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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo para a obtenção do Título de Doutor em Ciências, na área de Zoologia.

Orientador: Prof. Dr. Daniel José Galafasse Lahr

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Resumo

O sexo é inerente à vida eucariótica. Distante do senso comum, sexo pode ser definido como plasmogamia (fusão celular) seguida, eventualmente, de meiose. Ambos os processos são fundamentais para qualquer ciclo de vida sexual e requerem uma maquinaria específica. Muitos componentes desta maquinaria já foram identificados e permanecem altamente conservados entre as linhagens mais distantes, realizando basicamente as mesmas funções em todos os grupos. Esta caixa de ferramentas da meiose pode ser usada como uma indicação de processos meióticos, mesmo quando tais processos não foram observados ainda. Mesmo que o ancestral de todos os eucariotos seja presumidamente sexuado, alguns grupos são tratados como assexuais por muitos autores na literatura baseados na falta de ciclos sexuais observáveis em cultura. Entre estes 'assexuais' estão as amebas pertencentes a Amoebozoa, um super-grupo eucariótico. Muitas linhagens de Amoebozoa são consideradas assexuadas, mas alguns gêneros mais bem estudados dentro do super-grupo foram demonstrados como sendo plenamente sexuados, por exemplo, *Trichosphaerium*, *Physarum*, *Dictyostelium*. Alguns outros gêneros exibem evidências indiretas para processos sexuais e podem ser sexuadas também. A ocorrência de organismos sexuais dentro de Amoebozoa aponta para um ancestral sexuado para o super-grupo inteiro, de outra maneira o sexo teria que ter evoluído novamente em Amoebozoa, o que não seria uma visão parcimoniosa. Nós coletamos dados moleculares de várias linhagens de Amoebozoa com representativos da maior parte de sua diversidade conhecida e procuramos um caixa de ferramentas estendida, com adição da maquinaria da plasmogamia. Como resultado, pudemos encontrar todos os componentes desta maquinaria em basicamente todos os grupos de Amoebozoa. Estes resultados não só suportam a ocorrência de ciclos sexuais em todo o grupo Amoebozoa, mas também um provável alto nível de conservação para os principais processos sexuais. Adicionalmente, realizamos análises similares para toda a diversidade eucariótica conhecida com resultados similares, exceto para alguns grupos isolados. Reconstruções profundas também forneceram evidências para uma nova história evolutiva para o sistema de reparo de bases mal-pareadas (*mismatch-repair*) em eucariontes, um mecanismo de reparo de DNA que integra a maquinaria da meiose. Todos os resultados apontam para uma origem arqueal para a maquinaria meiótica e para uma ampla presença de processos sexuais em basicamente todos os eucariontes.

Palavras-chave: Amoebozoa, meiose, plasmogamia, sexo.

Abstract

Sex is an inherent part of eukaryotic life. Far from the common sense, sex can be defined as plasmogamy (cell fusion) eventually followed by meiosis. Both processes are fundamental to any sexual life cycle and require a specific machinery. Several components of this machinery have been already identified and remain highly conserved among the most distantly related eukaryotic lineages, performing basically the same functions in all groups. This meiosis toolkit may be used as an indication of sexual processes, even when such processes have not been observed yet. Even though the ancestor of all eukaryotes is assumed to be sexual, some eukaryotic groups are normally treated as asexuals by many authors in the literature based on the lack of observable sexual cycles in culture. Among these 'asexuals' are the amoebae belonging to Amoebozoa, a major eukaryotic supergroup. Several lineages of Amoebozoa are considered asexual, but some well studied genera inside this supergroup were demonstrated to be fully sexual, i.e., *Trichosphaerium*, *Physarum*, *Dictyostelium*. Some other genera exhibit indirect evidence for sexual processes and may be sexual as well. The occurrence of sexual organisms inside Amoebozoa points to a sexual ancestor for the whole supergroup, otherwise sex would have to evolve again in Amoebozoa, not exactly a parsimonious view. We collected molecular data from several amoebozoan lineages with representatives of most of its known diversity and searched for an expanded meiosis toolkit, adding the plasmogamy machinery to it. As a result, we were able to find every component of this machinery in basically all amoebozoan groups. These results support not only the occurrence of sexual processes in all Amoebozoa, but also a probable high level of conservation for mains sexual processes. Additionally, we performed similar analyses for all known eukaryotic diversity with similar results, except for a few isolated groups. Deep reconstructions also provided evidence for a new evolutionary history for the mismatch-repair system in eukaryotes, a DNA repair mechanism that is part of the meiotic machinery. All results point to an archaeal origin for the meiotic machinery and for a widespread presence of sexual processes in basically all eukaryotes.

Keywords: Amoebozoa, meiosis, plasmogamy, sex.

Índice

Capítulo 1: Introdução

Quando decidi iniciar um doutorado, vi-me diante de um novo grupo de eucariontes para realizar um trabalho em evolução molecular: Amoebozoa. Um desafio duplo: o primeiro seria abandonar meu antigo grupo de estudo – Apicomplexa – e me dedicar a um nova linhagem de eucariotos cuja circunscrição era mais recente – datando da década de 1990 (T. Cavalier-Smith 1998) – e sobre a qual muito pouco eu sabia; o segundo seria efetuar a transição de uma abordagem meramente taxonômica e morfológica para a evolução molecular com o uso de ferramentas de bioinformática aplicadas a sequências biológicas. A dúvida inicial pairava entre dois diferentes recortes no tema de evolução de Amoebozoa: evolução do flagelo no grupo e evolução da maquinaria molecular responsável pela meiose (e da meiose *per se*); optou-se pela última. Um grande volume de dados de transcriptomas havia sido anteriormente gerado como parte de uma colaboração internacional entre nosso laboratório e um grupo sediado em Mississippi (grupo de Matthew W. Brown). Tais dados, aliados aos genomas disponíveis, forneceriam um rico conjunto de dados para se explorar o tema do presente estudo iniciado em meados de 2015 com apoio da FAPESP.

Amoebozoa, um super-grupo de eucariontes, é composto por diversas linhagens ameboides, flageladas e ameboflageladas, tradicionalmente entendidas como grupos asexuais, capazes de mitose somente (Adl et al. 2019). Tal super-grupo inclui organismos-modelo e agentes infecciosos, a saber: *Dictyostelium discoideum*, *Physarum polycephalum*, *Entamoeba histolytica*, *Acanthamoeba castellani*, entre outros. Em reconstrução filogenética recente e compreensiva, três grandes linhagens foram estabelecidas: Tubulinea (inclui grupos majoritariamente aflagelados que se locomovem por pseudópodes lobosos ou tubulares podendo ser nuas ou tecadas; inclui *Amoeba proteus*, *Copromyxa* sp., *Arcella* sp., *Vermamoeba* sp., *Trichosphaerium* sp.), Evosea (grupo diverso contendo representantes flagelados ou não; inclui archamoebideos como *Entamoeba* sp e *Mastigamoeba*, Myxogastria, *Dictyostelium sp.*, protostelideos e outros), Discosea (constituído por

um conjunto de linhagens aflageladas diversas como *Acanthamoeba*, *Sappinia*, *Vannella* e *Vexillifera*) (Kang et al. 2017; Adl et al. 2019). Amoebozoa apresenta como grupos próximos os Opisthokonta e pequenos flagelados não-afiliados a quaisquer super-grupos (‘linhagens órfãs’) (Brown et al. 2013). Apesar da existência de linhagens com ciclos sexuais bem caracterizados dentro do grupo, a literatura se refere com frequência a Amoebozoa e muitos de seus sub-grupos como 'assexuados' (A. Smirnov et al. 2005; A. V. Smirnov et al. 2011; Thomas Cavalier-Smith et al. 2015). Este aparente paradoxo nos chamou a atenção, já tendo sido abordado previamente na literatura de forma mais teórica (Lahr et al. 2011). Os padrões filogenéticos de distribuição de ciclos sexuados em Amoebozoa são compatíveis com um ancestral plenamente sexuado, pois esta é a solução mais parcimoniosa para o grupo; a existência de diversas linhagens assexuadas espalhadas em diferentes regiões da árvore de Amoebozoa exigiria a perda do sexo e meiose várias vezes independentemente. São muito raros os exemplos de eucariotos assexuados na literatura e a perda dos ciclos sexuais diversas vezes independentemente não seria parcimoniosa. Em tese, a hipótese mais razoável seria a de que o grupo é constituído por linhagens sexuadas, mas artefatos de observação levaram à suposição de que diversos grupos seriam assexuados simplesmente porque ciclos sexuais jamais foram observados para tais grupos.

Entre as possíveis razões para se explicar esta suposta falta de observações, poder-se-ia citar, além das dificuldades inerentes de se observar processos em organismos unicelulares, a ausência de gametas complementares em cultura, a possibilidade da meiose ser modificada em relação a animais, plantas e fungos e não ser percebida mesmo quando observada (podendo até ser confundida com mitose), falta de estímulos ambientais específicos para a meiose ou plasmogamia etc. Ciclos sexuais são mais bem conhecidos em membros de Myxogastria. Este grupo foi por muito tempo considerado parte de Fungi e alvo de mais estudos que outras amebas em geral, o que pode explicar o fato dos ciclos sexuais serem descritos para este grupo e não para vários outros (Spiegel 2011). Indícios diretos e indiretos de processos sexuais, como plasmogamia, formação de cistos, formação de corpos de frutificação, cistos multinucleados, perfis de expressão gênica, entre outros

são sugestivos de que diversos processos sexuais podem ocorrer, mas carecem de mais atenção e estudo. Entre as evidências diretas ou indiretas de processos sexuais em amebas pode-se citar: ocorrência de complexo sinaptonemal em *Arcella* (Mignot and Raikov 1992), expressão de proteínas da meiose em cistos de *Entamoeba* (Ehrenkaufer et al. 2013), plasmogamia em *Paraquadrula* com formação de cisto (Lüftenegger and Foissner 1991), formação de cisto bicelular em *Sappinia* (Brown, Spiegel, and Silberman 2007). A confirmação da presença da maquinaria específica da meiose em diversas linhagens de Amoebozoa forneceria evidências adicionais para a ocorrência de processos sexuais no grupo.

