 are present in either of the two species. Mus81 are presented in both Chromerida species.

In Perkinsus, only two meiosis-specific genes, Dmc1 and Msh5, are present, while the other 9 are missing. Spo11, which initiates the meiotic crossover by generating the double-strand breaks, are also absent in Perkinsus. Of the other 40 meiotic gene, 19 are present.

Discussion

We inventoried 51 meiotic genes, including 11 meiosis-specific genes in the whole genome sequences of Apicomplexa, Chromerida and Perkinsus (Table 1).

Synaptonemal complex (SC) component proteins including Hop1 are present in Apicomplexa, nevertheless some other SC component proteins, such as Zip1, Red1, are missing. Their absence may due to the high variability of protein of SC among different eukaryotes. SC protein structure has been observed by TEM microscope in Apicomplexa *Grebnickiella gracilis* and *Eimeria tenella* (del Cacho et al., 2005; Molon-Noblot and Desportes, 1977). Combining the observation and our inventory results, we assumed that most, if not all, Apicomplexa has SC. Although it is still unclear if they undergo the

meiotic crossover pathway I, we hypothesize that they possess the potential ability of using it.

At least one Chromerida, *V. brassicaformis*, has Hop1 and the other one, *C. velia*, has a Hop1-like protein, whose the function is ambiguous. It is notable that, in other study, *C. velia* is considered as asexual due to the lack of observation of cell fusion and some core meiotic genes. However, we have the opposite opinion that, only from the standpoint of gene inventory, *C. velia* might be sexual with the ability of undergoing meiotic crossover pathway II. We suppose that the Hop1-like protein is nonfunctional and SC is absent in *C. velia*. It still possesses genes such as Mus81, which required in the crossover pathway II. Although sex has not been directly observed in *C. velia*, from the evolution point of view, *C. velia* might be secretively sexual or early asexual, whose core meiotic genes are starting to be lost. The other chromerida, *V. brassicaformis*, whose cell fusion are observed and most of core meiotic gene are found, are considered as sexual.

The loss of most meiosis-specific gene, especially Spo11 genes in Perkinsus indicate that it undergoes reproduction asexually. This result is supported by the fact that no sexual reproduction has been observed so far in Perkinsus. However, recent popular genetic analysis indicates that Perkinsus might employs multiple reproductive modes, both sexual and asexual reproduction, considering the genotypic diversity was great and recombination occurred between genetic loci (Thompson, 2010; Thompson et al., 2011). Since Spo11 is an ancient and ancestral core meiotic genes, its loss in Perkinsus would lead to following inferences: Perkinsus is a newly asexual lineage due to the genuine loss of Spo11, or the sequence divergence make it unrecognizable.

The present of most meiotic-specific genes, including SC-related genes, suggests that, most if not all, Apicomplexa and Chromerida have the potential ability of executing both canonical meiotic crossover pathway I and pathway II.

Our previous study of inventory of 51 meiotic genes in four ciliates reveals

that pathway I, as well as SC, are highly-reduced in ciliates. Ciliates probably depend largely on Mus81-dependent crossover pathway II. Another study of meiotic gene inventory in *Symbiodinium*, a diverse clade of dinoflagellates indicates that this putative asexual species are secretive sexual and the SC-dependent crossover pathway I are also reduced in Dinoflagellates. Dinoflagellates and Apicomplexa are more related to each other than either is to Ciliates, and Perkinsus are more related to Dinoflagellates while Chromerida are sister group of Apicomplexa (Cavalier-Smith and Chao, 2004; Fast et al., 2002; Gajadhar et al., 1991; Kuvardina Olga et al., 2005; Leander and J. Keeling, 2004; Saldarriaga et al., 2003). Thus, we infer that the loss of meiotic crossover pathway I in Dinoflagellates and Ciliates are independent, which may due to their specific genome architectures.

Dinoflagellates are characterized by its permanently condensed chromosomes that are composed of fibers organized without histones (Costas and Goyanes, 2005; Rizzo, 1991, 2003). It has been observed that during the chromosome segregation, the newly synthesized DNA is packed inside the original chromosome while the dividing chromosomes keeping condensed.

The ciliates has also peculiar chromosome architectures with nuclear dimorphism, containing a somatic macronuclear and a germline micronuclear, each with different genome. It is observed that, during the meiotic prophase, the micronuclear of Tetrahymena becomes extremely elongated, forming a "ultimate bouquet", during the meiotic prophase (Lukaszewicz et al., 2013).

It is already known that ciliates share the Mus81-dependent crossover pathway with fission yeast (*S. pombe*). The other common point of these two species is the substantial chromosome movement during the prophase of meiosis I (Chikashige et al., 1994; Robinow, 1977). In fission yeast, the nucleus undergoes elongation and the chromosomes are tethered to the spindle pole body associated with nuclear envelope, forming a "horsetail". SC, whose function is partly substituted by a linear elements called LinEs (Loidl, 2006; Wells et al., 2006), is also absent in fission yeast. Both the "horsetail" in

fission yeast and "ultimate bouquet " in Tetrahymena brings homolog chromosomes so adjacent to each other that only simple device or reduced SC with a few meiotic proteins are sufficient to combine the chromosomes for executing crossover. With condensed chromosomes during meiosis in Dinoflagellates, we would infer that the distances between homolog chromosomes might be a reason of the reduction of SC. Although few evidence support that SC is the premise of meiotic crossover pathway I, the interfering crossover and SC formation are always present/absent concomitantly. With jet obscure function, SC is inferred to facilitate the conversion of interfering crossover precursors into chiasmata (Loidl and Lorenz, 2016). Thus, we deduce that the reduction of SC would caused by the chromosome structure during the prophase of meiosis and led to the reduction of interfering crossover. However, whether the meiotic crossover are subject to interference need be tested in the future when a potential crossover-marker would be found.

Chapter III

Independent Reduction of Meiotic Crossover Pathway I in the Alveolates

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(Unpublished)

Abstract

The distribution and evolution of meiotic recombination pathways in alveolates would provide us clues of lost/gaining of pathways in early eukaryotes. Previous inventory for 11 meiosis-specific and 40 meiosis-related genes in four genomes of Ciliates already reveals their independence on crossover pathway I, which is mediated by Synaptonemal complex. In this study, we expand our research on the genome data of other three groups of alveolates, including three Dinoflagellates, seven Apicomplexa, two Chromerida and one Perkinsus. The results of inventory suggests that Apicomplexa might depend on canonical crossover pathway I, while Dinoflagellates undergo pathway II and Perkinsus are supposed to be asexual. It is still ambiguous that whether the pathway I in reduced in Chromerida. It is supposed that the loss of pathway I in Ciliates and Dinoflagellates are evolutionarily independent.

Key words: Alveolates, Ciliates, Dinoflagellates, Apicomplexa, meiosis pathways, gene inventory

Introduction

Sex is important for a species to survive in a changing environment by producing offspring with genetic diversity. Although the origin of sex is obscure, it is a consensus that sex is assumed to be in the common ancestor of all eukaryotes (Goodenough and Heitman, 2014). Most of eukaryotes show signs of having sex or sexual recombination, and the other asexual eukaryotes have evidences of evolving from sexual ancestors (Zimmer, 2009). However, the distribution of sex in specific lineage is debating. With the detection of almost full set of meiotic genes in the genomes of some putative asexual species, they are considered as secretively sexual (Chi et al., 2014b; Malik et al., 2008).

Playing a critical role in the faithful alignment and segregation of homolog chromosomes, meiotic recombination, or crossover are proved to have two pathways in eukaryotes (Argueso et al., 2004; Gottlieb et al., 1989; Mao-Draayer et al., 1996; Peoples-Holst and Burgess, 2005). In the predominate pathway I, a proteinaceous structure that juxtaposes homologs called synaptonemal complex (SC) is assembled at the interface between paired chromosomes (Moses, 1968). The pathway II involves the Mus81-Mms4 endonuclease complex and other genes that also have mitotic DNA repair functions (Schwartz et al., 2012). The existence of two crossover pathways was proposed by Zalevsky et al in 1999 (Zalevsky et al., 1999b). It is almost certain that meiosis evolved from mitosis (Wilkins and Holliday, 2009), caused by both the cytological novelties and the selective forces. Meiotic pathway II occurred before pathway I, since it shares nearly all regulators with mitosis, while pathway I applies more meiosis-specific proteins. Both meiotic crossover pathways are widely distributed in all eukaryotes, which leading to the hypothesis that they are also present in the ancestor of all eukaryotes.

The distribution of both meiotic crossover pathways is well studied in many model eukaryotes. Loss of one pathway is not rare. In some organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster,* the pathway I

are predominated (Lui and Colaiácovo, 2013; McKim et al., 2002; Zalevsky et al., 1999a), *Schizosaccharomycetes pombe*, however, uses only pathway II (Davis and Smith, 2001). In our previous study of discovering the two meiotic crossover pathways in ciliate, we hypothesis that ciliates have a slimmed pathway I, since most of proteins involved are missing in the genome of four ciliate genomes (Chi et al., 2014a).

Ciliates are in the larger groups of alveolates, together with Dinoflagellates, Apicomplexa and several other lineages such as Cshromerida, Perkinsus, Colponema, Ellobiopsids, Oxyrrhis, Rastrimonas and Parvilucifera, that do not neatly fit into the three above major subgroups (Leander, 2008). Alveolates is characterized by alveoli, that are sacs forming continuous layer of vesicles underneath their cell membrane (Stelly et al., 1991). Together with plasma membrane, membrane skeleton, microtubular structures, alveoli forms pellicular structure that maintains the around alveolates. Beyond the defining feature of the present of alveoli, very few unifying morphological character is detected in this group. It is reported that a large amount of alveolate-exclusive proteins, including a family of protein called Alveolins, are associated with the alveolate pellicle formed by in Ciliates, Apicomplexa and Dinoflagellates (Gould et al., 2011; Gould et al., 2008). It is reported that all Alveolates have similar ribosomal DNA sequences. However, the chromosomal architecture among Alveolates are highly diverse. In dinoflagellates, a permanently condensed nuclear matrix accommodates permanently condensed chromosomes that are composed of fibers organized without histones and nucleosomes in stacked rows of parallel nested arches (Costas and Goyanes, 2005). The Ciliates have also peculiar chromosome architectures with nuclear dimorphism, a somatic macronuclear and a germline micronuclear, each with different genome. Among all classes of ciliates, three of them (Spirotrichea, Phyllopharyngea and Armophorea) processed into “gene-sized” chromosomes (i.e., nanochromosomes) (Klobutcher et al., 1986; Maurer-Alcalá and Katz, 2016).

Discovering the present/absence of meiotic genes with the gene inventory approach would provide us evidences for inferring the evolution of meiotic pathways in particular species(Chi et al., 2014a). In our previous study of meiotic genes in *Symbiodium* (Dinoflagellates), we discussed only cryptic sex instead of analyze the pathways (Chi et al., 2014b). In this study, all available genome and EST data of alveolates are collected for meiotic inventory. Our aim is to uncover the distribution of both meiotic pathways in alveolates with the complementary of meiotic gene among species, to find out if independent loss of meiotic pathways occur, and to discover the influence of chromosome structure to meiotic pathways.

Results

In this study we used bioinformatic approach to search for homologs of 51 meiotic genes in whole genome of seven Apicomplexa (*Cyclospora cayetanensis*, *Eacervulina Houghton, Eimeria falciformis*, *Hammondia hammondi*, *Neospora caninum*, *Plasmodium falciparum*, *Toxoplasma gondii*), two Chromera (*Chromide velia*, *Vitralla brassicaformis*) and one Perkinsus (*Perkinsus marinu*). We also inventoried EST sequences of three Dinoflagellates (*Amphidinium carterae*, *Oxyrrhis marina*, *Karenia brevis*), whose inventory results are not presented due to the missing of too many genes (Table 1). The homologous protein search combined the results of both Blastp Search (Altschul et al., 1990) and HMMER Search (Eddy, 2011) and verified by reciprocal Blastp search against NCBI nr-protein database (version 2016January) and phylogeny analysis of RAxML (Stamatakis, 2014).

In Apicomplexa, 6 of 11 meiotic-specific genes are present in most species, including Spo11, Dmc1, Hop1, Hop2, Mnd1, Mer3. Those genes are particularly mediate meiotic crossover pathway I. Exceptions are the absence of Dmc1 in *H. hammondi* and *T. gondii*, Hop1 in *C. cayetanensis*, Hop2 in *C. cayetanensis* and *E. Houghton*, Mnd1 in *C. cayetanensis*, *H. hammondi*, *T. gondii*, Mer3 in *C. cayetanensis*, *N. caninum*, *P. falciparum*. The protein of Msh4 and Msh5 form heterodimer in yeast and human to bind and stabilize the double Holliday Junction and facilitate crossover during meiotic crossover pathway I (Nishant et al., 2010; Rakshambikai et al., 2013). These two genes, nevertheless, are largely absent in Apicomplexa. Three other meiosis-specific genes, Red1, Zip1 and Rec8, which are the participants in the forming of SC are absent in all Apicomplexa. Of the other 40 meiotic genes, 24 are present in most Apicomplexa. Mus81, which is required for meiotic crossover pathway II are presented in most species except *E. Houghton*.

In Chromerida, *V. brassicaformis* posses 8 of the 11 meiosis-specific genes, while Red1, Zip1 and Rec8 are absent. However, the other Chromerida,

C. velia lacks 7 of the 11 meiosis-specific genes, only Spo11, Dmc1, Msh4 and Msh5 are present. Of the other 40 meiotic genes, 16 are presented in both *V. brassicaformis* and *C. velia*, while 8 are present in either of the two species. Mus81 are presented in both Chromerida species.

In Perkinsus, only two meiosis-specific genes, Dmc1 and Msh5, are present, while the other 9 are missing. Spo11, which initiates the meiotic crossover by generating the double-strand breaks, are also absent in Perkinsus. Of the other 40 meiotic gene, 19 are present.

Discussion

Inventory of meiotic genes in Alveolates

We inventoried 51 meiotic genes, including 11 meiosis-specific genes in the whole genome sequences of Apicomplexa, Chromerida and Perkinsus.

Synaptonemal complex (SC) component proteins including Hop1 are present in Apicomplexa, nevertheless some other SC component proteins, such as Zip1, Red1, are missing. Their absence may due to the high variability of protein of SC among different eukaryotes. SC protein structure has been observed by TEM microscope in Apicomplexa *Grebnickiella gracilis* and *Eimeria tenella* (del Cacho et al., 2005; Molon-Noblot and Desportes, 1977). Combining the observation and our inventory results, we assumed that most, if not all, Apicomplexa has SC. Although it is still unclear if they undergo the meiotic crossover pathway I, we hypothesize that they possess the potential ability of using it. It is also notable that Mus81, the crossover junction endonuclease which facilitates the meiotic crossover pathway II, is missing in Apicomplexa *E. Houghton*, which, if not due to the problem of inventory methods, implies the insignificant role of Mus81 in crossover in *E. Houghton*.

At least one Chromerida, *V. brassicaformis*, has Hop1 and the other one, *C. velia*, has a Hop1-like protein, whose the function is ambiguous. It is notable that, in other study, *C. velia* is considered as asexual due to the lack of observation of cell fusion and some core meiotic genes. The detection of sexual behavior is problematic, giving the fact that the diploid stage of *C. velia* occurs only for short time and sporadically or within a coral host (Vazač et al., 2018). Although our gene inventory results are exactly the same with those in Vazač, et al, we have the opposite opinion that, only from the standpoint of gene inventory, *C. velia* might be sexual with the ability of undergoing meiotic crossover pathway II. Independent loss of one or a bunch of meiosis-specific genes in sexual eukaryotes is not surprising to see. We suppose that the Hop1-like protein is nonfunctional and SC is absent in *C. velia*. It still

possesses genes such as Mus81, which required in the crossover pathway II. Although sex has not been directly observed in *C. velia*, from the evolution point of view, *C. velia* might be secretively sexual or early asexual, whose core meiotic genes are starting to be lost. The other chromerida, *V. brassicaformis*, whose cell fusion are observed and most of core meiotic gene are found, are considered as sexual.

The loss of most meiosis-specific gene, especially Spo11 genes in Perkinsus indicate that it undergoes reproduction asexually. This result is supported by the fact that no sexual reproduction has been observed so far in Perkinsus. However, recent popular genetic analysis indicates that Perkinsus might employs multiple reproductive modes, both sexual and asexual reproduction, considering the genotypic diversity was great and recombination occurred between genetic loci (Thompson, 2010; Thompson et al., 2011). Since Spo11 is an ancient and ancestral core meiotic genes, its loss in Perkinsus would lead to following inferences: Perkinsus is a newly asexual lineage due to the genuine loss of Spo11, or the sequence divergence make it unrecognizable.

Spo11 is reported having three eukaryotic paralogs: Spo11-1, Spo11-2 and Spo11-3. The meiosis-specific protein Spo11-1 are widely found in all branches of eukaryotes; Spo11-2 forms a heterodimer with Spo11-1 in *Arabidopsis thaliana* and some other plants and required for meiotic double strand breaks (Vrielynck et al., 2016); while Spo11-3 is involved in DNA endo-reduplication as a part of the topoVI complex in and found in plant, red algae and a few protists (Malik et al., 2007). Although the gene duplication which resulting in Spo11-2 and Spo11-3 occurs priors to the command ancestor of eukaryotes, these two paralogs are not found in alveolates by our gene inventory. Supported by phylogenetic analysis, the spo11 proteins found by our methods are all spo11-1.

Gene inventory approach

The bioinformatic approach applying on inventory of meiotic genes was first described as "meiosis detection toolkit", which consist of a set of meiotic genes as the "best marker" of the present of meiosis(Schurko and Logsdon, 2008). This approach was used to detect meiotic genes in the genomes of several putative asexual organisms including Ciliates (Chi et al., 2014a; Dunthorn et al., 2017), Amoebozoans (Tekle et al., 2017), Diatoms (Patil et al., 2015), Symbiodium (Chi et al., 2014b), Monogonont rotifers (Hanson et al., 2013), Giardia (Ramesh et al., 2005), *Trichomonas vaginalis* (Malik et al., 2008), *Nasonia vitripennis* (Schurko et al., 2010) and etc. The maintaining of meiotic genes provide evidences for the cryptic sexuality in some species and the present/absence of particular genes indicate the "players" of meiotic crossover pathways (Fig. 1).

Although this approach is approved to be simple and direct in this time when genome data exploded, it has its disadvantage comparing to traditional molecular biology methods. First of all, the results depend largely on the completeness of genome sequencing. EST (Express Sequence Tag) sequences are from a cloned cDNA library and direct evidence for all the sampled transcripts. Although high-throughput technology enable the EST sequencing contribute significantly to the system biology approach, it still has its drawback in discovery homologous genes comparing to whole genome sequencing data. Genes which are not transcripted when sampled would not be sequenced, thus would not be considered as evidence for the present/absence of particular gene. In this study, the inventory of meiotic genes in EST sequences of *A. carterae*, *O. marina*, *K, brevis* would only be used as the supplement of gene inventory in other whole genome sequences. Secondly, the inventory of meiotic genes is based on homolog assortment but not testing. The sequences extracted from sequencing data could be incomplete or error-containing due to the limit of technology. The introns in nucleotide sequences are difficult to annotated and removed purely by bioinformatic methods. Finally, the gene inventoried might be involved in other

molecular processes besides meiosis.

Independent Reduction of Meiotic pathway I in Dinoflagellates and Ciliates

The present of most meiotic-specific genes, including SC-related genes, suggests that, most if not all, Apicomplexa and Chromerida have the potential ability of executing both canonical meiotic crossover pathway I and pathway II.

Our previous study of inventory of 51 meiotic genes in four ciliates reveals that pathway I, as well as SC, are highly-reduced in ciliates. Ciliates probably depend largely on Mus81-dependent crossover pathway II, which requires mostly mitotic repair genes. Another study of meiotic gene inventory in *Symbiodinium*, a diverse clade of dinoflagellates indicates that this putative asexual species are secretive sexual. Although the appliance of two meiotic crossover pathways are not discussed in that paper, the results shows that the SC-related genes (Hop1, Red1, Zip1) are all missing in the whole genome of *Symbiodinium*, which provides evidence that the SC-dependent crossover pathway one are also reduced in Dinoflagellates.

It has reached a consensus that the Dinoflagellates and Apicomplexa are more related to each other than either is to Ciliates, and Perkinsus are more related to Dinoflagellates while Chromerida are sister group of Apicomplexa (Cavalier-Smith and Chao, 2004; Fast et al., 2002; Gajadhar et al., 1991; Kuvardina Olga et al., 2005; Leander and J. Keeling, 2004; Saldarriaga et al., 2003). Thus, we infer that the loss of meiotic crossover pathway I in Dinoflagellates and Ciliates are independent, which may due to their specific genome architectures.

The chromosomal architecture among Alveolates are highly diverse. Dinoflagellates are characterized by its permanently condensed chromosomes that are composed of fibers organized without histones (Costas and Goyanes, 2005; Rizzo, 1991, 2003). Most Dinoflagellates contain only one nucleus (uninucleate), while a few others, which contain an endosymbiont alga, have

two (dinucleate) (Dodge, 1971). The nuclei of Dinoflagellates usually contain large amount of DNA (Rizzo, 2003). The genome size of dinoflagellates ranged from ~1.5 Gb DNA per haploid genome(*Symbiodinium* spp.) to ~250Gb (*Prorocentrum micans*) (Hou and Lin, 2009). Typical dinoflagellates are unusually larger comparing with other eukaryotes (*Homo sapiens* 2500~3500Mb, *Mus musculus* ~2700Mb, *Saccharomyces cerevisiae* ~12Mb, *Arabidopsis thaliana* ~135Mb). Due to their huge genome, genomic study of dinoflagellates are often stymied. Only a few species from *Symbiodium* has been sequenced (Aranda et al., 2016; Shoguchi et al., 2013), thus provide us material for evolution study of this mystic group of eukaryotes. It has been observed that during the chromosome segregation, the newly synthesized DNA is packed inside the original chromosome while the dividing chromosomes keeping condensed. Genetic analysis shows evidences for a usual meiosis in Dinoflagellates (*Crypthecodinium cohnii*) that may result from a centromere linkage or the absent of crossing over in traditional meiosis or an unusual one-division meiosis (Himes and Beam, 1975).