Durante as análises preliminares das famílias gênicas que compõem a maquinaria especializada da meiose, a topologia das árvores das recombinases nos chamou a atenção. Recombinases são enzimas altamente conservadas que realizam o processo de reparo de DNA em quebras de dupla fita através da recombinação entre regiões homólogas (Sherratt D. J. et al. 2004). Este tipo de reparo é mais preciso que outros (pela busca por homologia) e termina com uma menor probabilidade de ocorrência de mutação. Existem outros mecanismos de reparo não relacionados a este e que apresentam taxas maiores de mutação por ocasião do reparo, inclusive com a perda de trechos da fita de DNA na região da quebra de dupla-fita. Eucariontes apresentam três grupos básicos de recombinases de acordo com sua origem: as mitocondriais, oriundas de Alpha-Proteobacteria (recAmt), as plastidianas, oriundas de Cyanobacteria (recAp) e as eucarióticas ancestrais nucleares, de origem arqueal, grupos Radα e Radβ (Lin et al. 2006). Recombinases nucleares são essenciais para a ocorrência da meiose por causa da quebra de dupla-fita inerente ao processo de recombinação meiótica. Embora todas as recombinases nucleares possam participar da meiose, o grupo Radα (produzido por uma duplicação ancestral em algum ancestral dos eucariontes e formado por RAD51A e DMC1, esta última específica da meiose (Bishop et al. 1992)) tem papel mais relevante no processo. Durante o processo de busca de recombinases que ocorrem em Amoebozoa, uma base de dados com todos os proteomas disponíveis [derivados de genomas completos, rascunhos (*drafts*) de genomas e transcriptomas], um conjunto de recombinases formou

um grupo muito homogêneo e distante das outras recombinases; buscas de sequências deste clado em bases de dados públicas (como Genbank) revelou seu caráter bacteriano/mitocondrial. A reconstrução das relações filogenéticas entre as sequências encontradas em amebas e outros eucariontes revelou uma história complexa de aquisição e perda de recombinases bacterianas em eucariontes [**Capitulo 2**, (Hofstatter et al. 2016)]. Há uma forma mitocondrial adquirida por ocasião da endossimbiose que deu origem à mitocôndria, esta mantida em muitos grupos, porém perdida em Opisthokonta, Apicomplexa, Ciliophora e outros; há uma segunda forma, derivada de Cyanobacteria, adquirida durante o processo de endossimbiose que originou os cloroplastos em Archaeplastida, esta duplicada em plantas Angiospermae e transferida em endossimbioses secundárias de algas verde e vermelhas para Phaeophyta e Chlorarachniophyta. O entendimento da história destas recombinases foi importante para se isolar este grupo das recombinases nucleares, pois somente estas últimas são relevantes para o estudo dos processos meióticos em amebas, o objetivo central deste projeto de doutorado. O trabalho com a evolução da recombinase A (recA) em eucariontes foi apresentada em um congresso internacional de protozoologia em Moscou, Rússia, em meados de 2016.

Com o isolamento prévio de todo um grupo de recombinases não relacionadas a meiose, procedeu-se à busca geral por similaridade de homólogos específicos da meiose. A presente abordagem foi desenvolvida de forma a fornecer evidências moleculares e de bioinformática que suportassem a provável presença de ciclos meióticos/sexuais em Amoebozoa. A provável natureza sexual das amebas pode ser derivada teoricamente da presença de ciclos sexuais no ancestral dos eucariontes, nos ancestrais dos grupos atuais e na presença de linhagens sexuais derivadas dentro de Amoebozoa (**Figura 1**). A confirmação da existência da maquinaria de meiose em amebas forneceria uma evidência a mais para a ocorrência de meiose e sexo nos diversos grupos de amebas, além da previsão teórica estabelecida por parcimônia. Nesta abordagem, ferramentas de busca por similaridade foram usadas para a identificação das sequências candidatas a ortólogos de proteínas específicas da meiose, plasmogamia e cariogamia. A confirmação da ortologia transcende a simples

busca por similaridade pois a maioria das enzimas específicas da meiose é derivada de eventos de duplicação gênica ancestral e somente por meio de processos de reconstrução de árvores de proteínas pode-se concluir com maior segurança quais ortólogos de proteínas específicas da meiose estão presentes. Tais proteínas específicas foram anteriormente caracterizadas em organismos-modelo distantemente relacionados, a saber *Saccharomyces cerevisiae* (um fungo ascomiceto unicelular, a levedura), *Arabidopsis thaliana* (uma planta angiosperma modelo), *Homo sapiens* (modelo animal vertebrado), entre outros e compiladas na literatura sob o apelido de 'caixa de ferramentas da meiose' [*meiosis toolkit* (Schurko and Logsdon 2008)]. O presente trabalho se constituiu como uma aplicação que tal abordagem a toda a diversidade conhecida - e sequenciada - de Amoebozoa de forma compreensiva, com diversas amostras das três principais linhagens moleculares dentro do grupo, Tubulinea, Evosea e Discosea. Tais relações foram descritas em trabalho recente (Kang et al. 2017); o mesmo conjunto de dados forneceu material para o presente projeto.

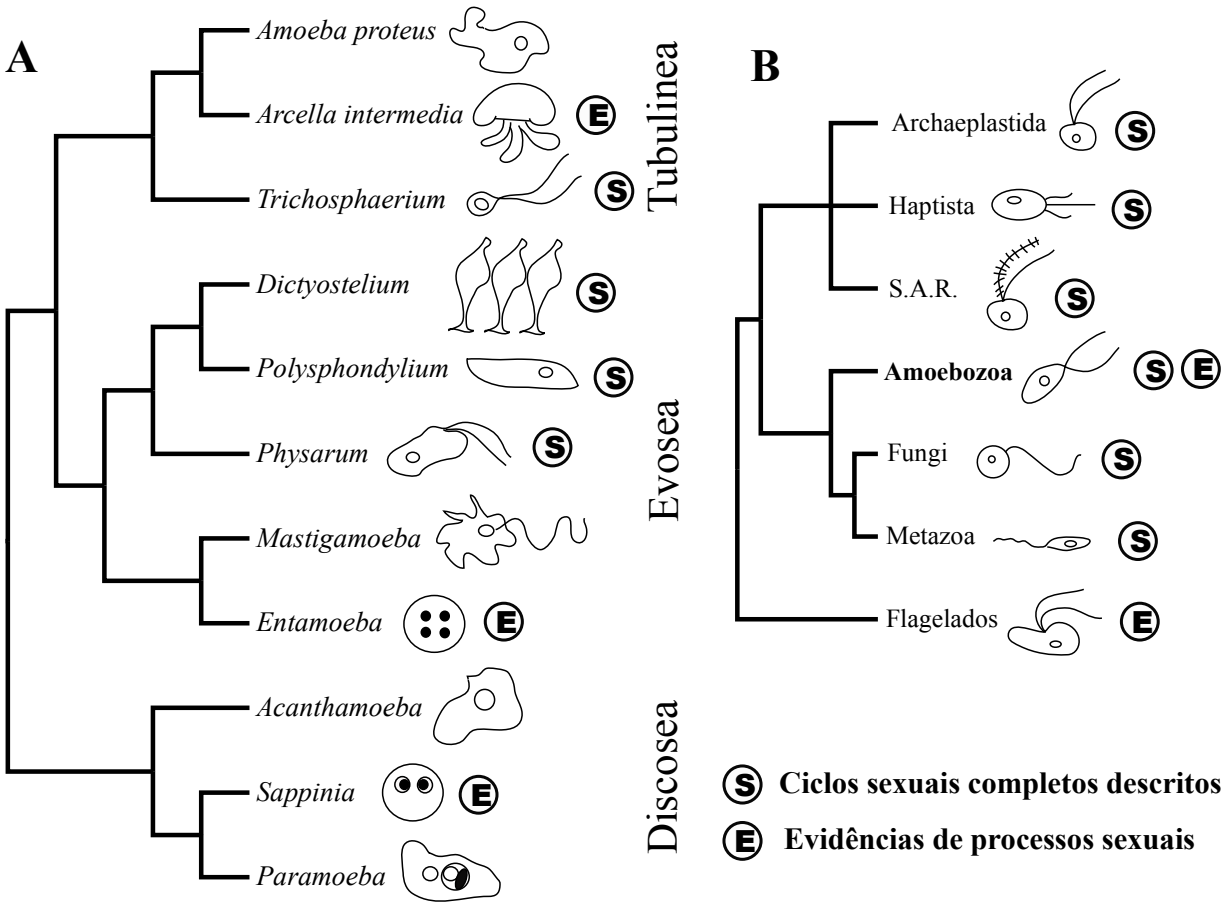

Figura 1. A. Relações filogenéticas entre representativos de Amoebozoa e a distribuição de ciclos sexuais ou evidências para processos sexuais nos diferentes grupos; B. Posição de Amoebozoa em relação a outros eucariotos e a distribuição de processos sexuais nos grandes grupos.

Para a busca de sequências candidatas, perfis de HMMER (Eddy 2011) foram construídos a partir do alinhamento de proteínas específicas da meiose caracterizadas em organismos modelos. Os alinhamentos foram produzidos por MAFFT (Katoh and Standley 2013). As sequências candidatas foram, a seguir, submetidas ao processo de reconstrução de árvores filogenéticas para posterior diferenciação dos ortólogos em questão. Candidatos para as seguintes proteínas foram buscados: HAP2 (fusão de gametas), GEX1 (fusão de núcleos), SPO11, MER11, RAD50 (introdução de quebras de dupla-fita de DNA), SMC1, SMC3, RAD21, REC8 (complexo de coesão de cromossomos/coesina), HOP1, PCH2 (pareamento de homólogos), DMC1, RAD51A, HOP2, MND1 (recombinação de homólogos), MER3, ZIP4, MSH4, MSH5 (complexo 'ZMM' de resolução de Junções duplas de Holiday/primeira via de resolução de crossing-over – específica da meiose), MLH1, MLH3, EXO1 (resolução da primeira via de crossing-over), MUS81, MMS4 (segunda de via de resolução de crossing-over – acessória nos principais modelos), MSH2, MSH6, PMS1, PMS2 (complexo de conversão gênica pós-meiotica baseada no processo de '*mismatch-repair*'). O processo de reconstrução consistiu de alinhamento com MAFFT (candidatos, sequências caracterizadas em modelos e grupos externos conhecidos – Bacteria e Archaea), remoção (*trimming*) de sítios não-alinhados ou não-homólogos e reconstrução com algorítmos de máxima verossimilhança (*maximum likelihood*) como RAxML (Stamatakis 2014) ou IQ-TREE (Nguyen et al. 2015).

As reconstruções obtidas desta forma revelaram a existência de todas as proteínas específicas da plasmogamia, cariogamia e meiose no grupo (**Capítulo 3**, (Hofstatter, Brown, and Lahr 2018)). Todas ocorrem nas três principais linhagens de Amoebozoa com exceção de REC8 cuja presença não pôde ser confirmada em Evosea até o momento. A presença do sistema ancestral de gametas complementares em Amoebozoa é fortemente sugestivo de ciclos sexuais heterotálicos

(sistema de reconhecimento mútuo entre gametas de tipos diferentes a fim de se evitar auto-fecundação por meio da expressão da proteína de membrana HAP2). A detecção de tal sistema sugere que ciclos sexuais dificilmente devem ocorrer em culturas monoclonais de laboratório por falta de gametas complementares na cultura. Em suma, a presença de todas as proteínas específicas de meiose indica não só a ocorrência do processo, mas que este é provavelmente canônico e inclui recombinação extensiva entre cromossomos homólogos com participação de DMC1 e ambas as vias conhecidas de resolução de *crossing-over* na meiose, a primeira via composta por MSH4, MSH5, MLH3 e portadora de interferência (específica da meiose) e a segunda via, mais simples e sem sinais de interferência meiótica, composta por MUS81 e MMS4. Tais evidências suportam a ocorrência de meiose em Amoebozoa, assim como ciclos sexuados, com implicações para o entendimento da biologia de todo o grupo. Os resultados obtidos neste projeto, o principal do doutorado, foram apresentados em congresso de protozoologia em Praga, Tchéquia em meados de 2017.

Apesar de amplo, o recorte proposto acima foi restrito a Amoebozoa e ao modesto conjunto de dados gerados para este grupo. Questões similares estão postas para os eucariontes em geral. Seria o ancestral de todos os eucariontes um ser sexuado capaz de realizar plasmogamia e meiose? Estaria o mesmo conjunto de proteínas presente em todos os grandes grupos de eucariontes e nas chamadas 'linhagens órfãs', conservado e executando as mesmas funções? A observação de que a maioria das proteínas específicas da meiose são resultantes de eventos de duplicação gênica forneceu pistas para um entendimento mais profundo de como a meiose poderia ter evoluído nos primeiros eucariontes a partir de mecanismos que já existiam nas formas ancestrais. Tais questões profundas, envolvendo Archaea (especialmente Asgard), nos levaram a um segundo projeto de pesquisa dentro do projeto inicial. Este projeto, que buscava fornecer indícios para o entendimento da evolução inicial da meiose em eucariontes em geral, aplicando uma abordagem semelhante àquela aplicada a Amoebozoa, mas desta vez a eucariontes em geral, para os quais dados moleculares estivessem disponíveis. Posteriormente, o projeto produziu dois trabalhos: um que

apresenta os resultados dos padrões de distribuição e evolução das proteínas da meiose em eucariontes, com o tratamento de alguns casos especiais de possíveis assexuados secundários [**Capítulo 4**, (Hofstatter and Lahr, 2019)]; e um segundo trabalho que apresenta os resultados das análises filogenéticas das famílias de proteínas que participam de processos de reparo de DNA do tipo '*mismatch-repair*', as quais fornecem subsídios para um melhor entendimento da origem e evolução deste sistema de reparo nos eucariotos (**Capítulo 5**). Em conjunto, ambas os trabalhos poderiam fornecer subsídios para se entender a origem da meiose e sexo em eucariontes e a própria evolução dos eucariontes (considerando-os como seres inerentemente sexuais).

Assim como no caso de Amoebozoa, a distribuição de proteínas específicas da meiose na árvore dos eucariotos é compatível com um ancestral plenamente sexuado (**Capítulo 4**, (Hofstatter and Lahr, 2019)). Além disso, todos os grandes grupos de eucariotos e 'linhagens órfãs', mesmo aquelas consideradas 'assexuadas', apresentam um distribuição de proteínas da meiose compatível com a ocorrência de ciclos sexuais em toda sua diversidade. No entanto, alguns pequenos grupos podem ter perdido a meiose e/ou sexo ao longo da evolução de forma secundária, como é o caso de *Malassezia*. Este gênero de fungos (Basidiomycota), que conta com genomas sequenciados para quatro espécies pelo menos, não apresenta praticamente nenhuma proteína da meiose e, paralelamente, não existem observações de processos sexuais ou meiose para o grupo. Um grupo próximo, *Ustilago*, é sabidamente sexual (O'Donnell and McLaughlin 1984; Banuett 1995). Estas evidências sugerem a perda secundária do sexo em *Malassezia*. Adicionalmente, um pequeno grupo de animais, os Bdelloidea, são considerados assexuados por diferentes abordagens e já foram referidos como "escândalos assexuados" na literatura (Judson and Normark 1996; Flot et al. 2013; Debortoli et al. 2016). Casos como esses (*Malassezia* e Bdelloidea) parecem ser raros. Há um forte tendência no sentido da manutenção de processos sexuais (ainda que teoricamente dispendiosos) em todos os grandes grupos. Inversamente, Glomeromycota, um grupo de fungos considerados totalmente assexuados, apresenta toda a maquinaria molecular da meiose sem nenhuma perda. Estes resultados indicam a ocorrência de ciclos sexuais canônicos no grupo (da mesma forma que em

Amoebozoa, como discutido previamente), mas prováveis artefatos de observação foram responsáveis pela descrição do grupo como uma linhagem assexuada. Glomeromycota é um grupo diverso e muito importante por suas associações ecológicas com plantas, com as quais formam extensas redes de micorrizas e a possibilidade de ciclos sexuais no grupo tem implicações para o entendimento de sua biologia. A presença da maquinaria de meiose já foi reportada previamente em *Glomus*, um membro do grupo (Halary et al. 2011). *Oenothera* (Angiospermae), *Cryptococcus* (Basiodiomycota), *Candida* (Ascomycota), Microsporidia e outros grupos tratados como assexuados foram também analisados. Resultados positivos para a presença da maquinaria sugerem a ocorrências de possíveis ciclos sexuais nestes grupos. Nucleomorfos (de Chlorarachniophyta e Cryptophyta) também foram analisados; a maquinaria da meiose desapareceu completamente neste caso.

O estudo da evolução das maquinarias de reparo de DNA na transição de Archaea para Eukarya (especialmente com a possibilidade de se utilizar dados de Asgardarchaea) oferece subsídios para se entender como o sexo e a meiose surgiram ao longo da evolução. Um melhor entendimento da evolução do sexo nos primeiros eucariotos nos permite entender a evolução dos próprios eucariotos. A maquinaria da meiose surgiu a partir de eventos de duplicação de componentes dos diversos processos de reparo de DNA já presentes em Archaea, entre eles recombinação homóloga e *mismatch-repair*. Em suma, toda a maquinaria de replicação e manutenção do genoma nuclear (quando apresenta homólogos rastreáveis) tem origem em Archaea, mesmo as famílias tipicamente bacterianas (mutS e mutL, componentes da maquinaria de *mismatch-repair*). A introdução de Asgardarchaea nos permitiu revisitar a história evolutiva do sistema de reparo de DNA *mismatch-repair* em eucariotos. Previamente considerada uma contribuição mitocondrial, é possível encontrar o sistema em Asgard e outros grupos de Archaea. Como o sistema é tipicamente bacteriano, é razoável se supor que tenha sido adquirido lateralmente de algum grupo de bactérias por Archaea e herdado posteriormente por Eukarya (seguido de diversos eventos de duplicação gênica). Mas as análises filogenéticas indicam três origens para o

sistema em eucariotos: uma ancestral obtido de Archaea e sujeito a diversos eventos de duplicação gênica e que realiza processos de reparo de DNA no núcleo e meiose; uma forma mitocondrial, a qual realiza reparo de DNA mitocondrial; uma forma cianobacterial, a qual realiza reparo de DNA nos cloroplastos. Alguns parálogos nucleares e as formas mitocondriais e dos cloroplastos foram perdidas em alguns grupos. É possível que outras famílias gênicas encontradas em eucariotos tenham sido adquiridas da mesma forma, especialmente quando ausentes em dados obtidos até o momento a partir de Archaea. A proposta de uma nova história evolutiva para o sistema mutS-mutL de *mistmatch-repair* em eucariotos (uma atualização de uma proposta anterior feita por outros autores (Lin, Nei, and Ma 2007)) foi apresentada em congresso de protozoologia evolucionária em Paphos, Chipre em meados de 2018 (**Capítulo 5**).

A própria abordagem bioinformática para o estudo do sexo e meiose em eucariotos diversos tem sido questionada. A principal crítica seria a de que a simples presença de proteínas de meiose em um dado genoma não seria demonstração suficiente da ocorrência de processos sexuais e meiose no grupo. Alguns revisores poderiam alegar que as proteínas específicas de meiose poderiam ter passado por processos de neofuncionalização e realizariam outras funções atualmente, sendo mantidas para este fim. Embora possível hipoteticamente, ainda não existe nenhuma demonstração de neofuncionalização de proteínas específicas da meiose que possa ser encontrada na literatura até o momento. Além do mais, mesmo que uma dessas proteínas tenha adquirido uma nova função, seria extremamente improvável que várias ou todas tenham adquirido novas funções ao mesmo tempo. Em caso recente, a abordagem bioinformática previu a existência de ciclos sexuais (meiose) em Choanoflagellata por meio da confirmação de proteínas específicas da meiose ocorrendo em genomas de espécies do grupo (Carr, Leadbeater, and Baldauf 2010); poucos anos mais tarde processos sexuais foram observados de forma inequívoca no grupo (Woznica et al. 2017). A presença de proteínas específicas da meiose foi demonstrada em Amoebozoa e outras linhagens de eucariotos neste estudo; o próximo passo seria a demonstração dos processos sexuais por meio de uma observação mais cuidadosa dos organismos considerados assexuados, cuja maquinaria da

meiose se encontre presente em seus genomas. Entre as possíveis abordagens estariam: observação sistemática de organismos em cultura, sujeitar os indivíduos a estímulos externos ou estresse (uma vez que a meiose e processos sexuais em geral estão normalmente associados a condições de estresse em diversos organismos), evitar culturas monoclonais, aplicação de técnicas de microscopia eletrônica para se visualizar modificações ultra-estruturais em cistos, contagem de cromossomos em diferentes estágios do ciclo de vida de um dado grupo por meio se cariótipos, medição de expressão gênica diferencial com foco em proteínas relacionadas à meiose etc.

Finalmente, propomos um texto de divulgação resumindo e esclarecendo os avanços no entendimento da meiose e sexo em eucariontes, sua evolução e sua importância central para a biologia do grupo (**Capítulo 6**). Neste texto, discutimos avanços no entendimento atual da evolução dos eucariontes, relações de parentesco entre os diferentes grupos e o papel do sexo eucariótico como algo inerente à própria vida eucariótica.

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Capítulo 2: Evolution of bacterial *recA* in eukaryotes explained by addition of genomic data of key microbial lineages. (*Publicado na Proceedings of the Royal Society: Series B, 2016*)

ABSTRACT Recombinase enzymes promote DNA repair by homologous recombination. The genes that encode them are ancestral to life, occurring in all known dominions: viruses, Eubacteria, Archaea, and Eukaryota. Bacterial recombinases are also present in viruses and eukaryotic groups (supergroups), presumably via ancestral events of lateral gene transfer. The eukaryotic *recA* genes have two distinct origins (mitochondrial and plastidial), whose acquisition by eukaryotes was possible via primary (bacteria-eukaryote) and/or secondary (eukaryote-eukaryote) endosymbiotic gene transfers (EGT). Here we present a comprehensive phylogenetic analysis of the *recA* genealogy, with substantially increased taxonomic sampling in the bacteria, viruses, eukaryotes, and a special focus on the key eukaryotic supergroup Amoebozoa, earlier represented only by *Dictyostelium*. We demonstrate that several major eukaryotic lineages have lost the bacterial recombinases (including Opisthokonta and Excavata) while others have retained them (Amoebozoa, Archaeplastida, and the SAR supergroups). When absent, the bacterial *recA* homologues may have been lost entirely (secondary loss of canonical mitochondria) or replaced by other eukaryotic recombinases. RecA proteins have a transit peptide for organellar import, where they act. The reconstruction of the RecA phylogeny with its EGT events presented here retells the intertwined evolutionary history of eukaryotes and bacteria, while further illuminating the events of endosymbiosis in eukaryotes by expanding the collection of widespread genes that may be useful for phylogenetic reconstruction.