The ciliates has also peculiar chromosome architectures with nuclear dimorphism, containing a somatic macronuclear and a germline micronuclear, each with different genome. Among all classes of ciliates, three of them (Spirotrichea, Phyllopharyngea and Armophorea) processed into “gene-sized” chromosomes (i.e.,nanochromosomes) (Klobutcher et al., 1986; Maurer-Alcalá and Katz, 2016). It is observed that, during the meiotic prophase, the micronuclear of Tetrahymena becomes extremely elongated, forming a "ultimate bouquet", during the meiotic prophase (Lukaszewicz et al., 2013). Meiotic recombination between homolog chromosomes occurs in a limited space in the "bouquet". If the prevalence of this cytological change during meiosis is verified in many ciliates, we would infer that the abandon or reduce of SC would caused by the chromosome structure change.

The genome of Apicomplexa are organized as normal linear chromates. The numbers of protein-coding genes of Apicomplexa, ranging from 3671 in

Babesia bovis to ~8000 in *Toxoplasma gondii*, is significantly reduced comparing to those in human (20000 to 25000), *Drosophila* (~13600), *Saccharomyces* (~6000) (Kissinger and DeBarry, 2011).

It is already known that ciliates share the Mus81-dependent crossover pathway with fission yeast (*S. pombe*). The other common point of these two species is the substantial chromosome movement during the prophase of meiosis I (Chikashige et al., 1994; Robinow, 1977). In fission yeast, the nucleus undergoes elongation and the chromosomes are tethered to the spindle pole body associated with nuclear envelope, forming a "horsetail". SC, whose function is partly substituted by a linear elements called LinEs (Loidl, 2006; Wells et al., 2006), is also absent in fission yeast. Both the "horsetail" in fission yeast and "ultimate bouquet " in Tetrahymena brings homolog chromosomes so adjacent to each other that only simple device or reduced SC with a few meiotic proteins are sufficient to combine the chromosomes for executing crossover. With condensed chromosomes during meiosis in Dinoflagellates, we would infer that the distances between homolog chromosomes might be a reason of the reduction of SC. Although few evidence support that SC is the premise of meiotic crossover pathway I, the interfering crossover and SC formation are always present/absent concomitantly. With jet obscure function, SC is inferred to facilitate the conversion of interfering crossover precursors into chiasmata (Loidl and Lorenz, 2016). Thus, we deduce that the reduction of SC would caused by the chromosome structure during the prophase of meiosis and led to the reduction of interfering crossover. However, whether the meiotic crossover are subject to interference need be tested in the future when a potential crossover-marker would be found.

Methods

A query data of 51 meiotic genes are collected from literatures and Keyword searching in NCBI protein database (http://www.ncbi.nlm.nih.gov/) from September 2012 to May 2015. For some genes that belong to a certain protein family or consist of certain conserved domains, full sequences from Pfam database (Finn et al., 2014).

The genome data of 14 alveolates are collected from NCBI genome database. Among all alveolates genome data, 10 are whole genome sequences and the other four are EST data.

The query database of meiotic genes was used to search the subject database constructed with amino acid sequence files from the three Labyrinthulomycete genomes using two complementary approaches: Blastp (Altschul et al., 1990), which returns the most similar protein sequences from the user-specified protein database, and HMMER v3.0. (Eddy, 2011) using HMMER search, which uses a profile hidden Markov model to detect remote homologs. We developed a customized Python program to integrate the results from both Blastp and HMMER together. For both methods, only hits with E-values $<10^{-}3$ for the full sequence were retained. All candidate homologs were then verified by reciprocal Blastp search against the nonredundant protein sequence database of NCBI.

Inference

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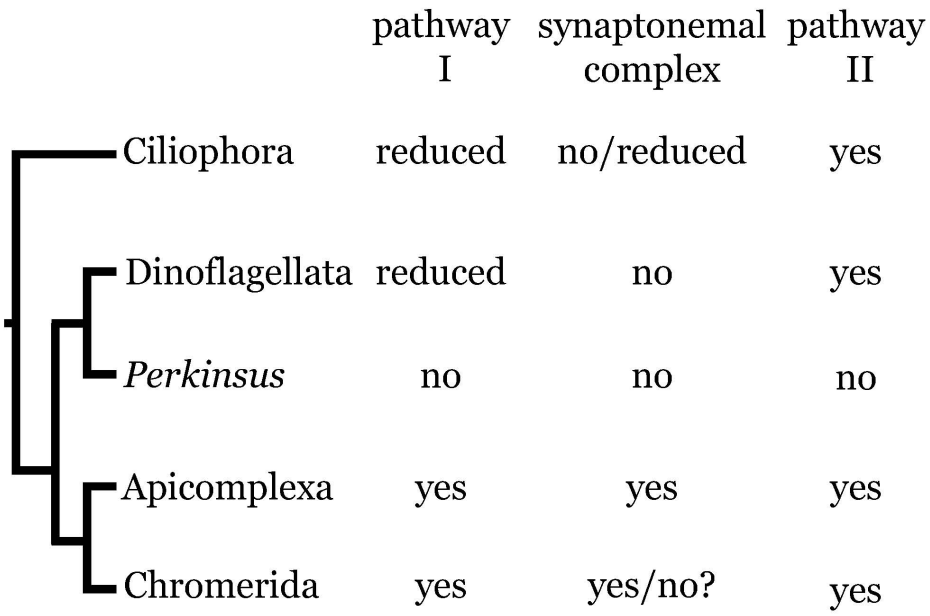
Table 1. Meiosis genes inventoried in four ciliate genomes. Genes are grouped according to functions. Meiosis-specific genes are bolded. Letters in parentheses after some gene names designates that name is used in specific organisms: a in *Arabidopsisthaliana*, c in *Caenorhabditis elegans*, d in *Drosophilamelanogaster*, m in *Mus musculus*, and s in *Schizosaccharomyces pombe*. “+” = genes identified in the genomes. “-” = genes not identified in the genomes

	Apicomplexa							Chromerida		Perkinsus	Ciliates				Dinoflagellates
Gene	*Cyclospora cayetanensis*	*Eimeria falciformis*	*Eacervulina Houghton*	*Hammondia hammondi*	*Toxoplasma gondii*	*Neospora caninum*	*Plasmodium falciparum*	*Chromera velia*	*Vitrella brassicaformis*	*Perkinsus marinus*	*Tetrahymena thermophila*	*Paramecium tetraurelia*	*Ichthyophthirius multifiliis*	*Oxytricha trifallax*	*Symbiodinium minutum*
Meiosis-specific genes															
SPO11/REC12 (s)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
DMC1	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+
HOP1	-	+	+	+	+	+	+	-	+	-	-	-	-	-	+
HOP2	-	+	-	+	+	+	+	-	+	-	+	+	+	+	+
MER3	-	?	+	+	+	-	-	-	+	-	-	-	-	-	+
MND1	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+
MSH4	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+
MSH5	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+
RED1/ASY3 (p)/*REC10* (s)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ZIP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
REC8	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Meosis related genes															
REC114/REC7 (s)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
MPS3/SUN-1 (c)/*SAD1* (v)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MEC1/ATR	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+
TEL1/ATM	+	+	+	+	+	+	-	-	+	-	-	-	-	-	+
MRE11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RAD17	-	-	-	+	+	+	-	-	+	+	+	+	+	-	+
RAD23	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
RAD24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RAD50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XRS2/NBS1 (v)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
KU70		?	+	+	+	+	-	+	-	-	+	+	+	+	+
KU80	+	?	-	+	+	+	-	-	-	-	+	+	+	+	+
LIG4/DNL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XRCC4/LIF1	-	-	-		-	-	-	-	-	-	-	-	-	-	+
BRCA1	-	-	-	-	-	-		-	-	-	+	-	-	-	+
BRCA2	+	-	+	-	+	+	-	-	-	-	+	+	+	+	+
DNA2	-	+		+	-	-	-	-	+	+	+	+	+	+	+
MMS4/EME1 (s)	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
EXO1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FEN1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MLH1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MLH3	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+
MPH1/FANCM (a,v)	-	-		-	-	-	-	-	-	-	+	+	+	+	+
MSH2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MSH3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
MSH6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MUS81	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
PMS1	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
RAD51	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
RAD52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
RAD54	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
RTEL1	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
SAE2/COM1 (c,a)/*CTIP* (v)	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
SGS1	+	-		-	-	-	-				+	+	+	+	+
SLX1	+	+	-	+	+	+	-	+	-	-	-	-	-	-	+
SLX4/HIM-18 (c)/*MUS312* (d)	-	-		-	-	-	-	-	-	-	-	-	-	-	+
SMC5	+		-				?	-	-	-	-	-	-	-	+
SMC6	?	+	-			-	?	-	-	-	-	-	-	-	+
YEN1/GEN1 (v)	+			-		-	-	-	-	-	-	-	-	-	+
CDC2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig 1. Summary of the inference of meiotic recombination pathways of alveolate species. The common tree of alveolates shows only the hierarchical relationship between taxa.



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Chapter IV

Meiotic Genes in Colpodean Ciliates Support Secretive Sexuality

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Summary

Background

Sex is thought to be maintained in animals and plants because it allows, for example, quicker escapes from parasites and quicker adaptations to changing environments (Bell 1982; Hamilton 2001; Maynard Smith 1978). Although the broad distribution of sex within eukaryotes has not been completely explained (Hartfield and Keightley 2012), putative asexual macro-organisms have long intrigued evolutionary biologists because they are theoretical anomalies (Normark, et al. 2003; Schön, et al. 2009). One of the largest putative asexual microbial eukaryotic groups are the Colpodea ciliates (Foissner 1993).

The putative asexual colpodean ciliates form a clade of over 200 described species that all have micronuclei and macronuclei. They have baroque morphologies in their somatic and oral regions, and are primarily found in terrestrial environments (Foissner 1993; Lynn 2008). Colpodeans have been consistent in their lack of conjugation in the laboratory even after more than forty years of observations, with the exception of only one species: *Bursaria truncatella* (Foissner 1993; Figure 1). Pseudoconjugation—where cells from clonal lines briefly fuse as if to mate—has been observed in other colpodeans while living in petri dishes, but there is no apparent exchange of haploid meiotic products (Foissner 1993).

In the absence of direct observations of sex in the Colpodea, the most powerful approach to evaluate secretive sexuality in this ciliate clade is to look for meiotic genes, as meiosis is the central aspect of eukaryotic sex (e.g., Chi, et al. 2014b; Malik, et al. 2008; Ramesh, et al. 2005; Schurko and Logsdon 2008). We therefore sequenced the genomes of the known sexual colpodean *B. truncatella* and the putative asexual *Colpoda magna*. With these genomic data, we inventoried meiotic genes to evaluate their presence or absence, and

we evaluated the rate of evolution of the inventoried genes relative to the same genes in known sexual ciliates from other clades (Chi, et al. 2014a) to look for evidence of relaxed selection.

Methods

Cells of *B. truncatella* were obtained from Carolina Biological Supply Company (Burlington, NC, U.S.A.) and cells of *C. magna* were obtained from the American Type Culture Collection (#50128). Ten starved cells from each species were individually whole genome amplified with REPLI-g Mini Kit (Hilden, Germany) following manufacturer's instructions. For each species, the ten whole-genome amplified products were combined in equal DNA concentrations. Amplified DNA from *B. truncatella* was sequenced with Illumina MiSeq v2 chemistry (13,288,644 2x250 bp reads) and Illumina HiSeq v3 chemistry 113,546,269 2x150 bp reads). Amplified DNA from *C. magna* was sequenced with MiSeq v3 chemistry (18,770,554 2x300 bp reads) and HiSeq v3 chemistry (28,298,554 2x150 bp reads). Genomes were then assembled with Minia v2.0.7 (Chikhi and Rizk 2012), setting the kmer minimal abundance to 5. The obtained contigs were then analyzed with AUGUSTUS v2.7(Stanke, et al. 2004) for a structural annotation.

A query database of 11 meiosis-specific genes and 40 meiosis- related genes from ciliate and non-ciliate eukaryotes was established using literature and keyword searches of the NCBI protein database was taken from Chi et al. (2014a). For REC8, we used canonical eukaryotic sequences and *T. thermophila*'s non-canonical *REC8*(Howard-Till, et al. 2013).The ORFs of the two colpodeans were searched by the query database using BlastP (Altschul, et al. 1990) and HMMER v3.0 (Eddy 2001). Hits with E-values $<10^{-4}$ for the full sequence were retained. Verification of candidate homologs used reciprocal BlastP search against the non-redundant protein sequence database of NCBI (Supplementary Files 1 and 2).

In order to determine the strength of purifying selection acting on the

inventoried meiotic genes, we measured ω = dN/dS. Sequences generated from this study were aligned with homologous sequences identified from *T. thermophila*, *P. tetraurelia*, *Ichthyophthirius multifiliis*, and *Oxytricha trifallax*by Chi et al. (2014a). Sequences were aligned in Geneious v4.8.3 (Kearse, et al. 2012) using Translation Align with ClustalW v2 (Larkin, et al. 2007). Maximum Likelihood genealogies of each gene were inferred with MEGA7 (Kumar, et al. 2016), and synonymous and nonsynonymous substation rates were estimated with PAML v4.8 (Yang 2007). ω was first calculated between *B. truncatella* and *C. magna*. Then all species were included to test whether the lineage leading to *C. magna* exhibited higher values of ω, which would be expected if these genes were no longer functional and thus experiencing relaxed selection. Using codeml, we compared a model of evolution with one value of ω for the whole tree (model = 0) to a model where the *C. magna* lineage had a separate value of ω (model = 2). A log-likelihood ratio test was used to determine whether the second model provided a significantly better fit to the data.

Results

To uncover the genes involved in meiosis in *B. truncatella* and *C. magna*, clonal cell lines were *de novo* genome sequenced. From these open reading frames, we evaluated the presence or absence of 11 meiosis-specific genes, and 40 meiosis-related genes that are also involved in mitosis. The complement of meiotic genes in the two colpodeans generally matched those from sexual ciliates(Table 1). Both *B. truncatella* and *C. magna* had *SPO11*, which causes the double-strand DNA breaks that initiate meiosis (Keeney 2001).Six other meiosis-specific genes that are involved in crossover regulation were uncovered: *DMC1*, *HOP2*(but not in *C. magna*), *MER3* (which was not found in other ciliates),*MND1*, *MSH4*, and *MSH5*. Like other ciliates, *HOP1*, *RED1*, and *ZIP1* were not found in the colpodeans, supporting the view of Chi et al. (2014a) that ciliates in general have a slimmed crossover pathway 1 that lacks a synaptonemal complex.*B. truncatella* and *C. magna*mostly had

the same complement of meiosis-related genes as found in the other ciliates, including *MUS81*; except that *CDC2*, *MPH1*/*FANCM*, *MPS3/SUN-1/SAD1*, *SGS1*, and *SLX1* were missing in one or both of them.

To look for evidence of relaxed selection in the uncovered meiotic genes from the colpodeans, we evaluated the rates of nonsynonymous relative to synonymous substitutions (ω; Table 1). Evolutionary rates were first measured in the genes contained in both *B. truncatella* and *C. magna*. In this pairwise comparison, all estimated values of ω were much less than one, indicating strong purifying selection in the meiotic genes. A second comparison was made of the two colpodeans with homologs from other ciliate species, where estimated values of ω were also very low. For this comparison between all of the colpodeans and other ciliates, two models were evaluated: M0, where all species were modeled to evolve at the same rate; and M2, where all evolve at the same rate except *C. magna*. Only three genes show a significant difference in evolutionary rates in *C. magna* (that is, the p-value for the log-likehood ratio test was ≤0.05), and of those three only one, *MUS81*, is in the predicted direction.

Discussion

The meiotic genes found in this inventory of the two colpodeans were the same as those found in known sexual ciliates. These meiotic genes would have been lost in *C. magna* if the colpodeans were asexual. In addition, there was no evidence of relaxed selection on these genes in *C. magna*, suggesting that they are under functional constraint just as in the sexual species. If these genomic data apply equally to the unsampled species, then, as predicted (Dunthorn and Katz 2010), the colpodean ciliates are likely sexual.

It should be noted, however, that having functional meiotic genes can also allow for activities other than what we defined here as sex (where there is a requirement of recombination between two individuals). These meiotic genes could be used in canonical genetic pathways such as automixis (= selfing).

They can also be used in non-canonical genetic pathways such as: diplomixis in the protist *Giardia intestinalis*, (Carpenter, et al. 2012; Poxleitner, et al. 2008); parasexuality in the fungus *Candida albicans* (Bennett and Johnson 2003; Forche, et al. 2008); and DNA repair inbdelloid rotifers from damages induced by desiccation and UV radiation that likely has allowed this large and ancient metazoan clade to be asexual(Bininda-Emonds, et al. 2016; Gladyshev and Meselson 2008; Hespeels, et al. 2014)

Beyond these genomic data, there are two additional lines of support for sexuality in *C. magna*andrest of the colpodean ciliates. One is cytological.

The other is behavioral. As micronuclei are only involved in sex(as far as we know), we propose that this cytological feature would have been lost over evolutionary time if the colpodeans were asexual. While the colpodeans have micronuclei, at least six species from two different subclades have micronuclei and macronuclei with shared outer nuclear membranes (Dunthorn, et al. 2008; Foissner 1993).

Pseudoconjugation has been observed in at leastfourcolpodean ciliates while living in petri dishes(Foissner 1993). As conjugation is only involved in sex (as far as we know), we propose that this behavioral feature would have been lost over evolutionary time if the colpodeans were asexual.

In conclusion, Our genomic analyses show that ciliates do not violate the macro-organismic theories against ancient asexuals and the loss-and-regain of complex characters: the Colpodea are sexual. This finding supports the increasingly accepted view that sex in microbial eukaryotes is ubiquitous although often secretive(Dunthorn and Katz 2010; Speijer, et al. 2015). Such secretive sex may result in long periods of mitotic division without meiosis, which could lead to the buildup of high mutational loads in the quiescent germline genomes of the colpodeans. However, rare sex could be tolerated if either the colpodeans have extremely low base-substitution mutation rates as found in the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila* (Long, et al. 2016; Sung, et al. 2012), or if the rare sex provides the same

benefits as does more frequent sex (D'Souza and Michiels 2010; Green and Noakes 1995).

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Chapter IV

Meiotic Genes in Colpodean Ciliates Support Secretive Sexuality

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Abstract

The putatively asexual Colpodean ciliates potentially pose a problem to macro-organismic theories of evolution. They are extremely ancient (although asexuality is thought to hasten extinction), and yet there is one apparently derived sexual species (implying an unlikely regain of a complex trait). If macro-organismic theories of evolution also broadly apply to microbial eukaryotes, though, then most or all of the colpodean ciliates should merely be secretively sexual. Here we show using *de novo* genome sequencing, that colpodean ciliates have the meiotic genes required for sex and these genes are under functional constraint. Along with these genomic data, we argue that these ciliates are sexual given the cytological observations of both micronuclei and macronuclei within their cells, and the behavioral observations of brief fusions as if the cells were mating. The challenge that colpodean ciliates pose is therefore not to evolutionary theory, but to our ability to induce microbial eukaryotic sex in the laboratory.

Key words: asexuality,Colpodea, genome sequencing, gene inventory

Introduction

There are many costs to sex (Lehtonen et al. 2012; Maynard Smith 1978), where there is a fusion of meiotic products from different individuals (Lehtonen and Kokko 2014; Normark et al. 2003). Sex is thought to be maintained in animals and plants because it allows, for example, quicker escapes from parasites and quicker adaptations to changing environments (Bell 1982; Hamilton 2001; Maynard Smith 1978). Although the broad distribution of sex within eukaryotes has not been completely explained (Hartfield and Keightley 2012), putative asexual macro-organisms have long intrigued evolutionary biologists because they are theoretical anomalies (Normark et al. 2003; Schön et al. 2009). By contrast, these theories have often been ignored in microbial eukaryotes, where many species and higher clades are considered to be asexual (Fenchel and Finlay 2006; Foissner et al. 2011; Schlegel and Meisterfeld 2003; Sonneborn 1957). One of the largest putative asexual microbial eukaryotic groups are the Colpodea ciliates (Foissner 1993).

Ciliates have dimorphic nuclei within each cell: micronuclei, which are transcriptionally inactive during vegetative growth; and macronuclei, which produce all mRNA required for protein synthesis(Lynn 2008). Ciliate sex—called conjugation—occurs by brief cell fusion of complementary mating types and mutual exchange of haploid products of micronuclear meiotic division (Bell 1988; Phadke and Zufall 2009; Zufall 2016).Although sex has been widely observed in almost all ciliate groups, some ciliates are asexual because they lack micronuclei(Zufall 2016). Many of these amicronucleate strains are closely related to known sexual ciliates, but some are potentially old (Doerder 2014).

The putative asexual colpodean ciliates form a clade of over 200 described species that all have micronuclei and macronuclei. They have baroque morphologies in their somatic and oral regions, and are primarily found in terrestrial environments (Foissner 1993; Lynn 2008). Colpodeans have been

consistent in their lack of conjugation in the laboratory even after more than forty years of observations, with the exception of only one species: *Bursaria truncatella* (Foissner 1993; Figure 1).Pseudoconjugation—where cells from clonal lines briefly fuse as if to mate—has been observed in other colpodeans while living in petri dishes, but there is no apparent exchange of haploid meiotic products(Foissner 1993).