Key words: Amoebozoa, DNA-repair, endosymbiotic gene transfer (EGT), mitochondria, recombinase, *recA*.

Abbreviations and gene/protein names

EGT – Endosymbiotic gene transfer;

pEGT – primary endosymbiotic gene transfer (from bacteria to eukaryote upon primary endosymbiosis);

sEGT – secondary endosymbiotic gene transfer (between eukaryotes upon secondary endosymbiosis);

recA – eubacterial recombinase homologue gene;

recAmt – eubacterial recombinase homologue gene of eukaryotes (mitochondrial origin);

recAp – eubacterial recombinase homologue gene of eukaryotes (plastidal origin);

UsvX – Viral recombinase homologue genes;

RADA and RADB – Archaeal recombinase homologue genes;

RAD51X – eukaryotic recombinase homologue genes;

DMC1 – eukaryotic recombinase associated with meiotic recombination;

SAR super-group – a diverse eukaryotic assemblage formed by Stramenopiles, Alveolata and Rhizaria.

Introduction

Recombinases are a family of enzymes responsible for DNA repair via homologous recombination (Hiom 2012). These proteins are widely common in genomes of diverse organisms, including bacteria, Archaea, eukaryotes and even viruses (Chintapalli et al. 2013). The most relevant homologous groups are referred to as RecA in bacteria, UvsX in viruses, RADA and RADB in Archaea and RAD51X in eukaryotes, collectively addressed as *recA* superfamily (Wu et al. 2011). Eukaryotes in general present a wide range of recombinases (RAD51A, DMC1, RAD51B, RAD51C, etc.), which arose by means of several duplication events, most of them probably occurring before the last eukaryotic common ancestor (Lin et al. 2006a). Due to its near universality, the recA superfamily has received significant attention and has been implicated in recent attempts to discover of new domains of life (Wu et al. 2011), as a protein model to research metagenomic data from oceans (Venter et al. 2004), and as a model for evolution by gene duplication and endosymbiotic gene tranfer (Chintapalli et al. 2013; Lin et al. 2006b).

The bacterial form of the *recA* gene is present in eukaryotic genomes because they were acquired via endosymbiotic gene transfer acquired in conjunction with the uptake of the mitochondrion and plastid (Lin et al. 2006b). Mitochondria are descendants of bacterial endosymbionts likely acquired before the last eukaryotic common ancestors, plastids being acquired later in evolution (Martin William F., Garg Sriram, and Zimorski Verena 2015).

During the processes of both primary endosymbioses, extensive lateral gene transfer (EGT) took place: from the bacterial genomes to the nuclear genome (Martin 2003; Adams and Palmer 2003). The resulting organelles have extremely reduced genomes, coding only a few proteins, rRNAs and tRNAs, probably because these entities cannot be easily imported by the organelle if synthesized outside the organellar space (Adams and Palmer 2003). As a result of EGT, eukaryotic RecA proteins are encoded in the nuclear genome, yet active inside organelles. These proteins are imported through the organellar membrane, after recognition by an N-terminus signaling transit

peptide, which is cleaved in the organelle yielding the active protein (Khazi, Edmondson, and Nielsen 2003; Hasegawa et al. 2004; Rowan, Oldenburg, and Bendich 2010).

Bacterial *recA* is widespread in eukaryotic genomes, but some lineages have secondarily lost the gene. An example is the Opisthokonta, since neither Metazoa nor Fungi have the genes (Lin et al. 2006b). Homologous recombination in the mitochondrial genome is carried out in humans by RAD51-group proteins (Sage and Knight 2013), which probably replaced the eubacterial homologue RecA.

Here we present a comprehensive phylogenetic reconstruction of the *recA* gene genealogy, including 225 taxa among bacteria, eukaryotes, and viruses. We show that, in the Amoebozoa, a sister-group to Opisthokonta, bacterial *recAmt* is ancestrally present in the nuclear genomes, in the same way as in *Thecamonas trahens*, greens plants and several SAR lineages, like Oomycetes, *Blastocystis*, *Cafeteria* and other groups. The most parsimonious interpretation of these data indicate that *recA* is ancestral in eukaryotes, being lost in a few lineages.

Material and Methods

Amoebozoan Sequences

Echinosteliopsis oligospora was isolated from dead leaf litter collected from Sam D. Hamilton Noxubee National Wildlife Refuge. *Schizoplasmodiopsis vulgaris* was isolated from dead leaf litter collected from North Vietnam. Other cultures were obtained from Culture Collection of Algae and Protozoa (CCAP) (Scotland, UK) or American Type Culture (ATCC) (Manassas, VA).

For *Echinosteliopsis oligospora*, *Clastostelium recurvatum*, *Cavostelium apophysatum*, *Schizoplasmodiopsis vulgaris*, *Crytodiffugia operculata*, *Vermamoeba vermiformis*, and *Echinoamoeba exudans,* cells were grown on weak malt yeast (wMY) (0.002 g malt extract, 0.002 g yeast extract, 0.75 g K_2HP0_4, 1.0 L deionized [DI] H_20) agar plate and *Rhizamoeba saxonica* was

grown on sterile artificial seawater wMY agar plate with various accompanying bacteria in culture. *Arcella vulgaris* was grown on sterile fresh water supplied with cereal grass media and accompanying bacteria. Once amoeboid cells reached the dense culture stage, 2-3ml of wMY liquid was poured over the agar plate. Subsequently cells were scrapped off and collected in sterile 15 ml falcon tube. The cells were centrifuged at 4000 x g at 4 °C for 5 min in centrifuge to pellet the cells. The pellet, which contained amoeboid cells, was subjected to cell lysis for RNA isolation. Total RNA was isolated using TRIzol reagent (Sigma-Aldrich, St Louis MO) according to the manufacturer's protocol (TRI Reagent RNA isolation reagent). To assess the quality of total RNA, 5 µl total RNA was assessed through electrophoresis in 1.8x Tris-Borate-EDTA (TBE) agarose gel (Bioexpress, Kaysville, UT). The quantity of total RNA was diluted (1:200) and measured with fluorometry using the QubiT® (Life Technologies, Carlsbad, CA) high sensitivity RNA assays. The total RNA was further cleaned through ethanol precipitation. Total RNA with 0.25 M NaCl was spun down at 14,000 x g for 20 min at 4 ºC. The final pellet was washed with freshly made 75% ethyl alcohol. Double stranded complementary DNA (dscDNA) synthesis were performed from 0.25 µg to 1.5 µg of total RNA using NEBNext® Poly(A) mRNA Magnetic Isolation Module followed by NEBNext® Ultra RNA kit (New England Biolab (NEB), Ipswich, MA) according to the manufacturer's protocol.

Amoeba proteus culture was obtained from Carolina Biological Supply. Because *Amoeba proteus* grows in association with a eukaryotic flagellate as a food source, *Chilomonas* sp., a single cell was washed free of any associated eukaryotes by serial washes with spring water and starving the individual cell overnight in sterile spring water. Similarly, *Difflugia* USP was isolated from nature at the University of São Paulo campus, and single cells were serially washed with sterile water and the individuals cells were starved overnight. Subsequently, the cleaned cell was picked using a micropipette into a 1.2µL drop of sterile spring water. The reaction tube was then subjected to six freeze-thaw cycles in -80 °C isopropanol and ~25 °C DI H_2O respectively. Total RNA was isolated and dscDNA was obtained using a modified version of Smart-Seq 2 [13].The dscDNA was

sheared using a Covaris S220 with the following settings: peak power 175 W, duty factor 10%, cycles per burst 200, mode frequency sweeping, and duration 30 sec. The sequencing library was then created from the sheared cDNA using NEBNext® Ultra DNA kit (New England Biolab (NEB), Ipswich, MA) according to the manufacturer's protocol.

Total RNA was extracted and converted to dscDNA from *Ceratiomyxa fruticulosa* using a modified version of Smart-seq2 (Picelli et al. 2014). Approximately 200 spores were collected from a fresh fructification using a .008" diameter platinum needle (Surepure Chemetals, Florham Park, NJ). Spores were then transferred into a PCR tube containing 1.2µl liquid wMY (.002 g yeast extract, .002 g malt extract, 0.75 g K_2PO_4/L ddH_2O) medium. After a 2.5 hr incubation period at room temperature (~21 °C) cells were lysed by the addition of the Smart-seq2 cell lysis buffer and 6 rounds of a freeze thaw cycle using -80 °C isopropanol (Picelli et al. 2014). The resulting dscDNA was prepared for sequencing using a NexteraXT DNA Library Prep kit (Illumina®, San Diego, CA).

Sequencing libraries was subjected to quality control (QC) using a combination of methods. The sequencing library concentrations were obtained with fluorometry using QubiT® high sensitivity dsDNA assays. First, the sequencing libraries were diluted (1:200) and then amplified using universal Illumina primers to estimate library sizes using electrophoresis in 1.8x TBE agarose gel. PCR reactions were composed of GoTaq® Green Master Mix (Promega, Madison, WI), IlluminaF (5' – AAT GAT ACG GCG ACC AC) at 10 µM and IlluminaR (3' – CAA GCA GAA GAC GGC AT) at 10µM (Oligonucleotide sequences © 2016 Illumina, Inc. All rights reserved), DNA template of adequate concentration and nuclease free water run under the following parameters: 5 min of initial denaturation at 94 ºC, followed by 20 cycles of 30 sec of denaturation at 94 ºC, 25 sec of annealing at 60 ºC, and extension of 1 min at 72 ºC. Library molarities were calculated using quantitative polymerase chain reaction (qPCR) of KAPA library Quant kit for Illumina (KAPA Biosystems, Boston MA) according to the manufacturer's protocol. Additionally, the average molecular weight (MW) of each library is calculated by MW=(Average Library Size in

basepairs * 607.4+157.9). The nanomolarity of each library is calculated by nM=(MW/Qubit Concentration (in ng/ul)*1,000,000). Libraries molarities were subsequently diluted in 0.1x Tris-HCl EDTA pH 8.0 (TE) to the lowest molarity concentration in the set of libraries to be pooled together in equal volumes. All libraries were sequenced using either the MiSeq or HiSeq 2000 platforms.

We passed the assembled transcriptome data through a series of quality control steps to remove rRNA and bacterial contaminants (Grant et al. 2012). The obtained reads were assembled using Trinity RNA-Seq de novo assembly Trinity software (Grabherr et al. 2011). TransDecoder (version number: v2.0.1; https://transdecoder.github.io/) was used to predict coding peptide sequences from the baseline transcriptome contig sequences. Resulting amino-acid sequences of 65 Amoebozoan representatives were concatenated to a single database for further analysis.

Dictyostelium discoideum RecAmt peptide sequence (GeneBank FAA00018) was used as the query in searches with tBlastn algorithm (Altschul et al. 1990) and an arbitrary expected value threshold of e-40 maximum was established. Sequences were deposited in the GenBank (Supplementary Material S3).

Sequences for diverse eukaryotes

The *D. discoideum* RecAmt protein was used as a query for searches in GenBank for similar proteins from other groups of organisms by tBlastn and Blastp algorithms (Altschul et al. 1990) with arbitrary e-value threshold of maximum e-40. The bacterial RecA representatives were chosen with a phylogenetic strategy. Big bacterial lineages were targeted in the construction of the data sets. We adopted the phylogenetic proposal of bacterial relationships as in Battistuzzi and Hedges (Battistuzzi and Hedges 2009). Another set of genes was obtained from MMETSP project (Marine Microbial Eukaryotic Transcriptome Sequencing Project) (Keeling et al. 2014). The translated databases were screened using *hmmsearch* tool of HMMER package (https://hmmer.org). Best hits

were captured from databases by FAST program (Lawrence et al. 2015). All sequences resulting from all different sources were gathered in a single matrix for further phylogenetic reconstruction.

Experimental Design and Phylogenetic reconstruction

The goal of this survey to determine the pattern of presence/absence of *recA* in major eukaryotic lineages, as well as clarify events of lateral gene transfer. While a number of methods have been proposed for efficient experimental design in phylogenetic reconstructions, there are no canonically accepted methods to reconstruct the deep history of a singles gene family. Some of the proposed approaches are restricted to nucleotide sequences (Goldman Nick 1998; Yang 1998) and would not be directly applicable for deep reconstructions where amino-acid sequences are used. Others might be employed when analyzing protein sequences, but more adequate for comparative analysis between two or more different candidate proteins (Townsend 2007; López-Giráldez and Townsend 2011). In order to better resolve the splits on the tree, we tried to sample the most diverse dataset as possible to avoid long branches and to ass taxa that would connect near internal nodes, following previously recommended practices (Geuten et al. 2007; Susko and Roger 2012).

Several rounds of alignments for RecA were constructed in Seaview (Galtier, Gouy, and Gautier 1996; Gouy, Guindon, and Gascuel 2010) with alignment algorithm MAFFT using the L-INS-I setting (Katoh, Asimenos, and Toh 2009). The resulting matrix had their least probable homologous sites and unpaired site removed by Gblocks algorithm (Castresana 2000) and fine adjusted manually. This strategy was followed by PhyML (Guindon et al. 2010) analysis using maximum likelihood as the optimality criterion in order to assess the quality of the sequences and visual inspections were done in order to reveal contaminants. For the final tree, a MAFFT alignment was used to construct a HMM-Profile with *hmmbuild* algorithm of HMMER and the whole set of homologues sequences was aligned with *hmmalign* algorithm of HMMER package (https://hmmer.org). The resulting matrix had their least probable homologous sites and unpaired

sites removed by Gblocks algorithm (Castresana 2000) and fine adjusted manually (only sites with probability of homology p≥0.8 were included). The resulting matrix of aligned and trimmed sequences was used as input for RAxML software (Stamatakis 2006; Stamatakis, Hoover, and Rougemont 2008), which performed a Maximum likelihood phylogenetic analysis with 120 independent initial searches using the PROTGAMMALGI molecular evolution model. Independently, to establish support, 1200 non-parametric bootstraps pseudoreplicates were performed using the PROTGAMMALGI model. The best fit model (LG+G4+I) was determined by online ProtTest software (Abascal, Zardoya, and Posada 2005; Darriba et al. 2011). Final matrix is available in Supplementary Material S4.

A bayesian analysis was performed with the same matrix subjected to PhyloBayes software (Lartillot, Lepage, and Blanquart 2009). For the analysis, five independent chains were run for 20000 cycles using default priors, CAT model and LG substitution model. A burn-in of 2000 cycles (10%) was applied after determining that likelihood values had stabilized. A maxdiff parameter <0.3 was attained as recommended by Phylobayes manual, which indicates that topologies on the five runs had converged acceptably to a single answer.

Results and discussion

The eubacterial *recA* type gene has been transferred to eukaryotic genomes in at least two occasions

Recombinases are a highly conserved group of enzymes. The *recA* genes are characteristic of Eubacteria; Archaeal *RADA* groups with eukaryotic *RAD51A* and meiosis specific *DMC1*, forming a well defined group, *RADα*; finally, Archaeal *RADB* groups with eukaryotic *RAD51B*, *RAD51C* and others, forming the *RADβ* group of genes (Chintapalli et al. 2013; Lin et al. 2006b).

Thus the most parsimonious interpretation for the presence of eubacterial *recA* in eukaryotes is that this event was a lateral gene transfer.

We performed a screening of both novel and available transcriptomes of microbial eukaryotes searching for previously unidentified *recA* in a wide range of deep level lineages. We have combined these into a broad bacterial taxonomic sampling and reconstructed a comprehensive gene genealogy of *recA*, upon which the general history of endosymbiotic gene transfer can be investigated (**Figure 1**; Supplementary Materials Figures S1, S2). The phylogenetic reconstruction reveals two independent primary endosymbiotic gene transfers (pEGT) from Eubacteria into the eukaryotic nucleus, one related to mitochondrial origin and the other to plastidial origin. The same topology still reveals the occurrence of secondary endosymbiotic gene transfers (sEGT), plastid-type bacterial genes being transferred from the red algal secondary endosymbiont to a lineage of Stramenopiles and from green algae to a group of dinoflagellates (**Figure 1**).

The tree obtained recovers several well-established deep relationships within Bacteria, plants, and Amoebozoa. The possibility of recovering such deep relationships, the universality of the recombinases among organisms and the abundance of available sequences suggest that the *recA* superfamily might be employed in helping resolving deep branching relationships, also along other genetic markers.

Our Bayesian and maximum likelihood (ML) analyses converged on most of the topologies obtained, with small differences observed: dinoflagellates are associated to Chlorophyta in ML and nest within Chlorophyta in Bayesian analysis; the glaucophyte *Gloeochaete wittrockiana* is a sister group to Cyanobacteria in ML analysis and nested within Cyanobacteria in the Bayesian (**Figure 1**. Supplementary Material Figure S2).

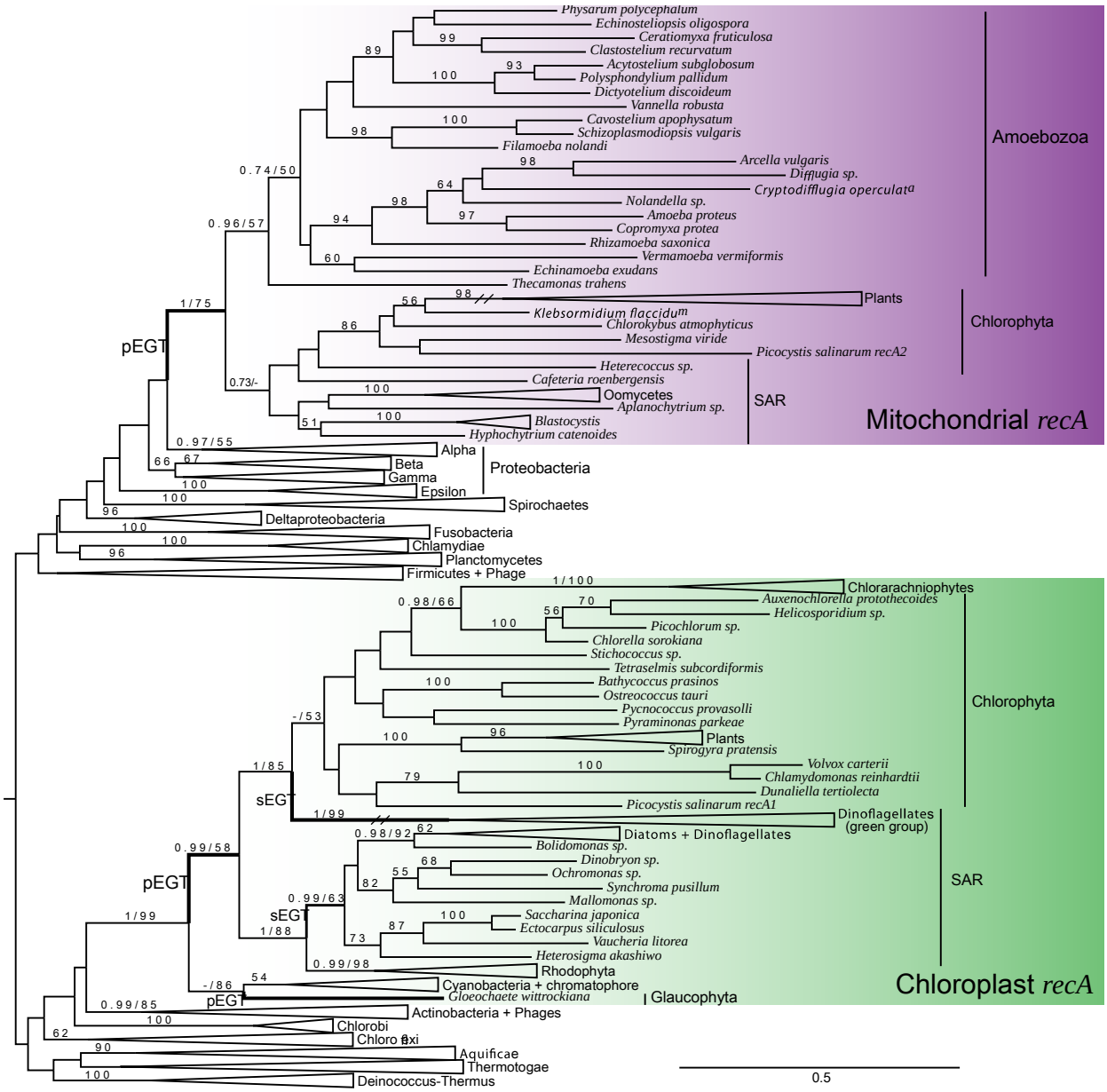

Figure 1. Phylogenetic tree of the bacterial homologue *recA*. Major group branches are collapsed. Eukaryotes received *recA* genes by two independent events of primary endosymbiotic gene transfers, with subsequent events of secondary endosymbiotic gene transfer. Full ML tree available as Supplementary Material Figure S1 and Tree S1; Full Bayesian tree available as Supplementary Material Figure S2 and Tree S2. Mitochondrial plant clade and dinoflagellate branches are represented as half-length.

The mitochondrial *recA* type (*recAmt*) was present in the genome of the last eukaryotic common ancestor, and has been lost in several lineages.