Because of macro-organismic theories about the maintenance of sex, two lines of evolutionary theory were previously used to argue that all, or almost all, of the colpodeans are secretively sexual (Dunthorn and Katz 2010). First, the colpodeans may have originated before the Phanerozoic(Wright and Lynn 1997), but ancient asexual lineages are extremely rare(Martens et al. 2003; Maynard Smith 1978; Normark et al. 2003). Second, *B. truncatella* is in a phylogenetically derived position (Dunthorn et al. 2011, 2008) implying a loss and then regain of sex, but reversing the loss of complex traits is thought unlikely to occur (Collin and Miglietta 2008; Gould 1970; Teotónio and Rose 2001).

In the absence of direct observations of sex in the Colpodea, the most powerful approach to evaluate secretive sexuality in this ciliate clade is to look for meiotic genes, as meiosis is the central aspect of eukaryotic sex (e.g., Chi et al. 2014b; Malik et al. 2008; Ramesh et al. 2005; Schurko and Logsdon 2008). We therefore sequenced the genomes of the known sexual colpodean *B. truncatella* and the putative asexual *Colpoda magna*. With these genomic data, we inventoried meiotic genes to evaluate their presence or absence, and we evaluated the rate of evolution of the inventoried genes relative to the same genes in known sexual ciliates from other clades (Chi et al. 2014a) to look for evidence of relaxed selection.

Meiotic Gene Inventory and Evolutionary Rates

To uncover the genes involved in meiosis in *B. truncatella* and *C. magna*, clonal cell lines were *de novo*genome sequenced. The aim of the sequencing was to produce open reading frames, not to resolve issues of chromosomal scaffolding or differences between micronuclei and macronuclei.From these open reading frames, we evaluated the presence or absence of 11 meiosis-specific genes, and 40 meiosis-related genes that are also involved in mitosis.

The complement of meiotic genes in the two colpodeans generally matched those from sexual ciliates(Table 1). Both *B. truncatella* and *C. magna* had *SPO11*, which causes the double-strand DNA breaks that initiate meiosis (Keeney 2001).Six other meiosis-specific genes that are involved in crossover regulation were uncovered: *DMC1*, *HOP2*(but not in *C. magna*), *MER3* (which was not found in other ciliates),*MND1*, *MSH4*, and *MSH5*. Like other ciliates, *HOP1*, *RED1*, and *ZIP1* were not found in the colpodeans, supporting the view of Chi et al. (2014a) that ciliates in general have a slimmed crossover pathway 1 that lacks a synaptonemal complex.*B. truncatella* and *C. magna*mostly had the same complement of meiosis-related genes as found in the other ciliates, including *MUS81*; except that *CDC2*, *MPH1*/*FANCM*, *MPS3/SUN-1/SAD1*, *SGS1*, and *SLX1* were missing in one or both of them.

To look for evidence of relaxed selection in the uncovered meiotic genes from the colpodeans, we evaluated the rates of nonsynonymous relative to synonymous substitutions (ω; Table 1). Genes showing evidence of relaxed selection (that is, elevated ω) would indicate the loss of functional constraints due to loss of use in sexual reproduction(Lahti et al. 2009). Evolutionary rates were first measured in the genes contained in both *B. truncatella* and *C. magna*. In this pairwise comparison, all estimated values of ω were much less than one, indicating strong purifying selection in the meiotic genes. A second comparison was made of the two colpodeans with homologs from other ciliate species, where estimated values of ω were also very low. For this comparison

between all of the colpodeans and other ciliates, two models were evaluated: M0, where all species were modeled to evolve at the same rate; and M2, where all evolve at the same rate except *C. magna*. If the genes in *C. magna* were experiencing relaxed selection, in contrast to purifying selection in the other taxa, we would expect to find evidence of larger values of ω in the *C. magna* lineage. However, only three genes show a significant difference in evolutionary rates in *C. magna* (that is, the p-value for the log-likehood ratio test was ≤0.05), and of those three only one, *MUS81*, is in the predicted direction.

Genomic Data Support Secretive Sex in Colpodeans

The meiotic genes found in this inventory of the two colpodeans were the same as those found in known sexual ciliates. These meiotic genes would have been lost in *C. magna* if the colpodeans were asexual. In addition, there was no evidence of relaxed selection on these genes in *C. magna*, suggesting that they are under functional constraint just as in the sexual species. If these genomic data apply equally to the unsampled species, then, as predicted (Dunthorn and Katz 2010), the colpodean ciliates are likely sexual.

It should be noted, however, that having functional meiotic genes can also allow for activities other than what we defined here as sex (where there is a requirement of recombination between two individuals). These meiotic genes could be used in canonical genetic pathways such as automixis (= selfing), which could allow for the purging of deleterious alleles, but would not provide the benefit of genetic exchange between individuals. They can also be used in non-canonical genetic pathways such as: diplomixis in the protist *Giardia intestinalis*, where there is homologous recombination between the two nuclei within each cell but no meiotic reduction in ploidy(Carpenter et al. 2012; Poxleitner et al. 2008); parasexuality in the fungus *Candida albicans*, where tetraploidy caused by cell fusion is non-meiotically reduced to diploidy (Bennett and Johnson 2003; Forche et al. 2008); and DNA repair in bdelloid rotifers from

damages induced by desiccation and UV radiation that likely has allowed this large and ancient metazoan clade to be asexual (Bininda-Emonds et al. 2016; Gladyshev and Meselson 2008; Hespeels et al. 2014).

Beyond these genomic data, there are two additional lines of support for sexuality in *C. magna*andrest of the colpodean ciliates. One is cytological. The other is behavioral.

All colpodean species that have been described in enough detail have micronuclei (e.g., Bourland et al. 2013; Dunthorn et al. 2009; Foissner 1993; Foissner et al. 2014; Quintela-Alonso et al. 2011). As micronuclei are only involved in sex(as far as we know), we propose that this cytological featurewould have been lost over evolutionary time if the colpodeans were asexual. While the colpodeans have micronuclei, at least six species from two different subclades have micronuclei and macronuclei with shared outer nuclear membranes (Dunthorn et al. 2008; Foissner 1993). This shared outer membrane could possibly chain the micronucleus to the macronucleus, and thereby prevent it from participating in sex. That is, having micronuclei chained to macronuclei could be analogous to having no micronuclei at all. However, it is unknown if this shared outer-nuclear membrane is present in most individuals within those six species or if the micronuclei can break freeat some point during the cell cycle (Dunthorn et al. 2008).

Pseudoconjugation has been observed in at least fourc olpodean ciliates while living in petri dishes(Foissner 1993). As conjugation is only involved in sex (as far as we know), we propose that this behavioral feature would have been lost over evolutionary time if the colpodeans were asexual. It should be noted, however, that pseudoconjugation can also allow for activities other than what we defined here as sex. For example, pseudocopulation occurs in the all female *Aspidoscelis uniparens*(desert grassland whiptail lizards), where females need to be mounted by other females to induce parthenogenesis(Crews and Fitzgerald 1980).

Whiles these genomic data and observations support sexuality in*C. magna*

and other colpodean ciliates, additional analyzes can be performed. For example, population genomics methods can be used to test for the presence and rates of outcrossing and recombination within and between populations in nature(Halkett et al. 2005; Ruderfer et al. 2006). Decisive evidence of sex in the colpodeans will have to come from direct observations of successful conjugationin the laboratory, but as with *Aspergillus fumigatus*(O'Gorman et al. 2009), finding the correct conditions to induce sex may be difficult.

Conclusions

Our genomic analyses show that ciliates do not violate the macro-organismic theories against ancient asexuals and the loss-and-regain of complex characters: the Colpodea are sexual. This finding supports the increasingly accepted view that sex in microbial eukaryotes is ubiquitous although often secretive(Dunthorn and Katz 2010; Speijer et al. 2015). Such secretive sex may result in long periods of mitotic division without meiosis, which could lead to the buildup of high mutational loads in the quiescent germline genomes of the colpodeans. However, rare sex could be tolerated if eitherthe colpodeans have extremely low base-substitution mutation rates as found in the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*(Long et al. 2016; Sung et al. 2012), or if the rare sex provides the same benefits as does more frequent sex (D'Souza and Michiels 2010; Green and Noakes 1995).

Materials and Methods

Cells of *B. truncatella* were obtained from Carolina Biological Supply Company (Burlington, NC, U.S.A.) and cells of *C. magna* were obtained from the American Type Culture Collection (#50128). Clonal cultures were established and grown in Volvic water with wheat grains and *Klebsiella* sp. *B. truncatella* cultures also included *Paramecium* sp. Individual cells were picked with a pipette and washed three times in sterilized Volvic water, then allowed to starve for 48 hours. Ten starved cells from each species were individually whole genome amplified with REPLI-g Mini Kit (Hilden, Germany) following manufacturer's instructions. For each species, the ten whole-genome amplified products were combined in equal DNA concentrations.

Amplified DNA from *B. truncatella* was sequenced with Illumina MiSeq v2 chemistry (13,288,644 2x250 bp reads) and Illumina HiSeq v3 chemistry 113,546,269 2x150 bp reads). Amplified DNA from *C. magna* was sequenced with MiSeq v3 chemistry (18,770,554 2x300 bp reads) and HiSeq v3 chemistry (28,298,554 2x150 bp reads). As the genome sizes of these two species are unknown, we could not estimate sequencing coverage. The optimal k-mer length for genome assembly was searched within a 21-201 range with Kmer Genie v1.6976 (Chikhi and Medvedev 2014), using the "diploid" parameter. Genomes were then assembled with Minia v2.0.7 (Chikhi and Rizk 2012), setting the kmer minimal abundance to 5. The obtained contigs were then analyzed with AUGUSTUS v2.7(Stanke et al. 2004)for a structural annotation, using the following parameters: search on both strands; genome is partial; predict genes independently on each strand, allow overlapping genes on opposite strands; report transcripts with in-frame stop codons; species set to the ciliate *T. thermophila*. Reads were deposited in GenBank's Sequence Read Archive under BioProject numbersPRJNA381863 andPRJNA382551.

A query database of 11 meiosis-specific genes and 40 meiosis- related genes from ciliate and non-ciliate eukaryotes was established using literature

and keyword searches of the NCBI protein database was taken from Chi et al. (2014a). For REC8, we used canonical eukaryotic sequences and *T. thermophila*'s non-canonical *REC8*(Howard-Till et al. 2013).The ORFs of the two colpodeans were searched by the query database using BlastP (Altschul et al. 1990) and HMMER v3.0 (Eddy 2001). Hits with E-values $<10^{-4}$ for the full sequence were retained. Verification of candidate homologs used reciprocal BlastP search against the non-redundant protein sequence database of NCBI (Supplementary Files 1 and 2).

In order to determine the strength of purifying selection acting on the inventoried meiotic genes, we measured ω = dN/dS, the number of nonsynonymous substitutions per nonsynonymous site divided by the number of synonymous substitutions per synonymous site. Sequences generated from this study were aligned with homologous sequences identified from *T. thermophila*, *P. tetraurelia*, *Ichthyophthirius multifiliis*, and *Oxytricha trifallax*by Chi et al. (2014a). Sequences were aligned in Geneious v4.8.3 (Kearse et al. 2012)using Translation Align with ClustalW v2(Larkin et al. 2007). Sequences that did not have sufficient overlap with the other genes, were unalignable, or were determined to be paralogous to the genes from these other species were excluded from further analysis (Supplementary Table 1). Maximum Likelihood genealogies of each gene were inferred with MEGA7 (Kumar et al. 2016), and synonymous and nonsynonymous substation rates were estimated with PAML v4.8 (Yang 2007). ω was first calculated between *B. truncatella* and *C. magna*. Then all species were included to test whether the lineage leading to *C. magna* exhibited higher values of ω, which would be expected if these genes were no longer functional and thus experiencing relaxed selection. Using codeml, we compared a model of evolution with one value of ω for the whole tree (model = 0) to a model where the *C. magna* lineage had a separate value of ω (model = 2). A log-likelihood ratio test was used to determine whether the second model provided a significantly better fit to the data.

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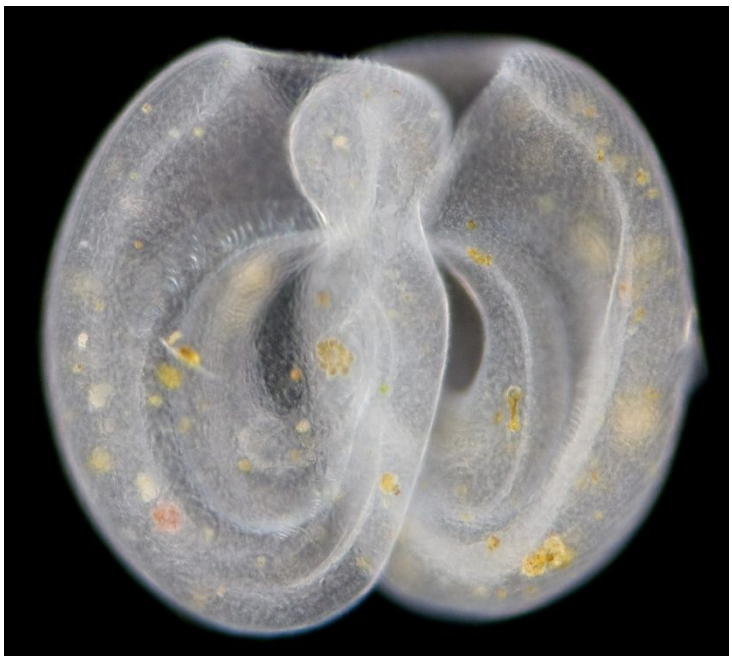
Table 1. Meiosis genes inventoried in two colpodean ciliates: *Bursaria truncatella* and *Colpoda magna*. Genes are grouped according to functions. Meiosis-specific genes are underlined. Data from *Tetrahymena*, *Paramecium*, *Ichthyophthirius*, and *Oxytricha* are from Chi et al. (2014a).ω(B+C) indicates the value of dN/dS between *B. truncatella* and *C. magna*. ω(M0) indicates the value of dN/dS for all taxa. The significance of the difference between a model with one parameter for ω vs. a model with an addition parameter for ω on the *C. magna* branch is shown by the p-value from a chi-squared test with one degree of freedom. In cases where this test is significant, ω(all) indicates the background rate and ω(Col) is the rate for *C. magna*.

	ciliate species						dN/dS				
Gene	*Bursaria truncatella*	*Colpoda magna*	Tetrahymena *thermophila*	*Paramecium tetraurelia*	*Ichthyophthirius multifiliis*	*Oxytricha trifallax*	ω(B+C)	ω(M0)	p-value	ω(all)	ω(Col)
DOUBLE STRAND BREAK FORMATION											
REC114/REC7	-	-	-	-	-	-					
SPO11/REC12	+	+	+	+	+	+	0.348	0.028	0.790		
CROSSOVER REGULATION											
DMC1	+	+	+	-	+	+	0.001	0.003	0.730		
HOP1	-	-	-	-	-	-					
HOP2	+	-	+	+	+	+					
MER3	+	+	-	-	-	-	0.008				
MND1	+	+	+	+	+	+					
MSH4	+	+	+	+	-	+	0.005	0.044	0.296		
MSH5	+	+	+	+	-	+	0.150	0.076	0.902		
RED1/ASY3/REC10	-	-	-	-	-	-					
ZIP1	-	-	-	-	-	-					
DOUBLE STRAND BREAK REPAIR AND MEIOTIC DIVISIONS											
REC8	-	-	+	-	-	-					
BOUQUET FORMATION											
MPS3/SUN-1/SAD1	-	+	+	+	+	+					
DNA DAMAGE SENSING/ RESPONSE											
MEC1/ATR	+	+	+	+	+	+	0.002	0.004	0.925		
TEL1/ATM	-	-	-	-	-	-					
MRE11	+	+	+	+	+	+	0.002	0.005	0.293		
RAD17	+	+	+	+	+	-	0.061	0.037	0.176		
RAD23	+	+	+	+	+	+	0.007	0.080	0.238		
RAD24	+	+	+	+	+	+	0.004	0.064	0.170		
RAD50	+	+	+	+	+	+	0.003	0.019	0.687		
XRS2/NBS1	-	-	-	-	-	-					
DOUBLE STRAND BREAK REPAIR (non-homology end join)											
KU70	+	+	+	+	+	+	0.007	0.008	0.656		
KU80	+	+	+	+	+	+	0.133	0.031	0.393		
LIG4/DNL1	+	+	+	+	+	+	0.007	0.018	0.687		
XRCC4/LIF1	-		-	-	-	-					
RECOMBINATIONAL REPAIR											
BRCA1	+	+	+	-	-	-	0.008	0.014	0.649		
BRCA2	+	+	+	+	+	+	0.003	0.029	0.025	0.038	0.004
DNA2	+	+	+	+	+	+	0.008	0.072	0.647		
MMS4/EME1	+	+	+	+	-	-	0.011	0.004	0.727		
EXO1	+	+	+	+	+	+	0.003	0.035	0.004	0.044	0.003
FEN1	+	+	+	+	+	+	0.202	0.009	0.434		
MLH1	+	+	+	+	+	+	0.032	0.102	0.193		
MLH3	+	+	+	-	+	-	0.039	0.017	0.782		
MPH1/FANCM	-	+	+	+	+	+					
MSH2	+	+	+	+	+	+	0.003	0.018	0.473		
MSH3	-	-	-	-	-	-					
MSH6	+	+	+	+	+	+	0.012	0.029	0.295		
MUS81	+	+	+	+	+	+	0.017	0.002	0.002	0.017	0.126
PMS1	+	+	+	+	+	+	0.004	0.010	0.992		
RAD51	+	+	+	+	+	+	0.078	0.020	0.894		
RAD52	-	-	-	-	-	-					
RAD54	+	+	+	+	+	-	0.005	0.038	0.223		
RTEL1	+	+	+	+	+	+	0.011	0.032	0.206		
SAE2/COM1/CTIP	-	-	+	+	-	-					
SGS1	-	-	+	+	+	+					
SLX1	+	-	-	-	-	-					
SLX4/HIM-18/MUS312	-	-	-	-	-	-					
SMC5	-	-	-	-	-	-					
SMC6	-	-	-	-	-	-					
YEN1/GEN1	-	-	-	-	-	-					
MEIOTIC ENTRY											
CDC2	+	-	+	+	+	+					

Figure 1. Two *Bursaria truncatella* cells during conjugation (= ciliate sex). Out of over 200 described species of colpodean ciliates, only *B. truncatella* has directly been observed to have sex. Copyright ©Charles Krebs.



Chapter V

Putatively asexual chrysophytes have meiotic genes: evidence from transcriptomic data

Diana Kraus, Jingyun Chi, Jens Boenigk, Daniela Beisser, Nadine Graupner and Micah Dunthorn

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Summary

Background

The Chrysophyceae Pascher 1914 are a morphologically diverse group of flagellates that are among the dominant protists in aquatic and terrestrial environments (Boenigk & Arndt, 2002; Foissner, 1987; Kristiansen & Preisig, 2001; Kristiansen & Škaloud, 2017; Sandgren, 1988). These protists serve as excellent models in ecology, ecophysiology, and evolution (Boenigk, 2008; Graupner et al., 2018), because of their wide range of nutritional strategies. Despite their known ecological importance, chrysophyte taxon richness and species boundaries are difficult to infer.

Chrysophytes are assumed to be capable of sex, even though conclusive evidence has not been demonstrated for either meiosis or the fusion of meiotic products from different individuals (Kristiansen & Škaloud, 2017). Possible formation of zygotes was observed in Dinobryon and Synura using morphological observations, but changes in ploidy were not evaluated (Bourrelly, 1957; Fott, 1959; Sandgren, 1983; Wawrik, 1972). These morphological studies are also restricted to a handful of taxa and the distribution of sex within the chrysophytes remains unknown.

Transcriptomic data from 18 chrysophyte isolates, representing 15 different species that were either photo-, mixo-, or hetero-trophic, were recently used to gain insights into nutritional strategies and phylogenetic relationships (Beisser et al., 2017). In this study we used those chrysophyte transcriptomes from Beisser et al. (2017) for a meiotic gene inventory to evaluate if these putatively asexual protists are capable of sex. Following Chi et al. (2014a), the presence and absence of these genes were placed into the context that there are two meiotic crossover pathways: class I pathway, which relies on meiotic-specific genes and can include a synaptonemal complex; and class II pathway, which uses meiotic-related genes that are also involved in

mitosis (Loidl, 2016).

Methods

From Beisser et al. (2017), sequenced and cleaned transcriptomic data were taken for 18 chrysophytes strains of 15 species: Acrispumella msimbaziensis (strain JBAF33), Apoikiospumella mondseeiensis (strain JBM08), Cornospumella fuschlensis (strain A-R4-D6), Dinobryon sp. (strain FU22KAK), Dinobryon sp. (strain LO226KS), Epipyxis sp. (strain PR26KG), Ochromonas or Spumella sp. (strain LO244K-D), Pedospumella encystans (strain JBMS11), Poterioochromonas malhamensis (strain DS), Poteriospumella lacustris (strain JBC07), Poteriospumella lacustris (strain JBM10), Poteriospumella lacustris (strain JBNZ41), Pedospumella sinomuralis (strain JBCS23), Spumella bureschii (strain JBL14), Spumella lacusvadosi (strain JBNZ39), Spumella vulgaris (strain 199hm), Synura sp. (strain LO234KE), and Uroglena sp. (strain WA34KE). The data are available at the European Nucleotide Archive accession PRJEB13662.