Although only one protein was used, canonical relationships were recovered, even if with low support in some cases. The Alphaproteobacteria was recovered in all reconstructions as sister-group to a monophyletic mitochondrial clade, which is the currently accepted relationship (Gray, Burger, and Lang 1999). Amoebozoa, Chlorophyta and non-photosynthetic SAR-supergroup members share a mitochondrial *recA* gene (bootstrap and bayesian support of 75/1, **Figure 1)**.

Through our deep sampling of genomic-level data of Amoebozoa, we find that *recAmt* is pervasive in the lineage (**Figure 1**). The presence of the gene was already assessed and documented in the model organism *D. discoideum* (Hasegawa et al. 2004; Eichinger et al. 2005). However, here we demonstrate that *Dictyostelium discoideum* is not an isolated amoebozoan in the *recA* tree as previously considered (Lin et al. 2006b; Chintapalli et al. 2013). On the contrary, it is only one instance within the entire Amoebozoa supergroup (**Figure 1**). The class of genes is robustly present in Amoebozoa, even though absent in a few lineages. For instance, *Entamoeba* likely lost *recAmt* due to the atrophy of mitochondrial organelles into anaerobic mitosomes (Müller et al. 2012) and in *Acanthamoeba* we infer that the gene was replaced by an alternative eukaryotic RAD51 homologue, as in Opisthokonta. Taken as a whole, our sampling demonstrates that *recAmt* is present in Tubulinea, Arcellinida, Flabellinida, Dictyosteliida, Myxogastria and other groups, which make up for the majority of the Amoebozoa clade (Lahr, Grant, and Katz 2013). Thus, the most parsimonious interpretation is that *recAmt* was present in the last common ancestor of the Amoebozoa.

Chintapalli *et al*. (2013) suggested a hypothetical transfer of the *recA* to Amoebozoa from Cyanobacteria. Our results show otherwise, the Amoebozoan *recA* are derived from Alphaproteobacteria, i.e., from mitochondria (**Figure 1**). Evidence supporting our hypothesis includes: i.) proteins are targeted to mitochondria, where they are active, and ii.) Amoebozoan RecA proteins group with Oomycetes+plant RecA in a well-supported, mitochondrially derived clade.

Another proposition by Chintapalli *et al*. is an EGT from brown, red algae and green plants *recA* to 'plants'. In fact, the EGT flux is the different: a gene influx from red algae to stramenopiles, brown algae and relatives (bootstrap and bayesian support of 88/1, **Figure 1**). The phylogenetic reconstruction provided by them lacks resolution, being unable to differentiate between *recAmt* and *recAp* (discussed below) which were available in their dataset. The mis-representation of relationships is a result of poor taxon-sampling as well as reconstruction of historical relationships using an optimality criterion that is widely-known to be prone to topology errors (i.e., the Neighbor Joining methodology, see Farris et al. (1996) for a discussion).

Several other major eukaryotic groups seem to have secondarily lost their bacterial *recAmt* homologues, as it can be seen in Opisthokonta, Excavata and Alveolata (**Figure 2**). Opisthokonta is part of a larger group, Obazoa, that includes anaerobic amoeboflagellates (Breviatea) and aerobic flagellates (Apusozoa) (Brown et al. 2013a). In our analyses, the genome of *Thecamonas trahens* (Apusozoa) has a *recAmt* that groups with Amoebozoa with moderate support (57/0.96, **Figure 1**). However, we were not able to recover *recAmt* in the transcriptome of *Pygsuia biforma* (Breviatea), which is likely due to its loss in the evolution of anaerobiosis within the breviates (Brown et al. 2013b). Opisthokonta, along with other obazoans are the sister-group to Amoebozoa and lack bacterial recombinases entirely. The loss probably occurred in the ancestral opisthokont, as neither Nucletmycea (Fungi + protistan relatives) nor Holozoa (Metazoa + protistan relatives) present any *recA* genes. Presumably, eukaryotic recombinases replaced the bacterial ones. For instance, RAD51C protein is imported by mitochondria and participates in mitochondrial DNA repair in *Homo sapiens* (Sage and Knight 2013). We performed extensive searches for *recA* among animals in GenBank returning only a handful of hits scattered through Metazoa. When analyzed in our phylogenetic framework, these appear to be contaminants in non-curated databases.

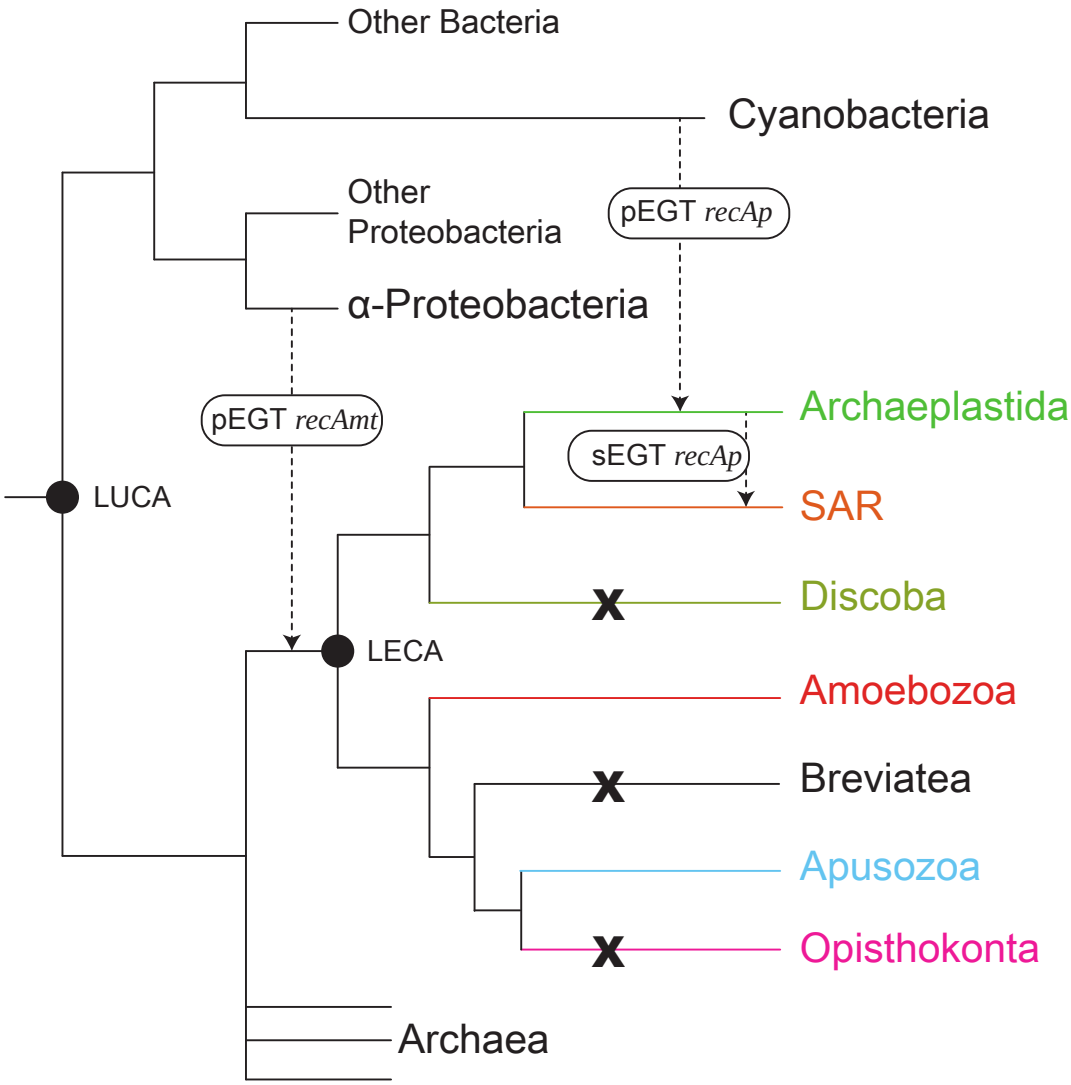

Figure 2. Three-domain depiction of the tree of life, with proposed acquisition and secondary loss events of bacterial *recA* homologues by eukaryotic groups. Major branches are based in Williams *et al*. (Williams et al. 2013) and eukaryotic relationships are based in Derelle *et al.* (2015).

Plants present a large group with retained *recAmt* genes. The green plants not only kept the mitochondrial recombinases, but also went through several rounds of gene duplication after EGT and diversification of eukaryotes (specially in the Angiosperms) (Supplement Figure S1). The

evolutionary history of land plants is marked by events of polyploidization by whole-genome duplication. One event of polyploidization has probably occurred in the ancestral of the angiosperms, prior to divergence of monocots and eudicots (De Bodt, Maere, and Van de Peer 2005; Jiao et al. 2011) and other events followed after the split of these lineages (Jiao et al. 2011). These facts would explain the pattern observed here, which is congruent with genome duplication events in plants. Presumably this substantial expansion correlates with the gains of new functions or maintenance of the original function with differential expression by tissue or life-cycle specificity (Miller-Messmer et al. 2012). Duplication of *recAmt* in Angiosperms may be an effect of genome-wide duplications in this lineage. The sampled species (*Zea mays, Oryza sativa, Arabidopsis thaliana, Populus trichocarpa* and *Ricinus comunis*) present 2-4 duplications of *recAmt* homologs in their genomes, at least one happening before the monocots/eudicots split, followed by subsequent lineage specific duplication events (Supplement Figure S1).

Heterotrophic stramenopiles show robust evidence for the presence of nuclear encoded *recAmt*. Their bacterial recombinases are clearly mitochondrial derived (**Figure 1**). Oomycetes and other several Stramenopiles lineages, like *Blastocystis* and *Hyphochytrium*, the flagellated bicoecid *Cafeteria roenbergensis*, the labyrinthulid *Aplanochytrium*, all of them SAR members, present mitochondrial originated *recA* genes. The photosynthesizing SAR lineages seem to have lost the *recAmt*, which was probably replaced by the plastid form (*recAp*).

A second paralog, the plastid *recA* type (*recAp*), was obtained in the endosymbiotic plastid event.

Further screening of the phylogeny reveals a second eukaryotic group of eukaryotic *recA* (**Figure 1**). These are the plastid-related *recAp*. Again, a highly supported clade emerges with a rich diversity of photosynthesizing organisms, that is sister to the Cyanobacterial *recA* (bootstrap and bayesian support 99/1, **Figure 1**). The grouping of green plants, dinoflagellates, red algae, brown

algae and diatoms indicates that these groups inherited RecA vertically from the single endosymbiotic origin of all known plastids, as earlier suggested (Rodríguez-Ezpeleta et al. 2005; Reyes-Prieto, Weber, and Bhattacharya 2007). However, the grouping of the glaucophyte *Gloeochaete wittrockiana* with Cyanobacteria, either as a sister-group or even nested within them, may be interpreted either as lack of phylogenetic resolution in the current reconstruction, or as an independent acquisition of this particular gene in the glaucophytes. Another known exception is the chromatophore of the rhizarian *Paulinella chromatophora* (Supplement Figure S1), which represents clearly an independent primary endosymbiotic event (Marin, Nowack, and Melkonian 2005; Nowack, Melkonian, and Glöckner 2008; Reyes-Prieto et al. 2010), in which the *recAp* gene has not been transferred to the nucleus.

The close proximity between Rhodophyta and the photosynthesizing lineages of Stramenopiles (SAR) (bootstrap and bayesian support 88/1, **Figure 1**) reinforces the secondary endosymbiosis hypothesis and more, demonstrates a secondary EGT (sEGT) as well (**Figure 2**), a eukaryote-eukaryote transfer of a bacterially originated gene. As it seems, the photosynthesizing Stramenopiles (*Bolidomonas*, Diatoms, Phaeophyceae, Xanthophyceae, and others) present functional forms of red algal derived *recAp,* putatively from a secondary endosymbiotic event. Noteworthy to note is the absence of *recAmt* in the red algae and in the lineages that acquired the *recAp* from them. The plastidal form seems to have replaced the mitochondrial one, potentially playing a role in both organelles simultaneously. This is possible by means of a dual target system, i.e., the same protein may be addressed to both organelles (Mackenzie 2005; Millar, Whelan, and Small 2006).

Chlorophyta maintained their *recAp*, but differently from *recAmt*, without further replications (**Figure 1**). This group, specially angiosperms, is the only one exhibiting both *recAmt* and *recAp* simultaneously, although either form may be lost in some lineages.

Dinoflagellates also present a *recAp,* but are divided in two groups: a diatom associated and a chlorophyte associated, with long branches in the latter. Presumably, these longer branches are

due to high evolutionary rates in dinoflagellates (Pochon, Putnam, and Gates 2014). The highly supported association between dinoflagellates and chlorophytes (bootstrap and bayesian support 85/1, **Figure 1**) does not support the red algal origin for a big part of dinoflagellate plastids. A parallel can be traced with euglenids: both groups present 3-layered chloroplasts, probably derived from secondary endosymbiotic events, involving chlorophytes in the case of euglenids (Archibald 2015). There is also a rhizarian group nested among unicellular chlorophytes, the chlorarachniophytes (**Figure 1**). These organisms clearly acquired their chloroplasts from the green group and even maintained a nucleomorph of the endosymbiont (Archibald 2015).

Multiple gene transfer of *recA* have occurred in the history of life by EGT, including multiple instances of bacteria to eukaryotic transfers and other instances of bacteria to virus transfers.

Amoebozoan, plants and Oomycetes *recA* proteins are encoded with a signaling sequence before the active sites of the enzyme. This sequence is crucial for the import mechanism into organelles and is not found in bacterial homologues. Another striking difference is the presence of several introns in the *recA* found in eukaryotes, all of which must have been acquired after the EGT event as the bacterial forms are devoid of any introns (**Figure 3**). Presumably, the organelle importing system must have been fully functional in the last eukaryotic common ancestor (Gray, Burger, and Lang 1999). Most of transferred genes are vital to the organelle and an importing system is a *sine qua non conditio* for successful EGT (Timmis et al. 2004). Once an importing system is fully functional, the organelle copy of the transferred gene may be lost by mutational decay.

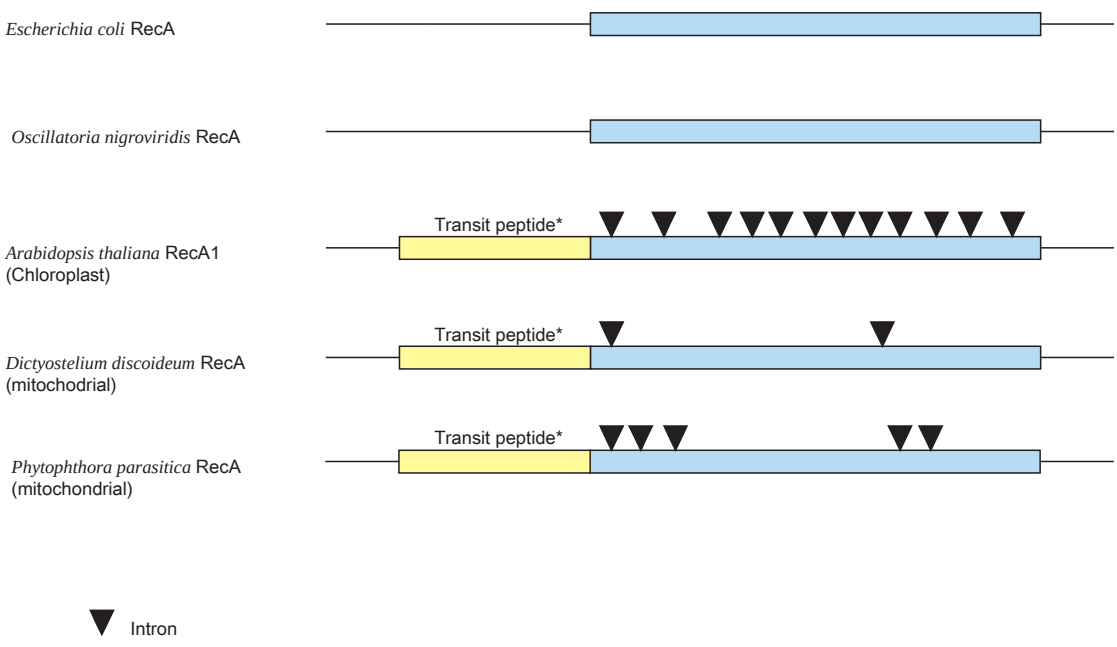

Figure 3. Comparison between sequence of original *recA* present in bacteria and their homologues transferred to eukaryotes. Eukaryotic forms have a ~50aa transit peptide in the N-terminal portion of the gene product, which is trimmed after import into the organelle; several introns were acquired after transfer to eukaryotes. Black triangles represent intron locations.

As a consequence, no organelle genome, from the ~7400 surveyed by us, keeps its original *recA*. This complete lack of recombinases in organelles suggests that EGT occurred only once in the ancestral of all eukaryotes for the *recAmt* and more than once for plastid homologues (at least a primary and a secondary EGT). Once established, the import mechanism paved a way to subsequent endosymbioses, most notably involving acquisition of photosynthesis by several groups. Additionally, it is possible also to verify the lateral gene transfer of *recA* from bacteria to some of their phage viruses, in this case *Mycobacterium* and *Bacillus* phages (Supplementary Material Figure S1). As viruses are intracellular parasites, they interact very intimately with their hosts and some genes are prone to be transferred and may be fixed in the viral genomes.

Lastly, *recA* is present in the genome of the chromatophore, the photosynthetic organelle, of *Paulinella chromatophora*. This endosymbiosis between a cyanobacterium and an amoeboid rhizarian occurred independently from other primary endosymbioses (Marin, Nowack, and Melkonian 2005). The same trend of EGT is observable in this case, as only about 26% of its genes remain in the organelle (Nowack, Melkonian, and Glöckner 2008), but the *recA* gene has not been transferred to the nucleus yet.

Supplementary material

Supplementary Material Figure S1 – Uncollapsed Maximum Likelihood reconstruction of *recA* genes surveyed.

Supplementary Material TreeS1 – Same tree as Figure S1, text format.

Supplementary Material Figure S2 – Uncollapsed Bayesian reconstruction of *recA* genes surveyed.

Supplementary Material Tree S2 – Same tree as S2, text format.

Supplementary Material S3 – Table listing all newly deposited GenBank accession numbers, as well as previously published sequences used in the study.

Supplementary Material S4 – Alignment matrix used for reconstructions.

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Capítulo 3: Comparative genomics supports sex and meiosis in diverse Amoebozoan (*Publicado na Genome and Biology Evolution, 2018*).