Here these data were compared to a query database of nine meiosis-specific and 30 meiosis-related genes established by Chi et al. (2014a). Using local scripts, two methods were used for comparing the transcriptomic data to the query database of meiotic genes: BlastP (Altschul et al., 1990) and HMMER v3.0 (Eddy, 2011). Reciprocal BLAST analysis was also performed using BLASTP against the non-redundant protein sequence database of NCBI. The parameters for BLASTp and HMMER are default, except sequences were retained if they had hits with E-values < $10E^{-4}$.

Results

Out of the 39 meiotic genes, 38 were identified in the transcriptomes of 18 chrysophytes strains (Table 1; File S1). For the nine meiosis-specific genes, all of them were found in at least six transcriptomes. In particular, SPO11, which initiates meiosis through double-strand DNA breaks in most eukaryotes

(Keeney, Giroux & Kleckner, 1997) except in some amoebae (Bloomfield, 2018), was found in seven strains. The following other meiosis-specific genes were found: DMC1 in 15 strains is important for recombination homolog bias (Bugreev et al., 2011); HOP2 in 18 strains stabilizes the association of the protein Dmc1 with DNA (Chen et al., 2004); MND1 in 12 strains also stabilizes the association of the protein Dmc1 with DNA (Chen et al., 2004); HOP1 in six strains, forms part of the synaptonemal complex (Hollingsworth, Goetsch & Byers, 1990); REC8 in 12 strains forms part of the sister chromatid cohesin complex (Howard-Till et al., 2013); MER3 in 16 strains is a DNA helicase (Nakagawa & Kolodner, 2002); and MSH4 in 14 strains and MSH5 in 13 strains, which are heterodimers that stabilize recombination intermediates (Nishant et al., 2010; Snowden et al., 2004).

For the 30 meiosis-related genes, 29 were found in at least five out of the 18 transcriptomes. Many of the missing meiotic genes could really be missing from the genomes, or the genes could be missing because of how the data were generated. In transcriptomes, just like in ESTs (Chi, Parrow & Dunthorn, 2014b), missing genes are expected because only genes being actively expressed will be sequenced.

Discussion

In this gene inventory of chrysophyte transcriptomes, we found evidence for the presence of many meiosis-specific and meiosis-related genes. If we assume a use-it-or-lose-it view of these genes (Normark, Judson & Moran, 2003; Schurko & Logsdon, 2008), then the chrysophytes are using the protein products of these genes to construct functional meiotic machinery. As with most other eukaryotes (Dunthorn & Katz, 2010; O'Malley, Simpson & Roger, 2013), the chrysophytes are therefore likely sexual, which supports earlier microscopic observations that potentially indicated sex (Bourrelly, 1957; Fott, 1959; Sandgren, 1983; Wawrik, 1972).

Additionally, we found meiotic genes involved in both crossover pathways,

including genes involved in making the synaptonemal complex in class I pathway. Although these pathways have been differentially lost in various eukaryotic groups (Chi et al., 2014a; Loidl, 2016), chrysophyte potentially use both of these pathways.

Chapter V

Putatively asexual chrysophytes have meiotic genes: evidence from transcriptomic data

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Abstract

Chrysophytes are a large group of heterotrophic, phototrophic, or even mixotrophic protists that are abundant in aquatic as well as terrestrial environments. Although much is known about chrysophyte biology and ecology, it is unknown if they are sexual or not. Here we use available transcriptomes of 18 isolates of 15 putatively asexual species to inventory the presence of genes used in meiosis. Since we were able to detect a set of nine meiosis-specific and 29 meiosis-related genes shared by the chrysophytes, we conclude that they are secretively sexual and therefore should be investigated further using genome sequencing to uncover any missed genes from the transcriptomes.

Key Words. Asexuality, Meiosis, Crossover pathways, Sex

Introduction

The Chrysophyceae Pascher 1914 are a morphologically diverse group of flagellates that are among the dominant protists in aquatic and terrestrial environments (Boenigk & Arndt, 2002; Foissner, 1987; Kristiansen & Preisig, 2001; Kristiansen & Škaloud, 2017; Sandgren, 1988). These protists serve as excellent models in ecology, ecophysiology, and evolution (Boenigk, 2008; Graupner et al., 2018), because of their wide range of nutritional strategies. The ecological importance of the chrysophytes is derived from the heterotrophic and mixotrophic taxa being important grazers of bacteria (Del Campo & Massana, 2011; Ekelund, Ronn & Griffiths, 2001; Finlay & Esteban, 1998), and the phototrophic and mixotrophic taxa being a large component of the primary producers in oligotrophic freshwaters (Kristiansen & Škaloud, 2017; Wolfe & Siver, 2013).

Despite their known ecological importance, chrysophyte taxon richness and species boundaries are difficult to infer. For example, there are some taxa with morphological characters of high diagnostic value such as in Paraphysomonas (Scoble & Cavalier-Smith, 2014) and Synura (Siver & Lott, 2016), taxa with morphological characters of uncertain taxonomic value such as in the Dinobryon divergens complex (Jost, Medinger & Boenigk, 2010), and taxa that are largely missing much morphological characters such as many colorless non-scaled taxa (Grossmann et al., 2016). Assessing reproductive isolation in these taxa may offer a starting point for a consistent taxonomic revision and recognition of species boundaries based on mating abilities. In general, chrysophytes are assumed to be capable of sex, even though conclusive evidence has not been demonstrated for either meiosis or the fusion of meiotic products from different individuals (Kristiansen & Škaloud, 2017). Possible formation of zygotes was observed in Dinobryon and Synura using morphological observations, but changes in ploidy were not evaluated (Bourrelly, 1957; Fott, 1959; Sandgren, 1983; Wawrik, 1972). These

morphological studies are also restricted to a handful of taxa and the distribution of sex within the chrysophytes remains unknown.

Meiotic sex is assumed to be retained in most macro-organismic eukaryotes because asexuality can lead to extinction over time (Bell, 1982; Maynard Smith, 1978). However, sex is often not easily observable in many microbial eukaryotic groups, which can lack distinctive morphological differences between the sexes or we do not know the right environmental conditions to induce sex in the laboratory (Dunthorn & Katz, 2010; Schurko, Neiman & Logsdon, 2009; Speijer, Lukeš & Eliáš, 2015). In the absence of direct observations of sex (O'Gorman, Fuller & Dyer, 2009) and in the absence of known sexual mating types (Corradi & Brachmann, 2017), one of the strongest molecular signatures of secretive sex in putative asexual protists is the presence of meiotic genes. If the meiotic genes are found in their genomes, then the protein products are likely being used for sex, otherwise they would have been lost over evolutionary time (Normark, Judson & Moran, 2003; Schurko & Logsdon, 2008). While genomic data are usually used for such meiotic gene inventories in protists (Chi et al., 2014a; Dunthorn et al., 2017; Malik et al., 2008; Patil et al., 2015; Ramesh, Malik & Longsdon, 2005; Hofstatter, Brown & Lahr, 2018), expressed sequence tag (EST) have also been used, although genes can be missing from an EST library if they are not being expressed at the time the protist was collected and analyzed for a secretive sexual stage (Chi, Parrow & Dunthorn, 2014b).

Transcriptomic data from 18 chrysophyte isolates, representing 15 different species that were either photo-, mixo-, or hetero-trophic, were recently used to gain insights into nutritional strategies and phylogenetic relationships (Beisser et al., 2017). Within the chrysophytes able to perform photosynthesis, the transcriptomes revealed a higher expression of genes participating in photosynthesis, photosynthesis-antenna proteins, porphyrin and chlorophyll metabolism, carbon fixation and carotenoid biosynthesis, while in the heterotrophic strains there was a higher expression of genes involved in

nutrient absorption, environmental information processing, and various transporters (e.g., monosaccharide, peptide, and lipid transporters). Here we used those same 18 chrysophyte transcriptomes from Beisser et al. (2017) for a meiotic gene inventory to evaluate if these putatively asexual protists are capable of sex. Following Chi et al. (2014a), the presence and absence of these genes were placed into the context that there are two meiotic crossover pathways: class I pathway, which relies on meiotic-specific genes and can include a synaptonemal complex; and class II pathway, which uses meiotic-related genes that are also involved in mitosis (Loidl, 2016).

Materials and Methods

From Beisser et al. (2017), sequenced and cleaned transcriptomic data were taken for 18 chrysophytes strains of 15 species: Acrispumella msimbaziensis (strain JBAF33), Apoikiospumella mondseeiensis (strain JBM08), Cornospumella fuschlensis (strain A-R4-D6), Dinobryon sp. (strain FU22KAK), Dinobryon sp. (strain LO226KS), Epipyxis sp. (strain PR26KG), Ochromonas or Spumella sp. (strain LO244K-D), Pedospumella encystans (strain JBMS11), Poterioochromonas malhamensis (strain DS), Poteriospumella lacustris (strain JBC07), Poteriospumella lacustris (strain JBM10), Poteriospumella lacustris (strain JBNZ41), Pedospumella sinomuralis (strain JBCS23), Spumella bureschii (strain JBL14), Spumella lacusvadosi (strain JBNZ39), Spumella vulgaris (strain 199hm), Synura sp. (strain LO234KE), and Uroglena sp. (strain WA34KE). The data are available at the European Nucleotide Archive accession PRJEB13662.

Here these data were compared to a query database of nine meiosis-specific and 30 meiosis-related genes established by Chi et al. (2014a). This database was originally established using literature and keyword searches of the NCBI protein database and the Uniprot Knowledgebase. Using local scripts, two methods were used for comparing the transcriptomic data to the query database of meiotic genes: BlastP (Altschul et al., 1990) and HMMER v3.0 (Eddy, 2011). Reciprocal BLAST analysis was also performed using BLASTP against the non-redundant protein sequence database of NCBI. The parameters for BLASTp and HMMER are default, except sequences were retained if they had hits with E-values < 10E-4. Following Saccharomyces cerevisiae nomenclature, gene names are signified in italic capital letters, and proteins in lowercase except first letter.

Results

Out of the 39 meiotic genes, 38 were identified in the transcriptomes of 18 chrysophytes strains (Table 1; File S1). For the nine meiosis-specific genes, all of them were found in at least six transcriptomes. In particular, SPO11, which initiates meiosis through double-strand DNA breaks in most eukaryotes (Keeney, Giroux & Kleckner, 1997) except in some amoebae (Bloomfield, 2018), was found in seven strains. The following other meiosis-specific genes were found: DMC1 in 15 strains is important for recombination homolog bias (Bugreev et al., 2011); HOP2 in 18 strains stabilizes the association of the protein Dmc1 with DNA (Chen et al., 2004); MND1 in 12 strains also stabilizes the association of the protein Dmc1 with DNA (Chen et al., 2004); HOP1 in six strains, forms part of the synaptonemal complex (Hollingsworth, Goetsch & Byers, 1990); REC8 in 12 strains forms part of the sister chromatid cohesin complex (Howard-Till et al., 2013); MER3 in 16 strains is a DNA helicase (Nakagawa & Kolodner, 2002); and MSH4 in 14 strains and MSH5 in 13 strains, which are heterodimers that stabilize recombination intermediates (Nishant et al., 2010; Snowden et al., 2004).

For the 30 meiosis-related genes, 29 were found in at least five out of the 18 transcriptomes. The only gene that was not found in any transcriptome was REC114. The meiosis-related gene MMS4 was found in the smallest amount of five transcriptomes. The seven meiosis-related genes MPH1, PMS1, RAD23, RAD50, SGS1, SMC5, and SMC6 were found in all 18 transcriptomes. Nine of the other meiosis-related genes were only not present in two or three chrysophyte transcriptomes.

Many of the missing meiotic genes could really be missing from the genomes, or the genes could be missing because of how the data were generated. In transcriptomes, just like in ESTs (Chi, Parrow & Dunthorn, 2014b), missing genes are expected because only genes being actively expressed will be sequenced. These differences between the sequences of

strains of the same species here suggest that indeed the transcriptomes are likely missing a lot of non-expressed genes. For example, HOP1 is only found in two of three strains of Poteriospumella lacustris, and MSH4 and MSH5 are only found in one of two stains of Dinobryon sp.

Discussion

In this gene inventory of chrysophyte transcriptomes, we found evidence for the presence of many meiosis-specific and meiosis-related genes. If we assume a use-it-or-lose-it view of these genes (Normark, Judson & Moran, 2003; Schurko & Logsdon, 2008), then the chrysophytes are using the protein products of these genes to construct functional meiotic machinery. As with most other eukaryotes (Dunthorn & Katz, 2010; O'Malley, Simpson & Roger, 2013), the chrysophytes are therefore likely sexual, which supports earlier microscopic observations that potentially indicated sex (Bourrelly, 1957; Fott, 1959; Sandgren, 1983; Wawrik, 1972). If this is the case, and even if sex has not yet been directly observed, the genetic diversity and adaptive evolution of the chrysophytes would benefit from this secretive sex. And this benefit could occur even if sex was a rare event in the chrysophytes (D'Souza & Michiels, 2010; Green & Noakes, 1995).

Additionally, we found meiotic genes involved in both crossover pathways, including genes involved in making the synaptonemal complex in class I pathway. Although these pathways have been differentially lost in various eukaryotic groups (Chi et al., 2014a; Loidl, 2016), chrysophyte potentially use both of these pathways. Given the phylogenetic placement across the chrysophyte tree of life of the 15 species sampled here (Beisser et al., 2017), these results supporting secretive sex and the presence of both crossover pathways should be applicable for all, or most, other chrysophyte species.

Here we used transcriptomic data to show that there are meiotic genes in the putative asexual chrysophytes. These genes are likely being used for sex. This finding suggests that more thorough de novo genome sequencing of different chrysophyte species should be performed to uncover the meiotic genes possibly missed in the transcriptomes. This finding also suggests that targeted mating attempts of different chrysophyte species in the laboratory should be attempted, as these observations will offer the best evidence that

the chrysophytes are truly sexual in nature and that meiosis in these protists is not being used just for automixis.

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Table 1: Meiosis genes inventoried in the transcriptomes of 18 strains of 15 species of chrysophytes.

Gene	Chrysophyte species																	
	Acrispumella msimbaziensis	*Apoikiospumella mondseeiensis*	*Cornospumella fuschlensis*	*Dinobryon* sp. (strain FU22KAK)	*Dinobryon* sp. (strain LO226KS)	*Epipyxis sp.*	*Ochromonas or Spumella sp.*	*Pedospumella encystans*	*Pedospumella sinomuralis*	*Poteriochromonas malhemensis*	*Poteriospumella lacustris (strain JB07)*	*Poteriospumella lacustris (strain JBM10)*	*Poteriospumella lacustris (strain JBNZ41)*	*Spumella bureschii*	*Spumella lacusvadosi*	*Spumella vulgaris*	*Synura sp.*	*Uroglena sp.*
Double-strand break formation																		
REC114	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SPO11	−	−	+	−	−	+	−	+	+	−	−	−	−	+	−	+	+	−
Crossover regulation																		
DMC1	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−
HOP1	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−	+	−	+
HOP2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MER3	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
MND1	−	−	+	+	−	+	−	+	−	+	+	+	+	+	+	−	+	+
MSH4	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	−
MSH5	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	−
Double-strand break repair																		
REC8	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	−	−
Bouquet formation																		
SAD1	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	−	+
DNA damage sensing/response																		
MRE11	−	−	+	+	−	+	−	+	+	+	+	+	−	+	−	+	−	−
RAD17	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−
RAD23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RAD24	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RAD50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NBS1	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+	−	−	+
Double-strand break repair (nonhomology end join)																		
KU	−	−	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	−
LIG4	+	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	−	+
LIF1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Recombinational repair																		
DNA2	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+
MMS4	−	−	−	−	−	+	−	+	−	−	−	−	−	+	−	+	+	−
EME1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
EXO1	−	−	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	+
FEN1	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MLH1	+	−	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+	−
MLH3	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
MPH1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MSH2	−	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	−
MSH6	+	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+
MUS81	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−
PMS1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RAD51	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−
RAD52	−	−	−	−	+	−	−	−	−	−	+	+	−	+	−	−	−	−
RAD54	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RTEL	−	−	+	+	−	+	−	+	+	+	+	+	+	+	−	+	−	−
SAE2	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
SLX1	−	−	+	+	−	+	−	+	+	−	+	+	+	+	−	−	+	−
SLX4	+	−	+	−	+	+	+	+	+	−	−	−	−	+	+	+	−	−
SMC5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SMC6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GEN1	−	−	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	−

Conclusion and Outlook

The genes that enable meiotic sex has been just begin to be explored using bioinformatic methods. Inventory of meiotic genes and, accordingly analyzing meiotic pathways, could cover the shortage of observation in study the sex in microbial to a large extent. The evolution of meiotic recombination pathways in alveolates could help discovering its origin and distribution in the very early stage of eukaryotes.

In my dissertation, a bunch of meiosis-specific and meiosis-related genes from all kinds of eukaryotes are collected, and using bioinformatic methods, inventoried in genomes of alveolates and other microbial (Chrysophytes, also known as golden algae). According to the function of each gene in meiotic recombination, the meiotic pathways could be implied. The results shows that 1) not all eukaryotic meiosis-specific and meiosis-related genes are needed in every group. 2) Our data suggest that ciliates and Dinoflagellates are capable only of a slimmed meiosis, using a set of mitotic repair proteins for meiotic recombination. We speculated that the result of the abandonment of the more classic pathway I might due to the abnormal chromosome structure of both Ciliates and Dinoflagellates. 3) Phylogenetic analysis of the distribution of meiotic pathways within alveolates suggest that the reduction of meiotic pathway I in Ciliates and Dinoflagellates are independent. Considering that Apicomplexa are capable of both meiotic pathways, we would infer that those two pathways exist in the common ancestor of alveolates. 4) The judgment of asexual organisms by only observation is inaccurate, since several putative asexual species, including Symbiodinium (Dinoflagellates), Colpodean (Ciliates) and Chrysophytes, are proved to be secretively sexual in our study.

With the development of bioinformatics, the whole genome of more microbial from different groups could be sequenced and the accuracy of inventory methods could be improved, the origin of meiotic pathways be better uncovered. With RNA-seq technology, more meiotic genes could be recognized and their functions could be uncovered to optimize the meiotic pathways in molecular level.

Summary

Most of eukaryotes show signs of having sex or sexual recombination, and the other asexual eukaryotes have evidences of evolving from sexual ancestors. Meiotic recombination, or crossover are proved to have two pathways in eukaryotes, whose distribution was well studied in many model eukaryotes. However, the distribution of sex in specific lineage is debating. The distribution and evolution of meiotic recombination pathways in alveolates would provide us clues of lost/gaining of pathways in early eukaryotes and fill the gaps between protist and more complex multicellular organisms (fungi, animals and plants) .

In this dissertation, we designed a customized program with Python, which integrated Blastp and HMMER v3.0, to search for homologs of 51 meiotic genes (11 meiosis-specific and 40 meiosis-related genes) in the whole genome sequences or EST data of five Ciliates, seven Apicomplexa, two Chromerida, one Perkinsus, one Dinoflagellates and Chrysophytes (golden algae). All candidate homologs were then verified by reciprocal Blastp search against the nonredundant protein sequence database of NCBI and phylogeny analysis of RAxML.

The gene inventory results shows that several eukaryotic meiosis-specific and meiosis-related genes are missing in every group. However, the presents of meiosis initiate protein Spo11 in some putative asexual lineage (Symbiodinium, Colpodean and Chrysophytes) suggest that they might be cryptically sexual. Within alveolates, Apicomplexa are capable of both pathways, while Ciliates and Dinoflagellates using a set of mitotic repair proteins for meiotic recombination. We speculated that the result of the abandonment of the pathway I might due to the abnormal chromosome structure of both Ciliates and Dinoflagellates. Phylogenetic analysis of the distribution of meiotic pathways within alveolates suggest that the reduction of meiotic pathway I in Ciliates and Dinoflagellates are independent. Considering that Apicomplexa are capable of both meiotic pathways, we would infer that those two pathways exist in the common ancestor of alveolates.

Appendix

Supplementary material: Chapter I

Meiosis Gene Inventoryof Four Ciliates Reveals the Prevalence of a Synaptonemal Complex-Independent Crossover Pathway

Chi J[1], Mahé F[2], Loidl J[3], Logsdon J[4], Dunthorn M[5].

Mol Biol Evol. 2014 Mar;31(3):660-72. doi: 10.1093/molbev/mst258. Epub 2013 Dec 13.

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4 Department of Biology, University of Iowa, Iowa City, USA.
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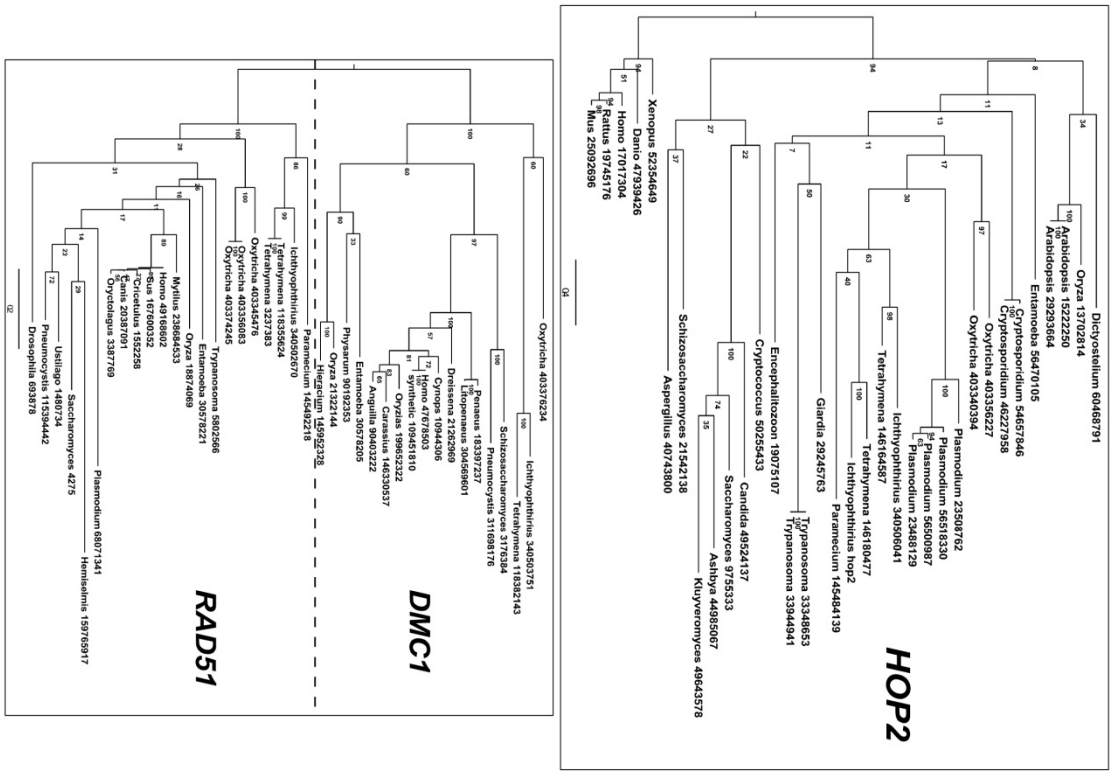
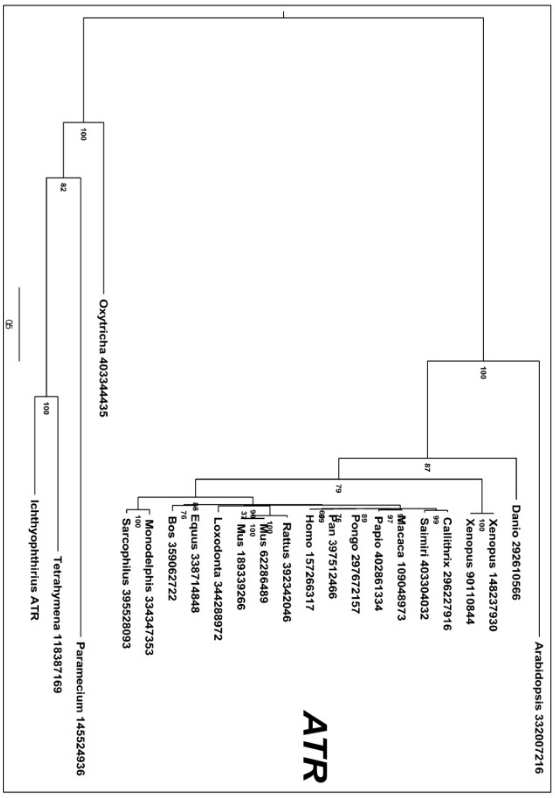
Supplementary file S1: Maximum likelihood analyses for all genes inventoried in the four ciliate genomes, as well as all bipartition support values.

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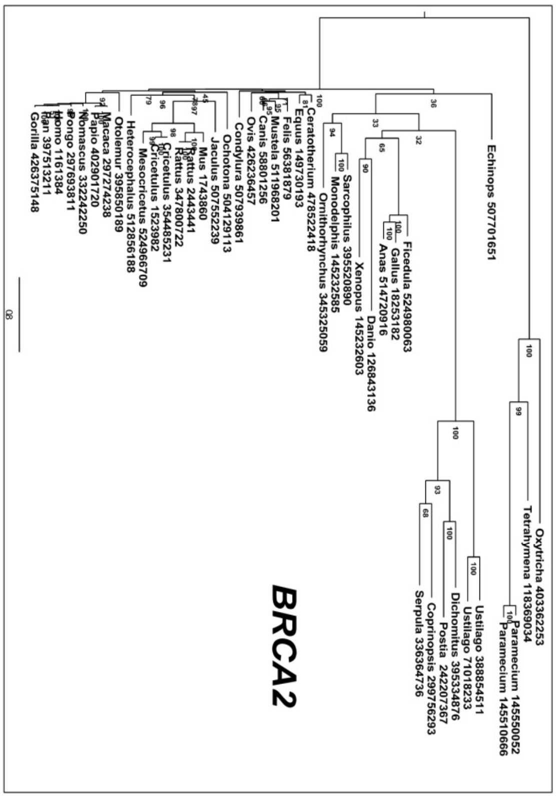
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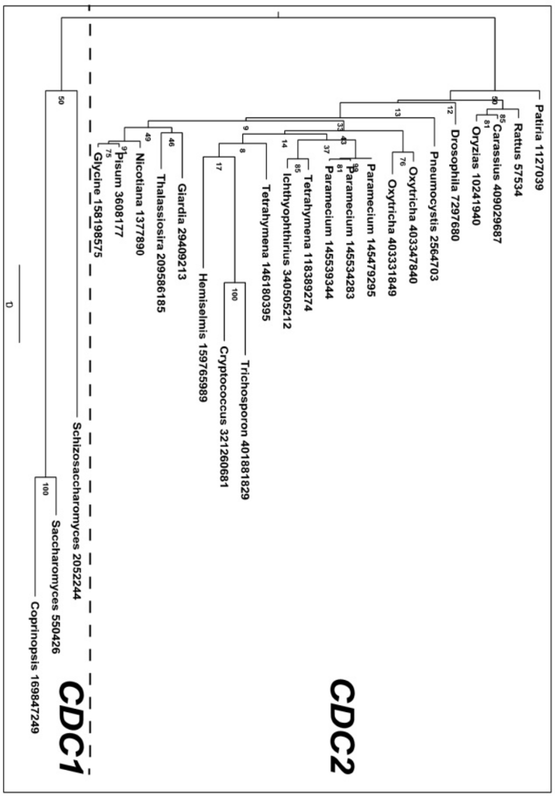
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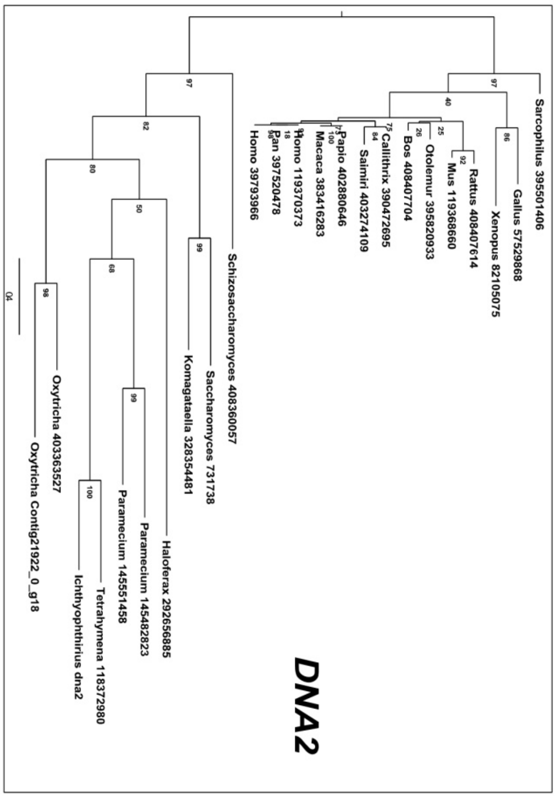
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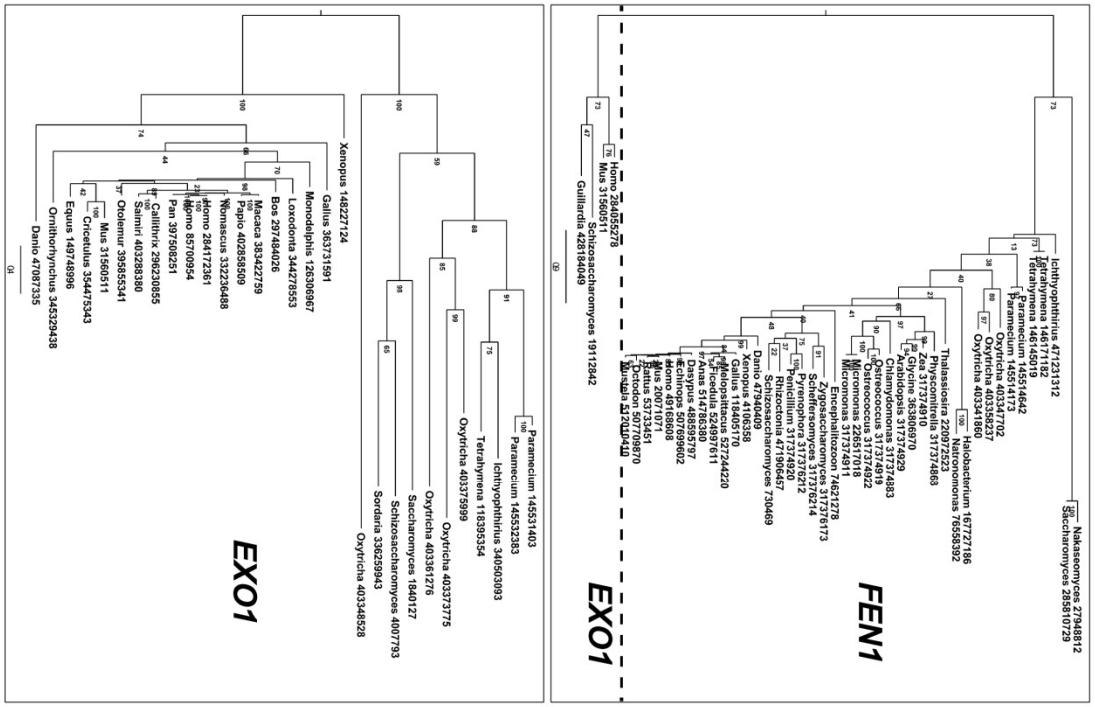
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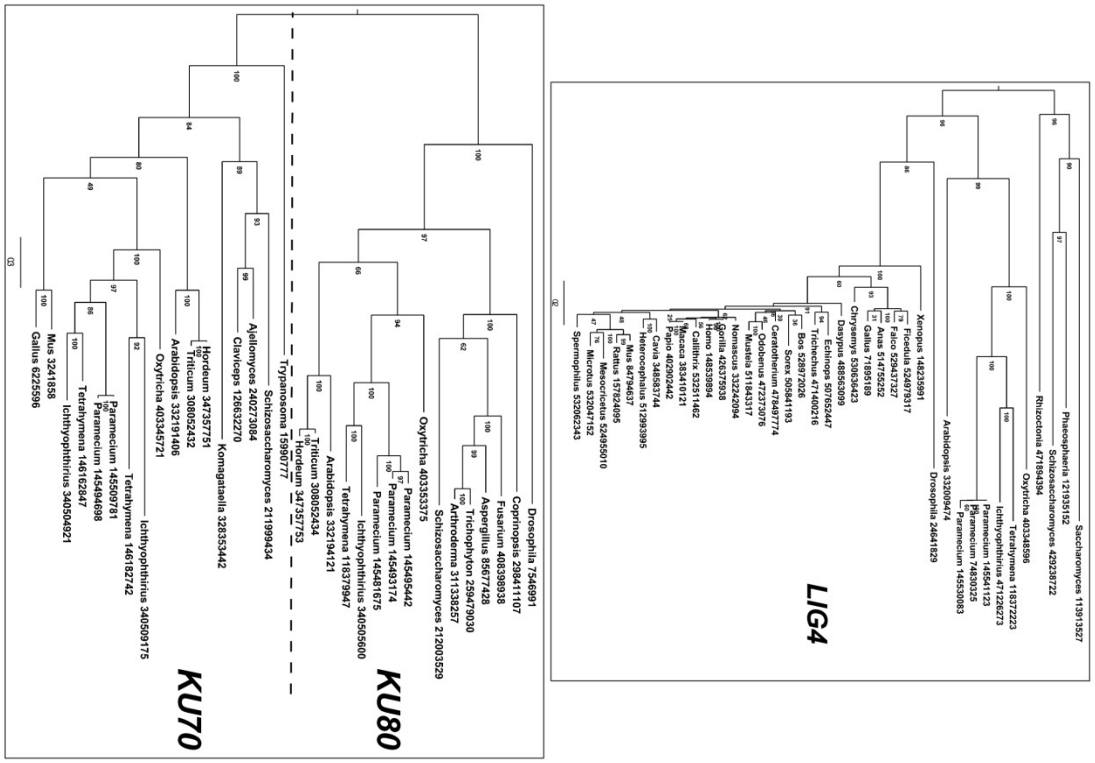
FEN1
EXO1
EXO1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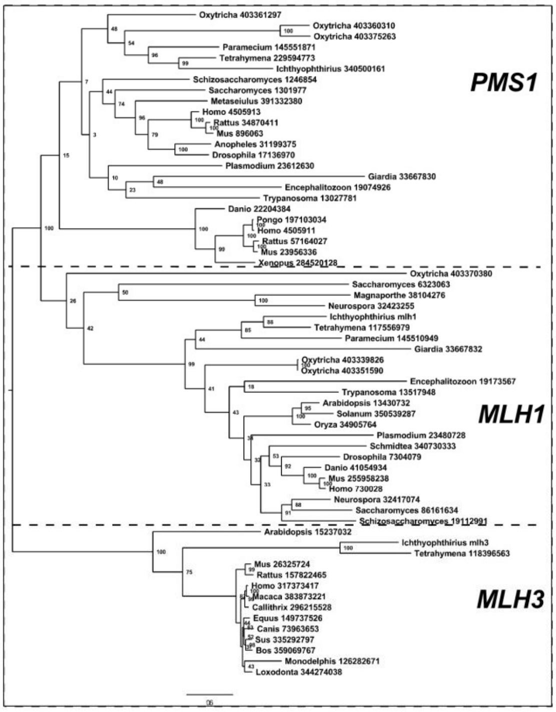
KU80
KU70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Drosophila 7304079
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Homo 730028
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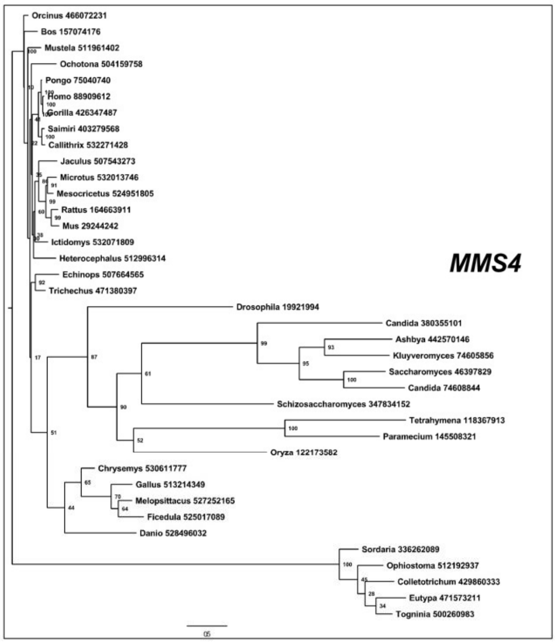
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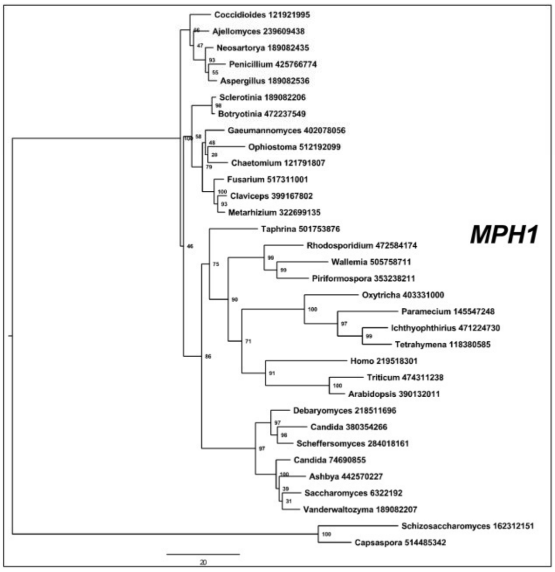
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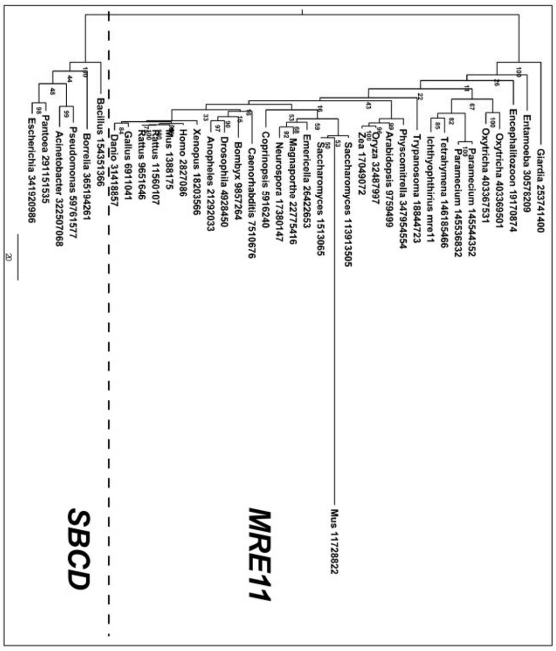
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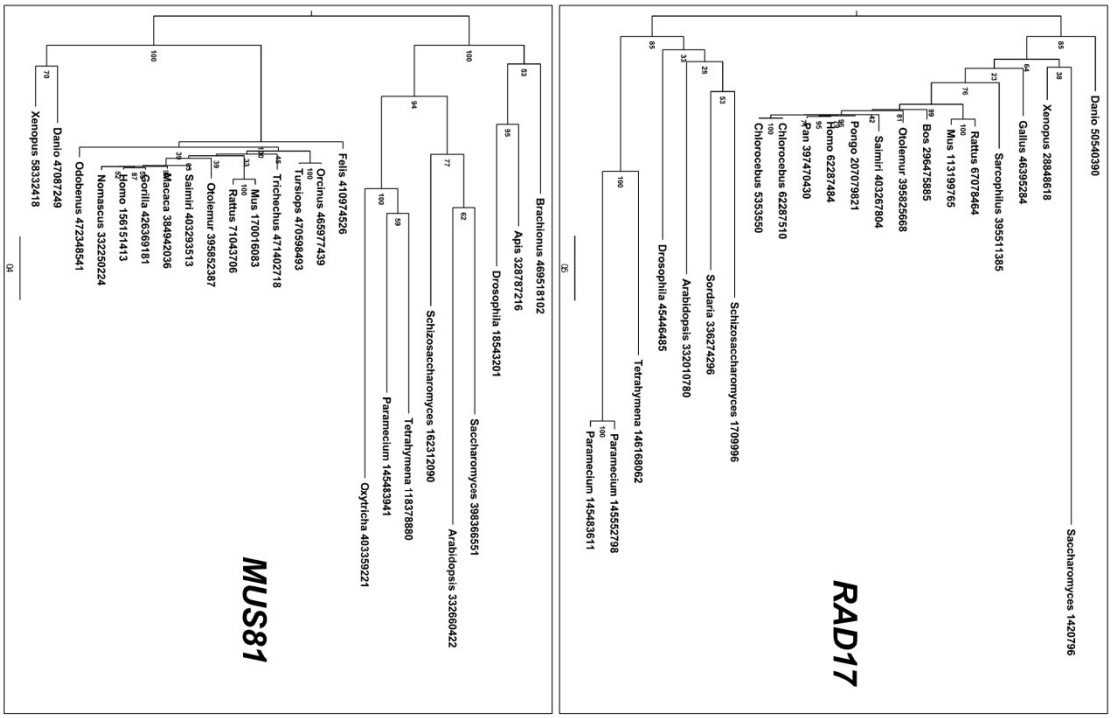
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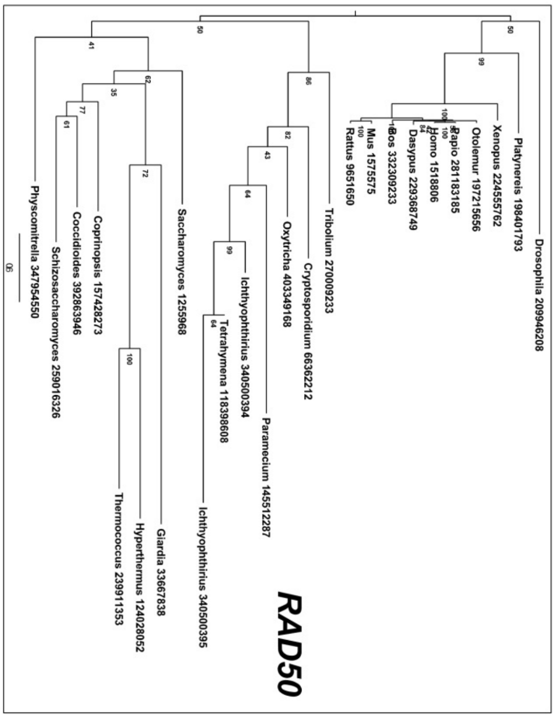
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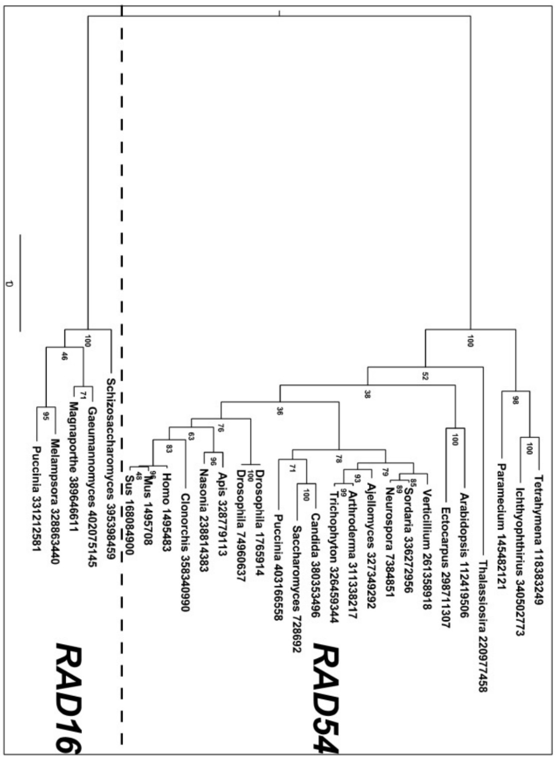
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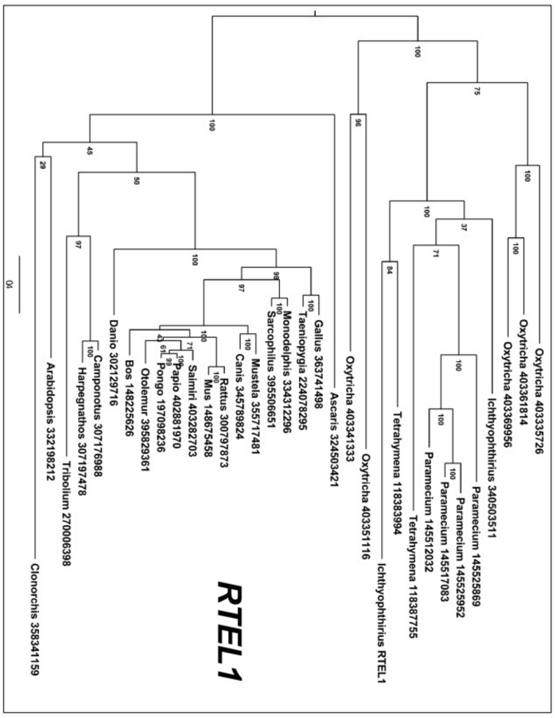
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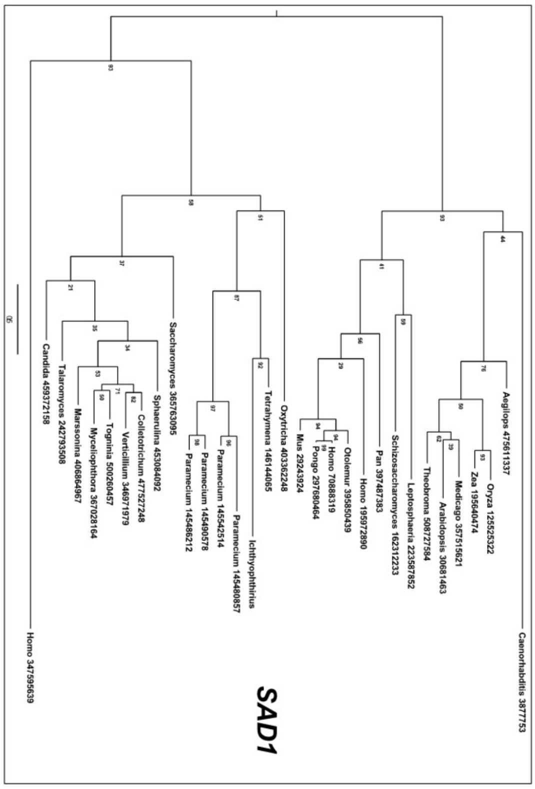
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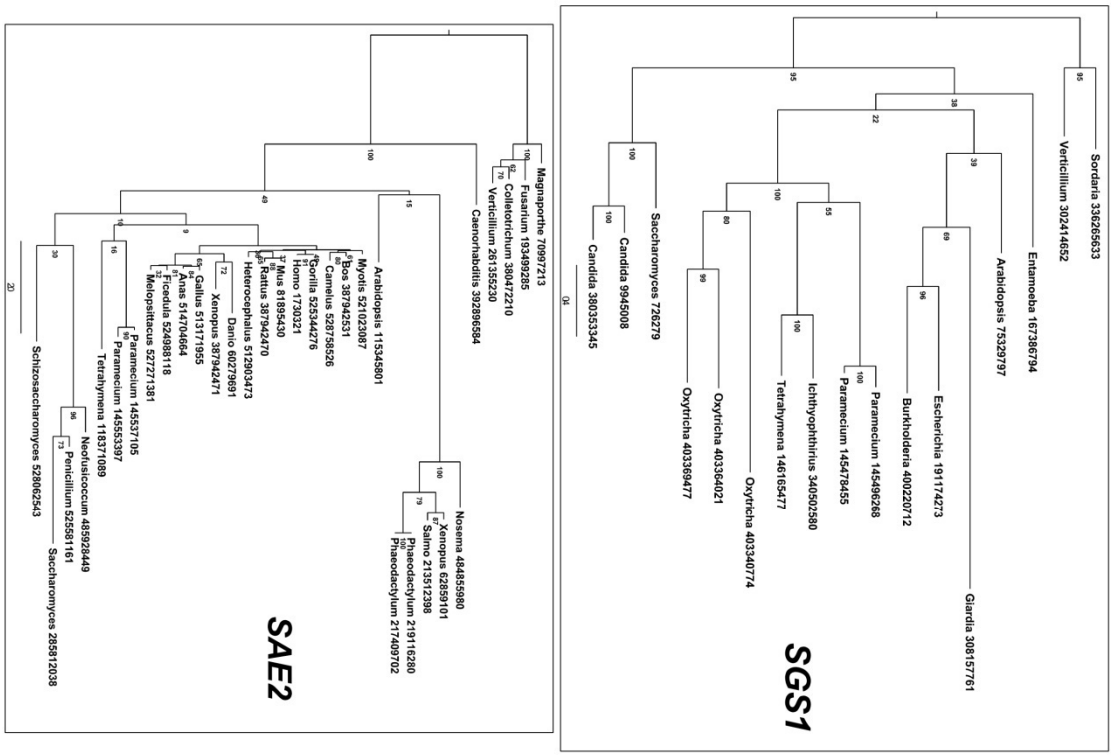
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Tetrahymena 146165477
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Candida 9945008
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SGS1
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Colletotrichum 380472210
Verticillium 261355230
Caenorhabditis 392896584
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Bos 387942531
Camelus 528758526
Gorilla 525344276
Homo 1730321
Mus 81895430
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Heterocephalus 512903473
Xenopus 387942471
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Gallus 513171955
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Ficedula 524988118
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Tetrahymena 118371089
Neofusicoccum 485928449
Penicillium 525581161
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Saccharomyces 285812038
Nosema 484855980
Xenopus 62859101
Salmo 213512398
Phaeodactylum 219116280
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SAE2

Supplementary File S2: Twelve unannotated genes were found in *Ichthyophthirius multifiliis*, which were translated from the ORFs.

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QSNYRFYIQHYIHIYNYLYNQKIFQNFLFIMIYVIHFSFFYINYIIQYIRKKKIFINIYYIFQIFRLFQF
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QMVLKYDLIDLNPLKVLEKLKNLYFQIFTGILECKTALILNNNNEIEYNFEYLMYMENNCQKNKE
KNLSHQNTCDYLLRLLYIIEYISKCIIEQRICSKREIYYSNIQLFQENQKTVNDSIKELQLLLQIPR
FYLNIGASNKGLLSGKIQYKIQNRDDIINLGGNIGEFRPISSFEKIEWVKIGAKRHVLIVEKESVF
QKILGEKNMEFFEVFVVFTGKGNGDYSTKSFVKQIWMENQNCLVYYLGDCDPFGFFIFLNYL
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KKIYLIIFYYILKNNYVNNSFFNNYIINGYQLEQFFFISINIKLYIRLSVNQNNQQINNHISFLFFLFF

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CYQFKRRYQYKRFFNNKQRILFIKCQKYISKKLNKINIQKKLIQKIYGSNEYPVKKWLELGNKK
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G

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HDNFFSKLIMNIKIQQNIEFINVILSKIISNEKIIIFKTSYNTFINIL

Supplementary material: Chapter II

Cryptic sex in Symbiodinium (Alveolata, Dinoflagellata) is supported by an inventory of meiosis genes

Jingyun Chi[1] , Matthew W. Parrow[2], Micah Dunthorn[3]

J. Eukaryot. Microbiol., 61: 322-327. doi:10.1111/jeu.12110

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2 Department of Biology, University of North Carolina at Charlotte, North Carolina 28223, U.S.A.
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Figure S1. Phylogenetic placement of the Symbiodinium isolates used in this study. Modified from Coffroth and Santos (2005), and Bayer et al. (2012).

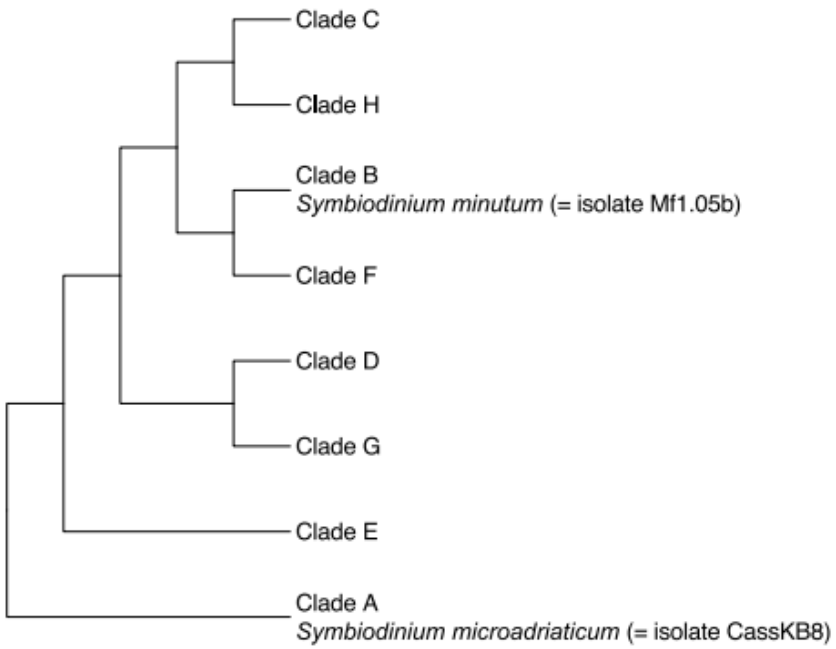
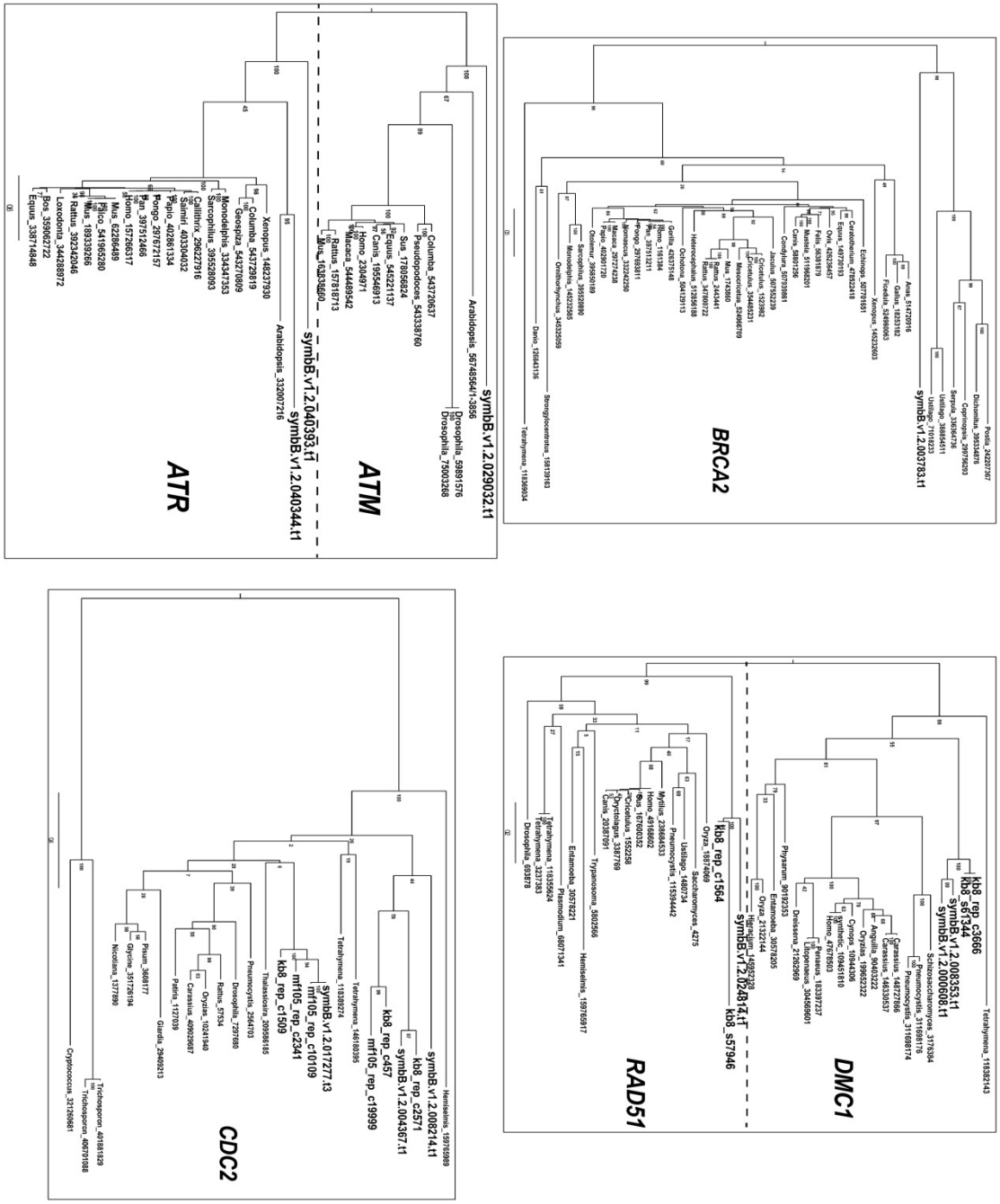
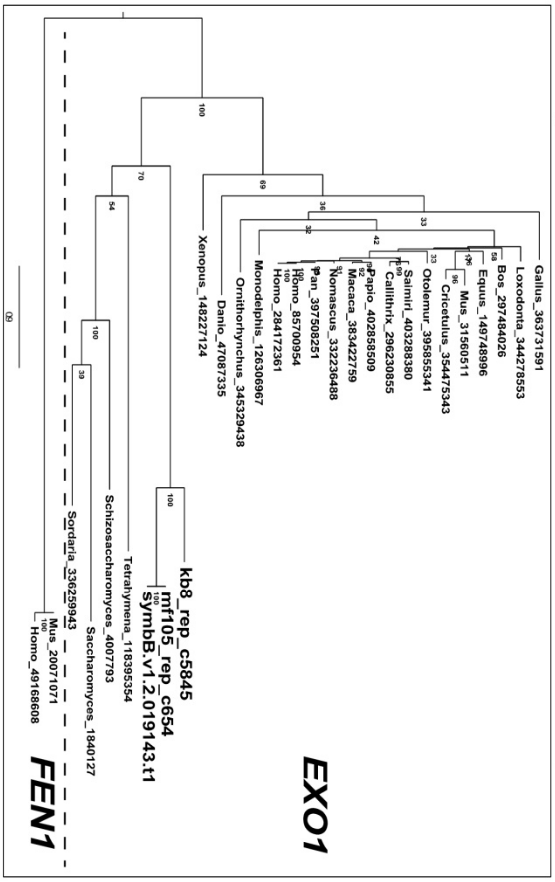


Figure S2. Maximum likelihood inferences of 31 meiotic genes inventoried in *Symbiodinium*. Bootstrap values are shown. Genes containing similar domains or belonging to the same protein families were analyzed together. For some genes, homologs were chosen as outgroups. Detailed figures could be downloaded from: https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fjeu.12110&file=jeu12110-sup-0001-FigureS1-S2.pdf

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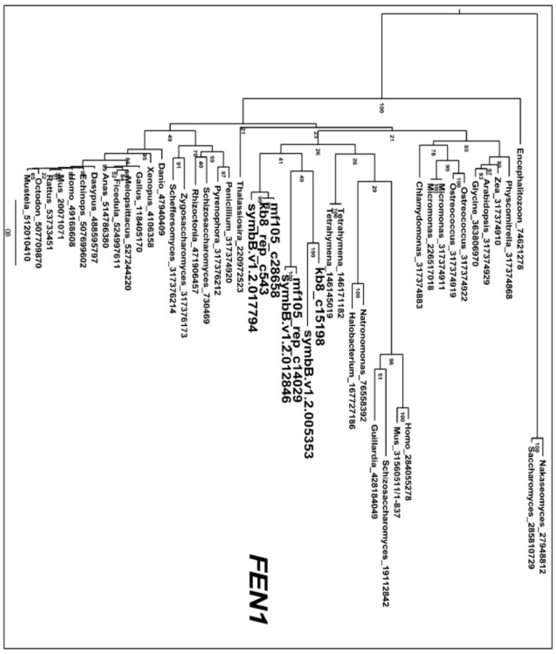
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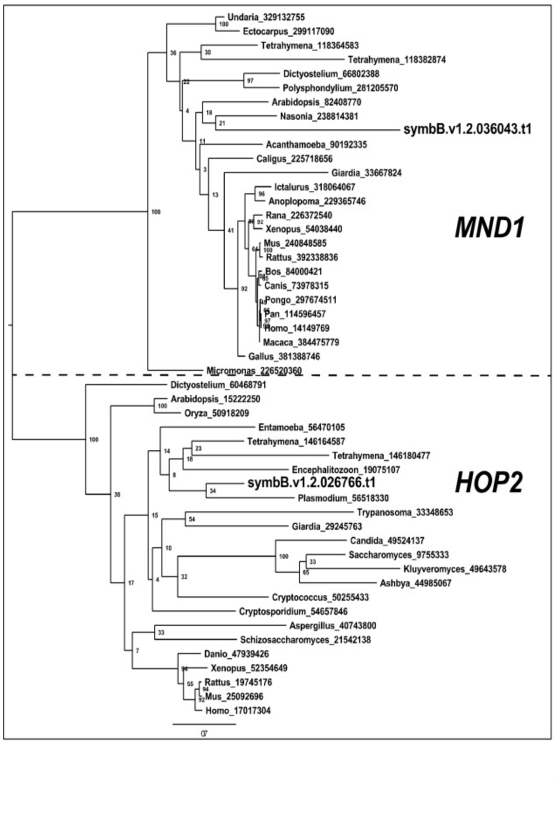
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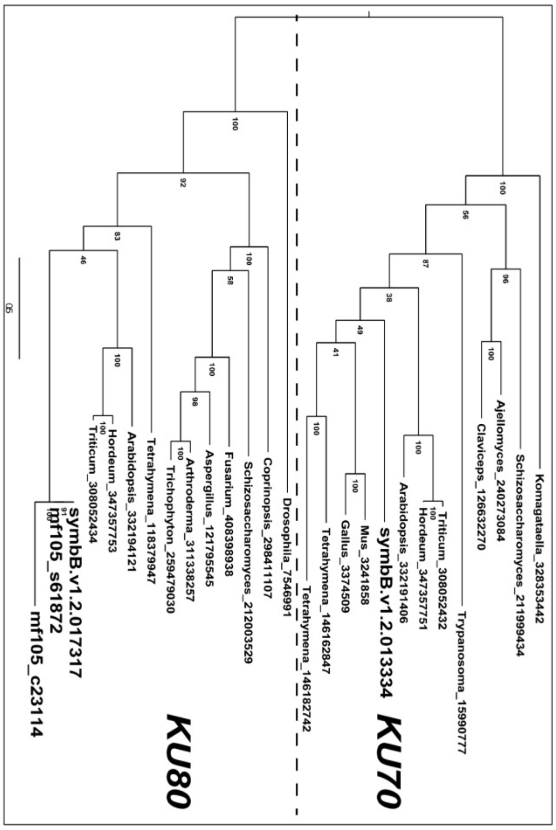
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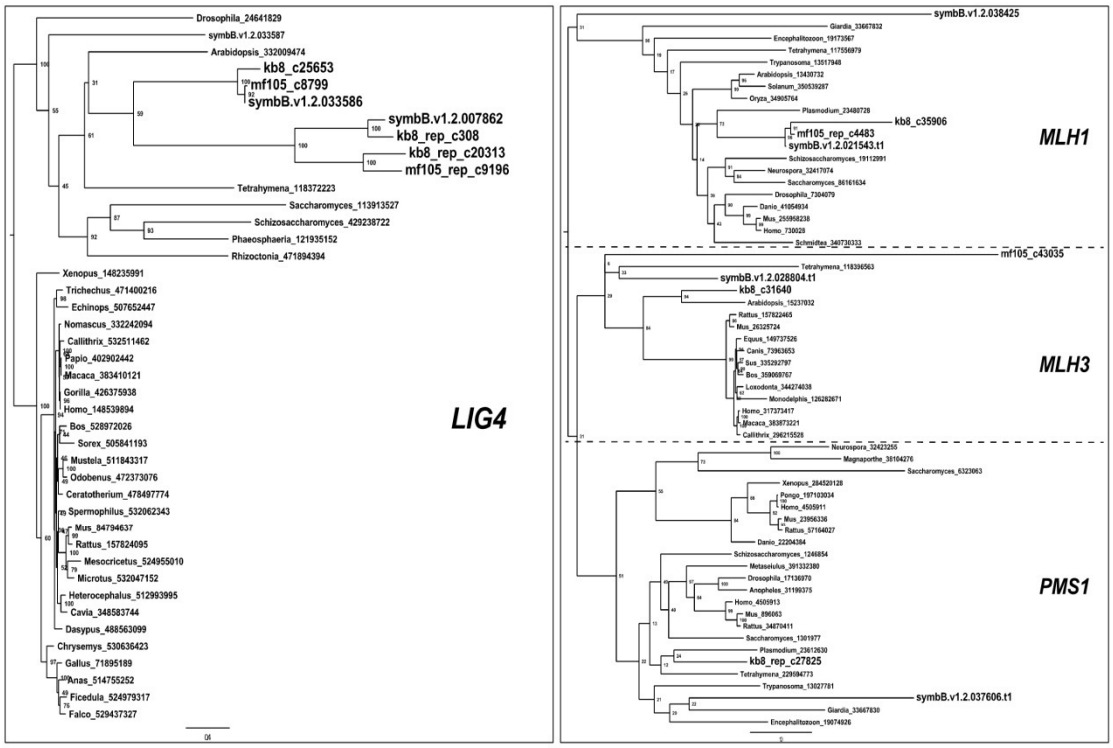
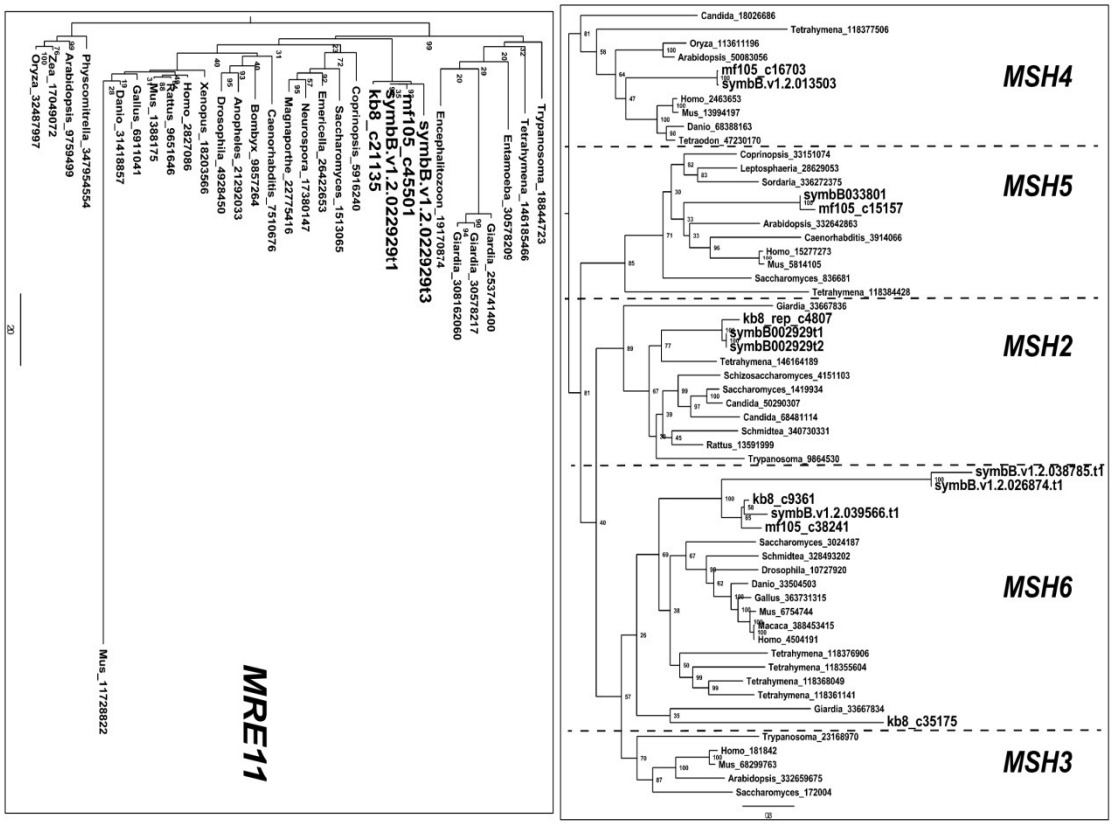

LIG4
MLH1
MLH3
PMS1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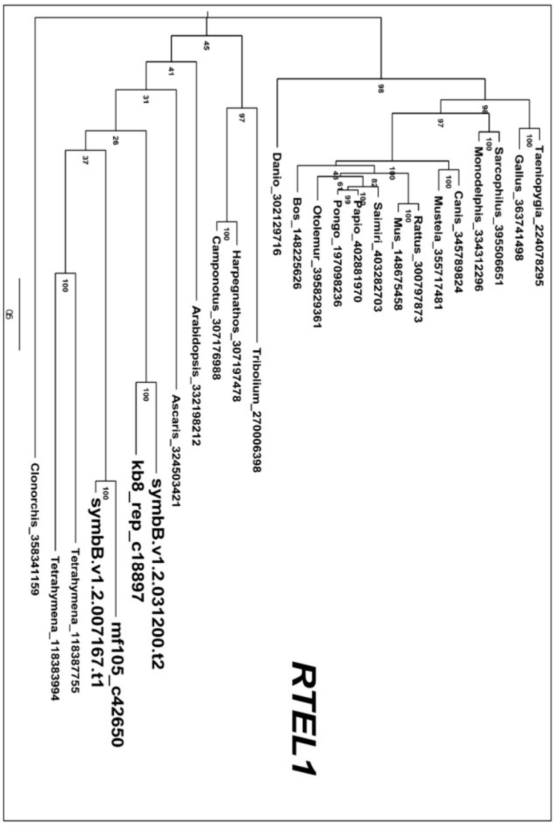
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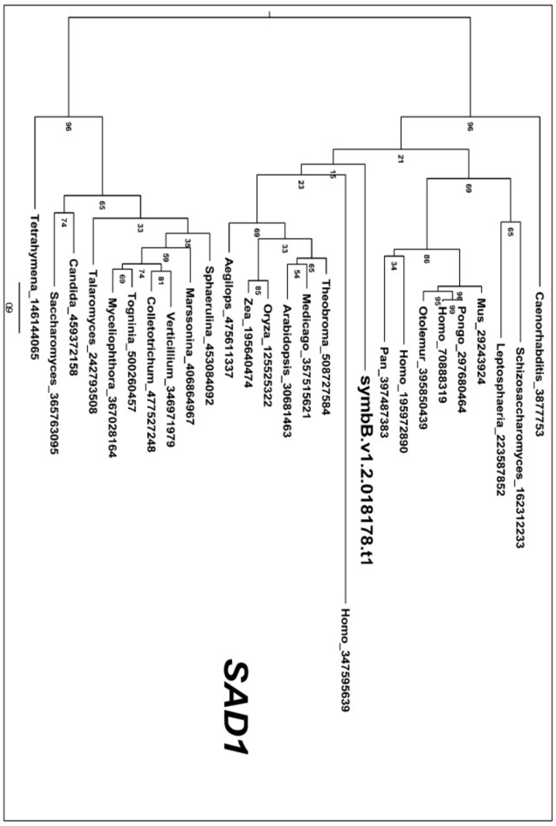
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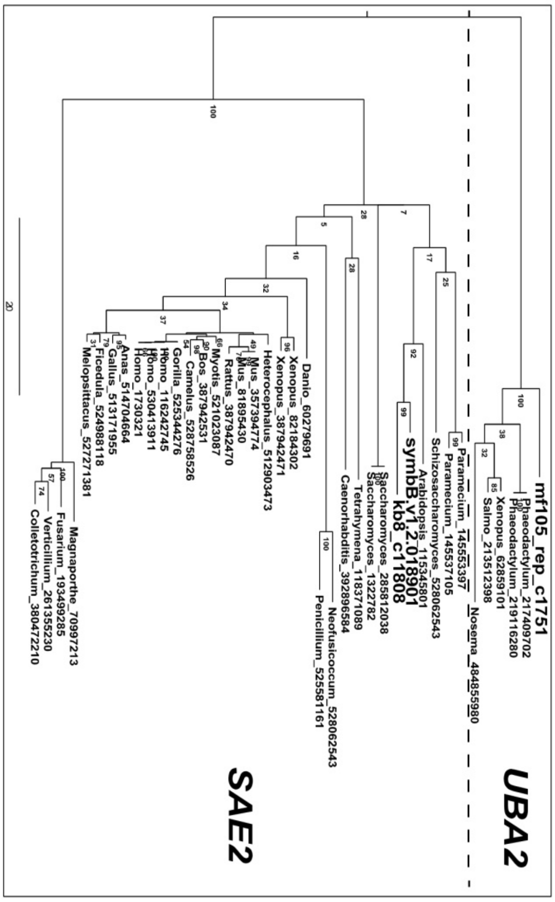
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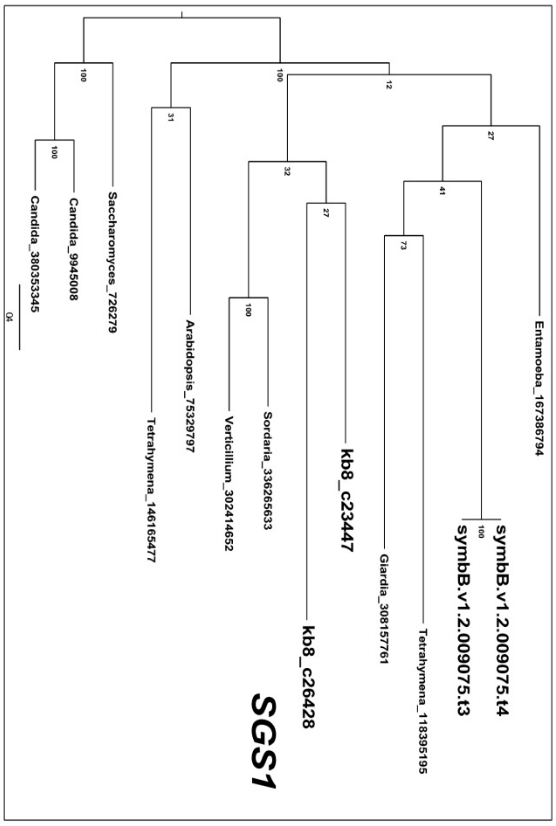
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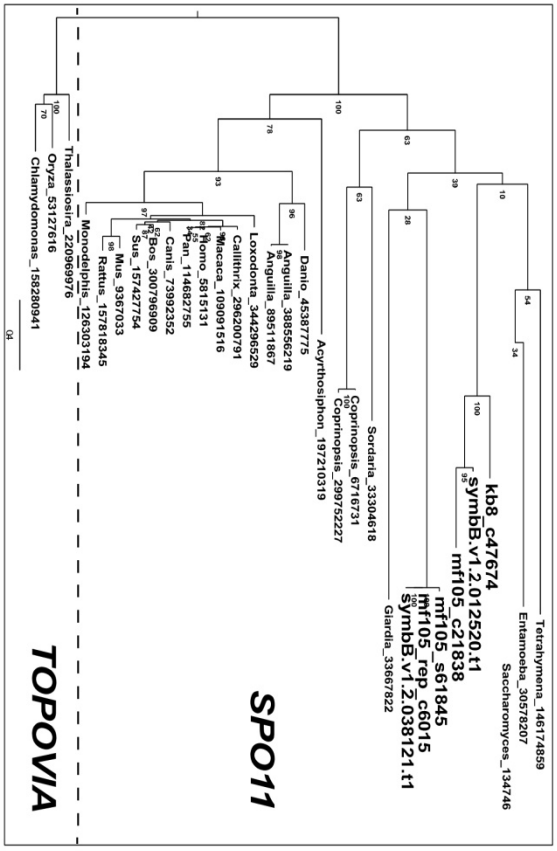
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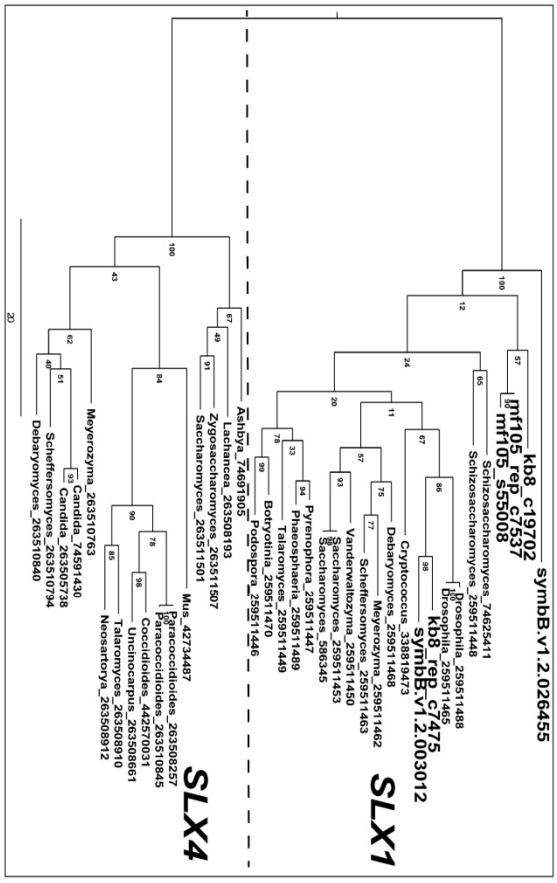
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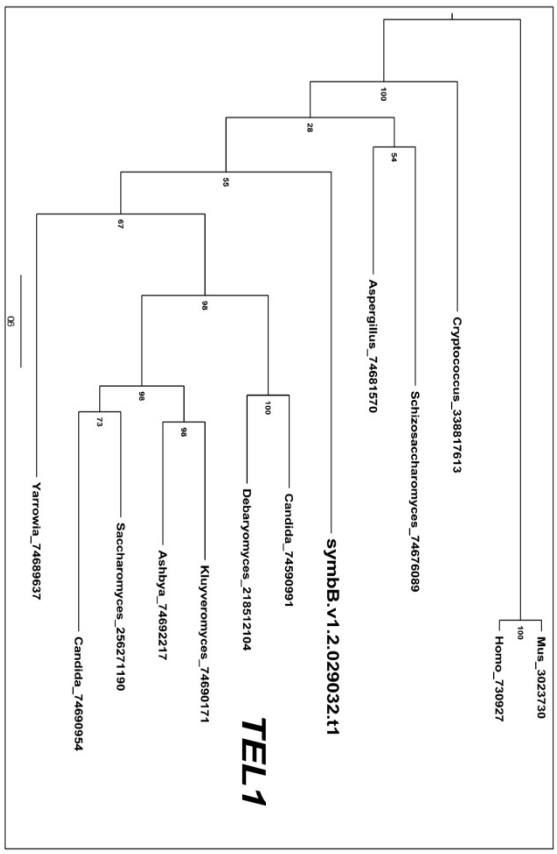
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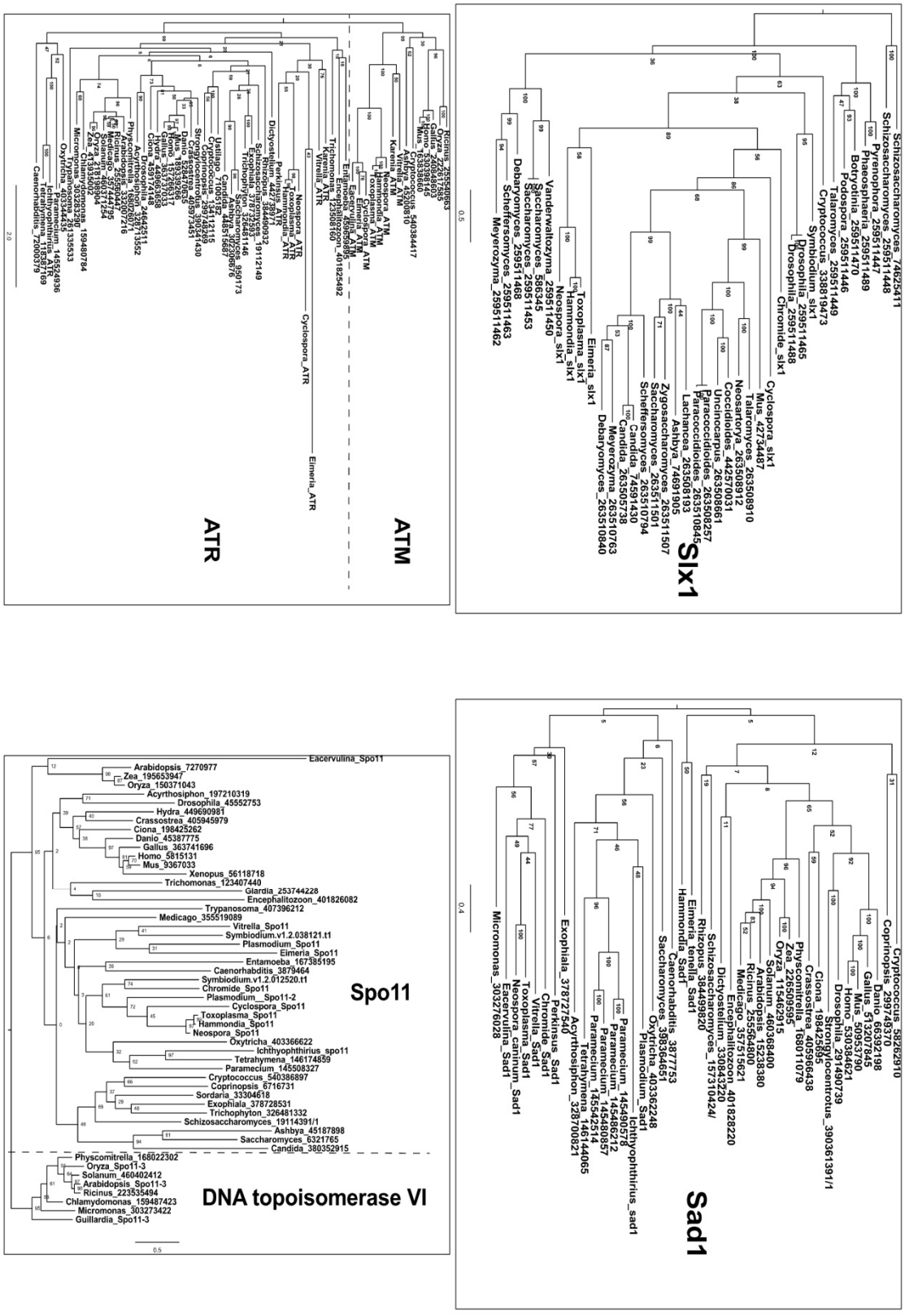
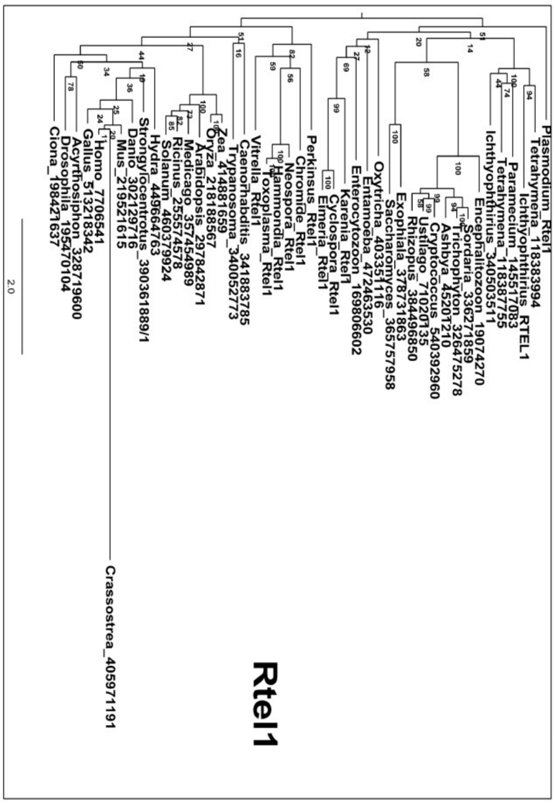
Supplementary material: Chapter III

Meiotic gene inventory in Alveolate genomes

Supplementary figure 1: Maximum likelihood inferences of 27 meiotic genes inventoried in Alveolates.

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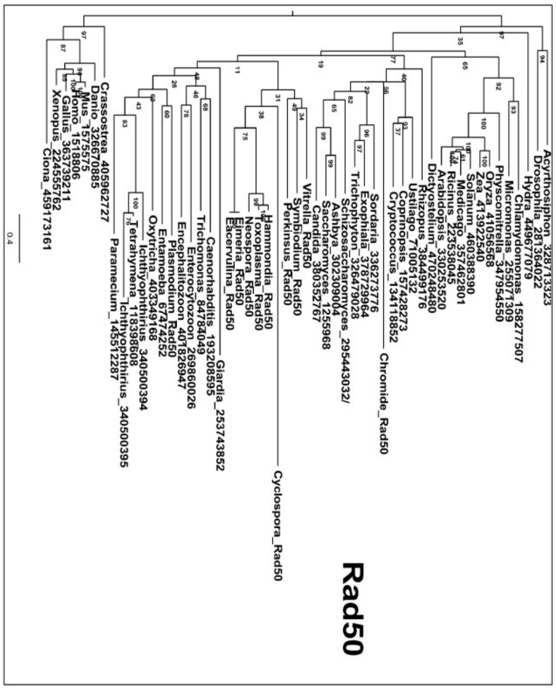
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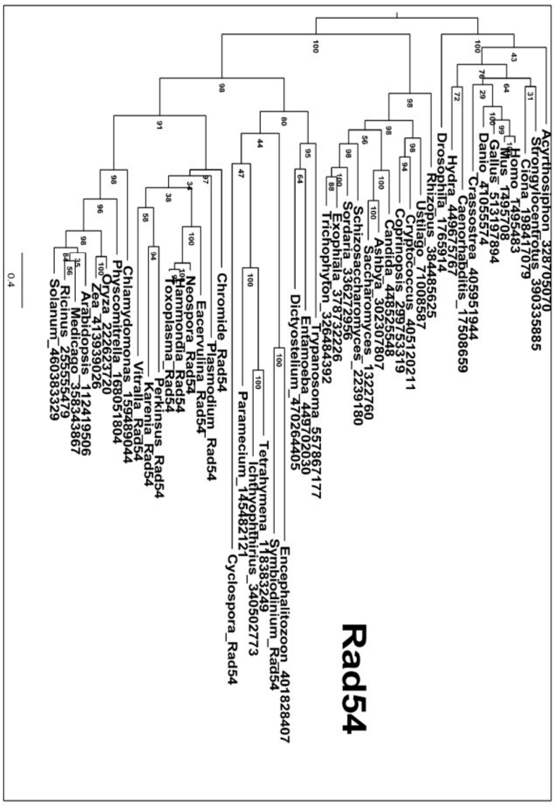
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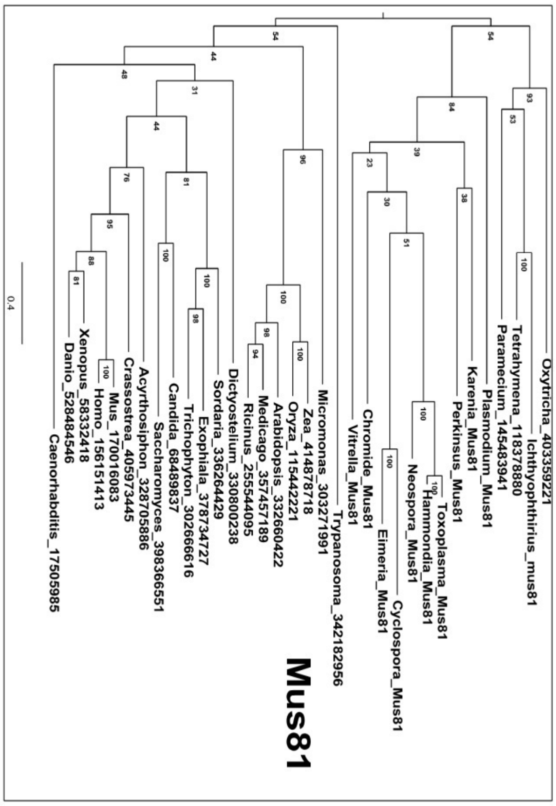
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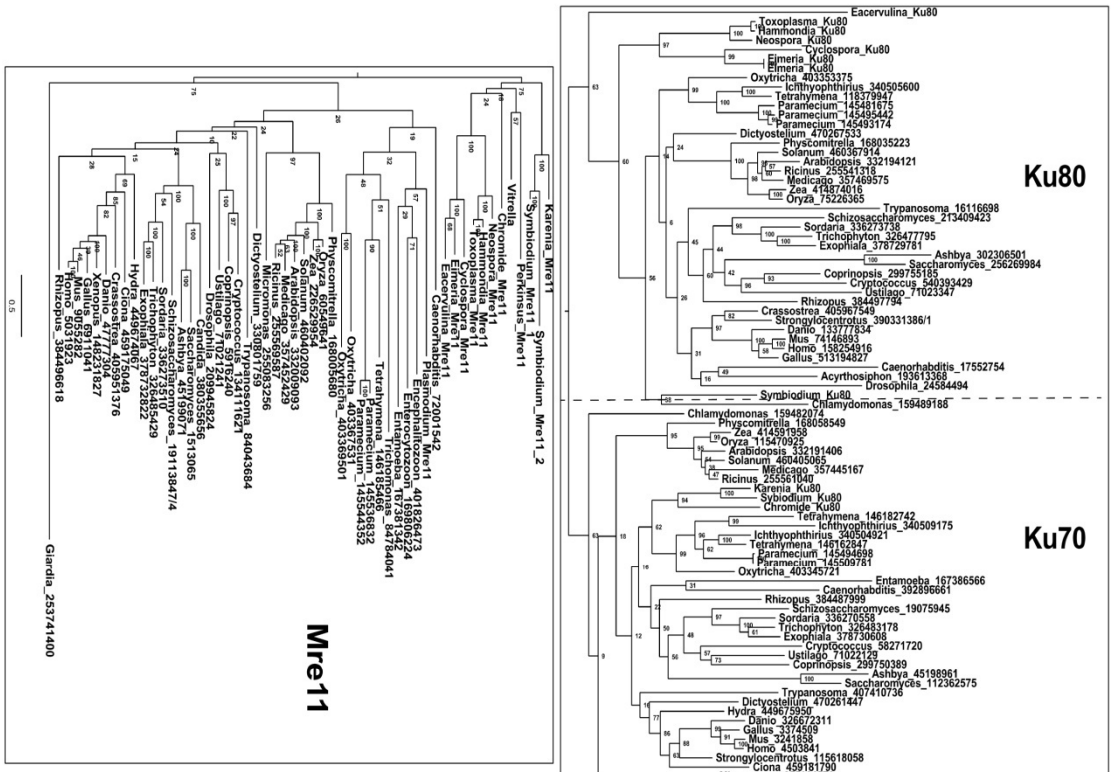
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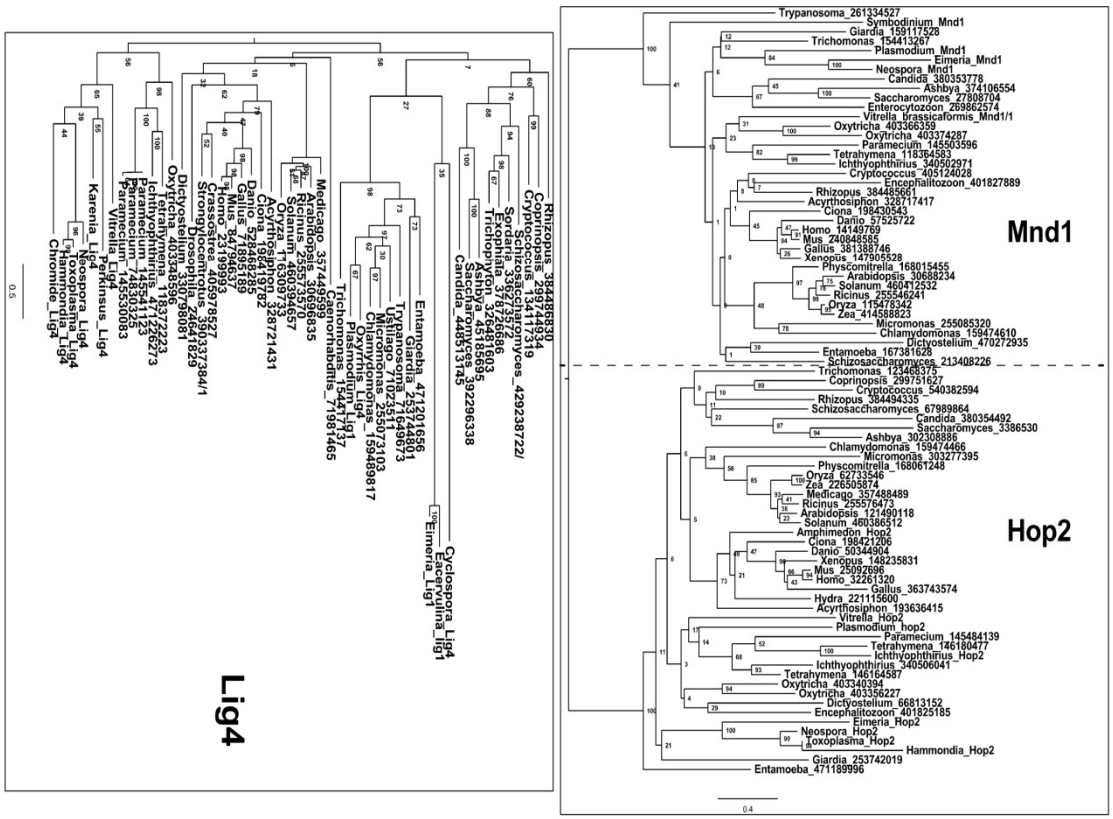
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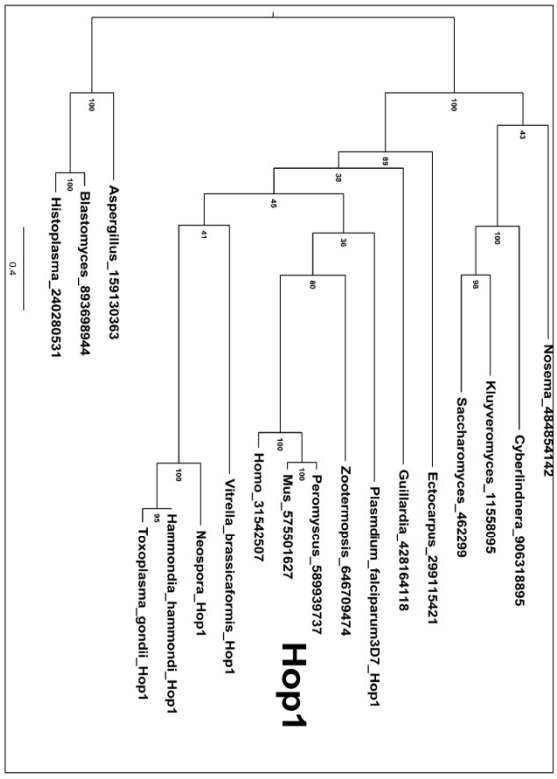
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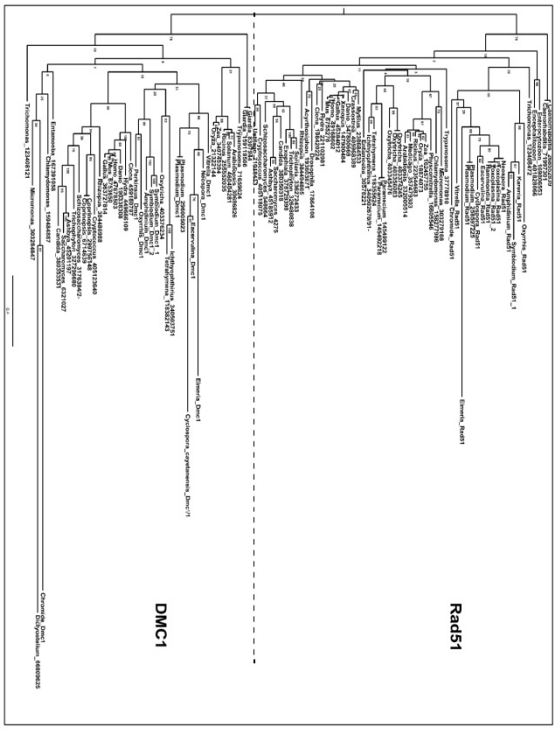
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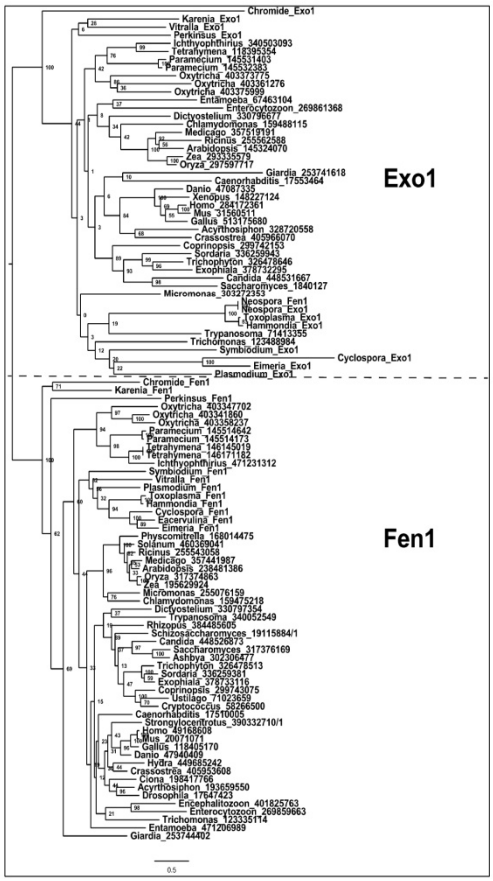
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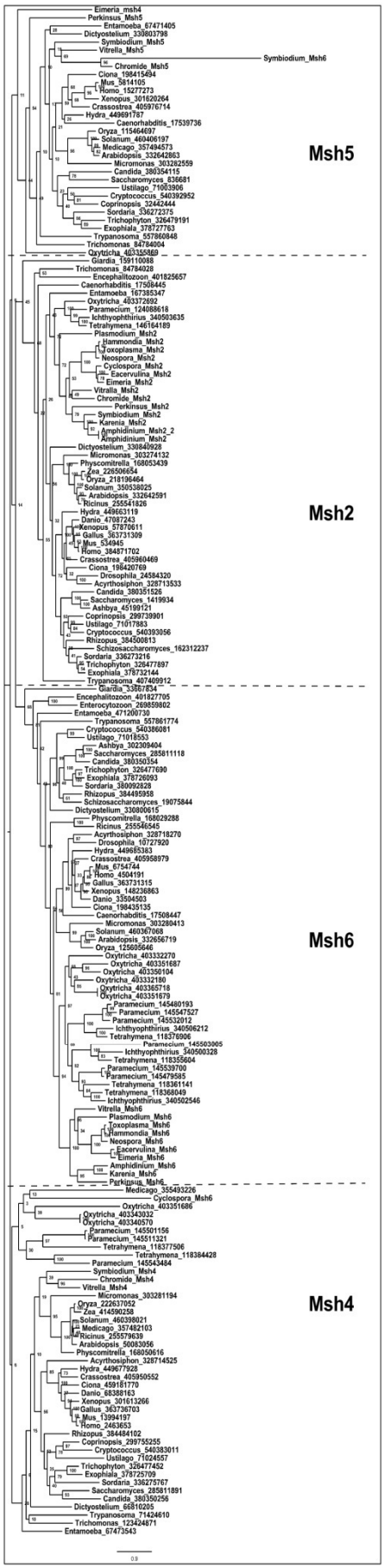
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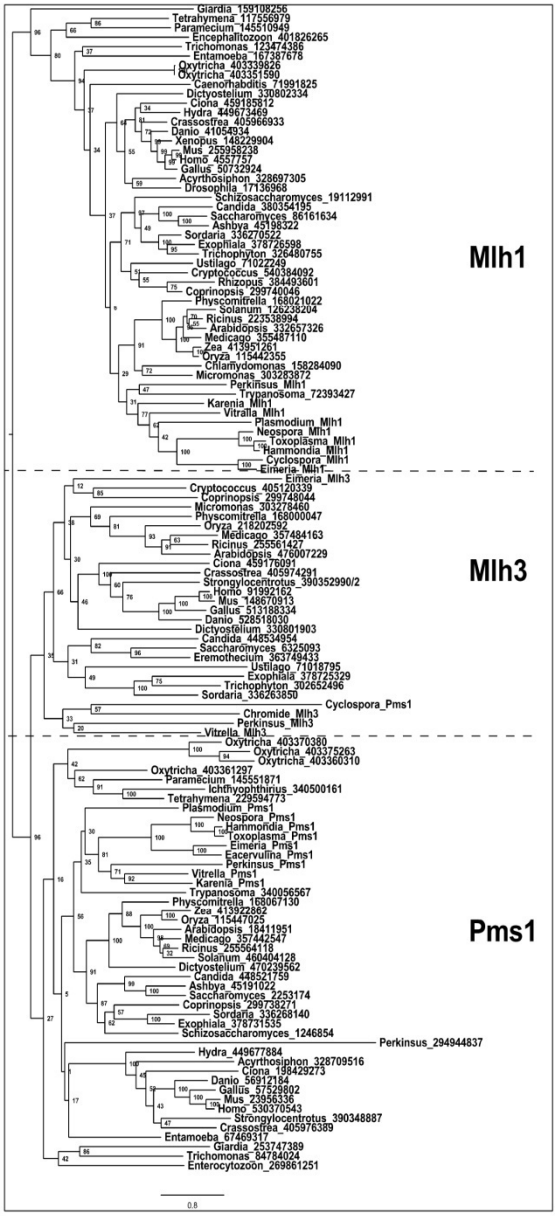
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Supplementary material: Chapter IV

Meiotic Genes in Colpodean Ciliates Support Secretive Sexuality

Micah Dunthorn[1],*, Rebecca A. Zufall[2], Jingyun Chi[3], Konrad Paszkiewicz[4], Karen Moore[4], Frédéric Mahé[5]

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2 Department of Biology and Biochemistry, University of Houston, Houston, U.S.A.
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Exeter, United Kingdom
5 CIRAD, UMR LSTM, Montpellier, France

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Supplementary Table 1. Details on molecular evolutionary rate calculations, noting when not all identified paralogs were included in analyses. Gen names refer to thoses in Supplementary Files 1 & 2.

Gene	Notes
MND1	bursaria_truncatella_Mnd1_1 and colpodea_magna_Mnd1_1 are paralogous, thus ω cannot be calculated
MSH4	bursaria_truncatella_Msh4_1 was excluded due to insufficient overlap with other sequences
MRE11	bursaria_truncatella_Mre11_1 was excluded due to insufficient overlap with other sequences
KU70	bursaria_truncatella_Ku70_1 and bursaria_truncatella_Ku70_3 were excluded because they are paralogous with the rest of the sequences
DNA2	colpodea_magna_Dna2_2 was excluded from ω_{BC} calculation due to insufficient sequence overlap
EXO1	Includes only bursaria_truncatella_Exo1_2 and colpodea_magna_Exo1_3; other Bursaria and Colpodea sequences are paralogous
FEN1	bursaria_truncatella_Fen1_1 and colpodea_magna_Fen1_2 were excluded
MLH1	For ω_0 calculation, bursaria_truncatella_Mlh1_1, bursaria_truncatella_Mlh1_2 (i.e. both Bursaria sequences) and colpodea_magna_Mlh1_1 are excluded because they don't align well and are probably paralogous
MSH2	Low confidence in ω_{BC} due to short overlap between sequences
MSH6	bursaria_truncatella_Msh6_4 and bursaria_truncatella_Msh6_5 were excluded from ω_{BC} calculation due to paralogy and insufficient overlap, respectively; bursaria_truncatella_Msh6_5 was excluded from ω_0 calculation due to insufficient overlap with other sequences
MUS81	colpodea_magna_Mus81_2 was excluded due to insufficient overlap with other sequences
PMS1	bursaria_truncatella_Pms1_2 was excluded due to poor alignment
RAD51	Includes only bursaria_truncatella_Rad51_3 and colpodea_magna_Rad51_1; other sequences are overlapping contigs, but show less overlap with other species sequences
RTEL1	colpodea_magna_RTEL1_3 and Oxytricha locus AMCR01017421 were excluded due to insufficient overlap and low similarity, respectively

Acknowledgment

First of all, I would like to thank Prof. Dr. Micah Dunthorn and Prof. Dr. Thorsten Stoeck for the opportunity to work in their research group and provide support, guidance and advise for my research. Without your patience and encouragement, this dissertation will not be possible.

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I am deeply grateful to the entire group of the Department of Ecology at the University of Kaiserslautern for provide a relaxing work environment. Special thanks for Dr. Frédéric Mahé, who offered me useful advice in programming and other bioinformatics problems, Dr. Dominik Forster, who helped me a lot by solving many problems in research and writing, and Hans-Werner Breiner, who shared valuable experience in the lab. I would also like to thank all the experts and students involving in my PhD dissertation. Meanwhile, I am also grateful to all the RHRK specialists who provide supercomputers for running programs and other technology support.

Finally, I must express my very profound gratitude to my parents, husband and daughters, for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Curriculum Vitae

Jingyun Chi

Master of Science

Education

10/2012~2019 **P.h.D student**, Department of Ecology, University of Kaiserslautern.
Thesis: " Meiosis genes inventory in alveolates and other Protist provide evidence for cryptic sex and the prevalence of a synaptonemal complex-independent crossover pathway."
Supervisor: Prof. Dr. Micah Dunthorn

10/2009~01/2012 **M.Sc.,** Department of Genetic, University of Kaiserslautern
Thesis: "Bioinformatics analysis of Diversity Generating Retroelement."
Supervisor: Prof. Dr. John Cullum

08/2005~07/2009 **B.Sc.,** Biopharmacology, Soochow University
Thesis: "Preparation and pharmacodynamics property of chitosan nanoparticles of Diammonium Glycyrrhizinate."
Supervisor: Prof. Dr. Jinhao Cui

Awards & Grants

2013 Travel Award, International Research Coordination Network for Biodiversity of Ciliates.

2005~2006 Excellent Students Scholarship, Soochow University

Contributed Talks

2015 "An inventory of meiotic genes in alveolates reveals an independent loss in ciliates and dinoflagellates."
German Protozoology Society, Magdeburg, Germany.

2014 "Inventory and Analysis of sex-associated Genes in four Ciliate Genomes."
German Protozoology Society, Essen, Germany.

2013 "Inventory and Analysis of sex-associated Genes in four Ciliate Genomes."
Ciliate Molecular Biology, Steamboat Springs, USA.

Publications

Chi J, Dunthorn M. 2019. Meiotic gene inventory in Alveolate genomes. Protists (unpublished)

Kraus D, **Chi J**, Boenigk J, Beisser D, Graupner N, Dunthorn M. 2019. Putatively asexual chrysophytes have meiotic genes: evidence from

transcriptomic data. PeerJ 6:e5894 DOI 10.7717/peerj.5894

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