Abstract

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

Keywords: amoebae, Amoebozoa, meiosis, mixis, sex

Introduction

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

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

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

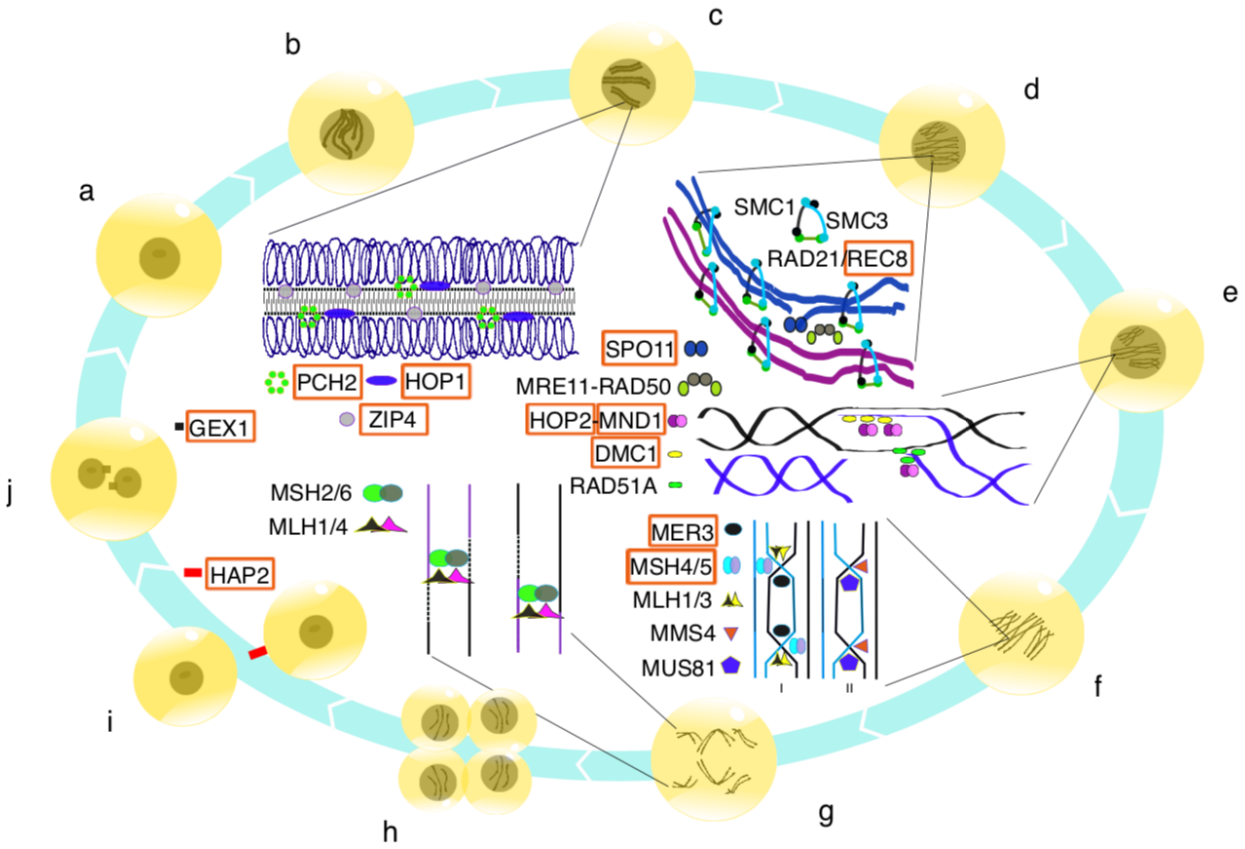

Figure 1: Cell cycle highlighting main processes happening upon meiosis and plasmogamy/karyogamy: **a**. duplication of DNA during interphase (synthesis phase); **b**. meiosis-specific bouquet formation, promoted by BQT1 and BQT2 in *Schizosaccharomyces pombe* (Scherthan 2001; Chikashige et al. 2006); **c**. the assembly of synaptonemal complex (Zickler and Kleckner 1999; Fraune et al. 2012) involves many meiosis-specific structural proteins, some of them high conserved, PHC2 and HOP1 (Anuradha and Muniyappa 2004; Farmer et al. 2012) and ZMM complex protein ZIP4/SPO22 (Lynn, Soucek, and Börner 2007); **d**. sister chromatids are kept close together by cohesin complexes, composed by SMC1, SMC3, RAD21 or it meiotic paralogue REC8 (Uhlmann, Lottspeich, and Nasmyth 1999; Haering and Nasmyth 2003; Revenkova and Jessberger 2005; Peters, Tedeschi, and Schmitz 2008), which keep together sister chromatids until anaphase II when they are finally cleaved by separases (Nasmyth 2005); double-strand breaks are introduced onto DNA by SPO11 and TopoVIB-like proteins working as dimers or tetramers (Malik et al. 2007; Keeney 2008; Robert et al. 2016); before the activation of the homologous recombination machinery SPO11 is removed and DNA strands are processed (resection) by MRN complex (MRE11, RAD50 and NBS1) resulting in the single 3' strand used for invasion of the homologous chromosome, where it is extended by a DNA polymerase forming a D-loop (Borde 2007; Williams, Williams, and Tainer 2007; Berchowitz and Copenhaver 2010); **e.** homologous recombination mediated by RAD51A and it meiotic paralogue DMC1, HOP2 and MND1 (Petukhova et al. 2005; Lin et al. 2006); **f.** the

chiasmata contain double-Holiday junctions, which can be resolved in order to promote cross-overs by two main pathways: the main interference bearing pathway I, which involves MER3, MSH4-5, MLH1-3, EXO1, and SGS1 (Wang, Kleckner, and Hunter 1999; Nakagawa and Kolodner 2002; Snowden et al. 2004; Zakharyevich et al. 2012) and pathway II, which involves MUS81 and MMS4 (de los Santos et al. 2003; Higgins et al. 2008); the correct assortment of chromosomes depends on the occurrence of cross-overs (Chakraborty et al. 2017); both pathways work at the same time, but pathway I is responsible for most cross-overs in *Saccharomyces* and *Arabidopsis*; however, some organisms relay completely on pathway II for cross-over resolution (*Schizosaccharomyces pombe* and *Tetrahymena thermophila*) (de los Santos et al. 2003; Higgins et al. 2008; Lukaszewicz, Howard-Till, and Loidl 2013); **g.** the mismatches formed are corrected by the nuclear mismatch repair system composed basically by MSH2-6 and MLH1-PMS1 (in yeast) (Wang, Kleckner, and Hunter 1999); **h.** canonical meiosis results in four haploid cells; **i.** Gametes of a single mating type express the transmembrane HAP2, that facilitates cell membrane fusion (Wong and Johnson 2010; Liu et al. 2015); **j.** GEX1 is nuclear membrane protein involved in karyogamy (Ning et al. 2013). Proteins considered to be meiosis-specific are highlighted with a red box.

Traditionally considered asexuals, amoeboid organisms are scattered in several eukaryotic lineages, e.g., Rhizaria, Excavata, Stramenopiles, Opisthokonta, and Amoebozoa (Lahr et al. 2011). Among them, Amoebozoa is a very ancient (more than 750 Ma old (Porter and Knoll 2000) monophyletic assemblage of diverse amoebae and amoeboflagellates (see (Kang et al. 2017)). Some important human pathogens such as *Entamoeba histolytica* and *Acanthamoeba castellanii* as well as the model organism *D. discoideum* are amoebozoans. Phylogenetically, the lineage is closer to Obazoa (the group that includes animals and fungi) than to any other eukaryotic super-group (Brown et al. 2013). Due to the lack or rarity of observable sexual processes, most amoebozoans are considered ‘asexuals’. The emended description of Amoebozoa does not mention sex or meiosis in the group (T. Cavalier-Smith 1998). Most literature on Amoebozoa (or some of its groups) refers to them as “presumably asexual”, “sexual or asexual” (sexual referring to Myxogastria and Dictyosteliida) or simply does not mention sexual processes at all (A. Smirnov et al. 2005; A. V. Smirnov et al. 2011; Adl et al. 2012; Thomas Cavalier-Smith et al. 2015). Kang and colleagues (Kang et al. 2017) point out a handful lineages out of the entire amoebozoan diversity as sexual

(three members of Tubulinea, Myxogastria, Dictyostelia, and only *Sappinia* inside Discosea) basically depicting the whole diversity of Discosea as asexual. However, Myxogastria and Dictyostelia represent exceptions among amoebozoan lineages as their life cycles are well known and their sexual processes (including details of syngamy and meiosis) have been described, including occurrence of SC, a meiosis-specific structure, in cysts or spores of *Physarum*, *Dictyostelium*, *Echinostelium*, *Ceratiomyxa* and *Microglomus* (Aldrich 1967; Furtado and Olive 1971; Haskins et al. 1971; Erdos et al. 1972; Erdos et al. 1975; Szabo et al. 1982; Olive et al. 1983). Furthermore, microscopic evidence suggests the occurrence of meiosis in Tubulinea based on the observation of SC in *Arcella* (Mignot and Raikov 1992); *Paraquadrula* was convincingly demonstrated to perform plasmogamy and karyogamy with subsequent cyst formation (Lüftenegger and Foissner 1991); *Copromyxa* was also observed to fuse and encyst in a putative sexual process (Brown, Silberman, and Spiegel 2011). Among Discosea, *Sappinia* makes a bicellular cyst, where sexual processes are hypothesized to happen (Brown, Spiegel, and Silberman 2007; Walochnik, Wylezich, and Michel 2010). *Cochliopodium* was also proposed to have sexual processes base on described fusions of cells and karyogamy (Wood, Heidari, and Tekle 2017). *Echinosteliopsis* produces two kinds of spores with different germination rates, what may be interpreted as evidence for sexual processes, in this case, meiosis, even though the author himself asserted that no evidence for sex could be found then (Reinhardt 1968). Among Archamoebae, transcriptomic and microscopic evidence strongly suggest the occurrence of meiosis in *Entamoeba invadens* during the encystation process, when meiotic genes are up-regulated in the first hours after cyst formation resulting in a mature cyst with four nuclei (Ehrenkaufer et al. 2013).

The current general understanding depicts amoebozoan groups mostly as asexuals despite scattered evidence on the contrary. The issue of sex in Amoebozoa was addressed once before through bioinformatics (Tekle et al. 2017). The authors aimed to evaluate the presence of meiosis related proteins in several Amoebozoan lineages. However, the molecular machinery for

plasmogamy and karyogamy was not investigated, and the large and diverse lineage of Tubulinea was severely under-sampled. Here we assess the occurrence of proteins associated to both syngamy and meiosis across all Amoebozoa lineages. We employ a comparative genomics analysis based on molecular genomic and transcriptomic data obtained from a wide phylogenetic sampling of the group (a dataset of 52 taxa).

Materials and methods

We have sampled 52 different Amoebozoan species covering the whole known diversity of this super group, including both species known to perform sexual cycles as well as those with unknown sexual processes. All data was obtained exclusively from public databases. *Entamoeba histolytica*, *Dictyostelium discoideum*, *Polysphondylium pallidum* are represented by genomic sequences obtained from public databases. All other species are represented by transcriptomic data and are derived from sequences which have been deposited in NCBI, mostly under BioProject PRJNA380424 among others (**Suppl. Table 1**). Raw sequence data were subjected to TRIMMOMATIC (Bolger, Lohse, and Usadel 2014) for cleaning and trimming of adaptors for posterior assembly with TRINITY (Grabherr et al. 2011). Translation of nucleotide sequences was performed by Transdecoder (https://github.com/TransDecoder/TransDecoder/wiki) in order to establish protein datasets used for further analyses.

Sequences of meiotic proteins characterized in model organisms (*H. sapiens*, *S. cerevisiae*, *A. thaliana* and others) serving as guides for trees were obtained from Genbank. Sequences from diverse Archaea and Bacteria strategically sampled were used as outgroups for trees, the proteomes being obtained from Genbank as well. Outgroups are important to determine more easily different paralogs in the analyses. In order to build profiles for the search of candidate sequences model organism sequences were aligned using the *mafft-linsi* tool of MAFFT (Katoh and Standley 2013).

Alignments thus obtained were employed for the construction of profiles with *hmmbuild* tool of HMMER (Eddy 2011). The only exception was the profile for Gex1/Kar5 because this protein is not well conserved. For this, we constructed a HMMER profile according to (Ning et al. 2013). All amoebozoan proteomes either from genomic or transcriptomic sources were combined in a single database and screened with protein profiles using *hmmsearch* tool of HMMER. Best hits (e-value < e-6 for most proteins and e-value < 0.001 for GEX1) were extract from the local database using the tool HMMER *esl-sfetch* for further processing. As the simple occurrence of similar or homologous sequences is not enough to determine a candidate sequence, all sequences obtained were subjected to phylogenetic reconstruction to confirm *bona-fide* orthologues. For this, sequences from a strategic sampling that could provide both wide phylogenetic coverage and outgroups were provided. We aligned matrices using default *mafft* tool from MAFFT; multiple sequences alignments (MSA) were subjected to trimming using BMGE (Criscuolo and Gribaldo 2010) with relaxed parameters as matrix BLOSUM30 given the divergent feature of the sequences and all steps inspected visually. The trimmed MSA files were used as input for phylogenetic reconstructions with IQ-TREE (Nguyen et al. 2015). The substitution models were evaluated and set automatically by ModelFinder based on the input data (Kalyaanamoorthy et al. 2017) and 1000 ultrafast bootstrap (Hoang et al. 2018). All candidate orthologues were compiled to a single table used as input for the Coulson Plot Generator (Field, Coulson, and Field 2013) in order to make the results easier to understand and expose possible evolutionary patterns.

Results

The present study was proposed in order to investigate the molecular machinery required for syngamy and meiosis in most of the known diversity of Amoebozoa. The data obtained from amoebozoan lineages were organized and interpreted based on the most recent comprehensive

phylogenomic reconstruction of the evolutionary relationships of the group according to Kang et al. (Kang et al. 2017). Genes required for plasmogamy, karyogamy, and main meiotic steps, either specific or not, were analyzed using a phylogenetics approach. In general, every protein surveyed yielded positive results for most amoebozoan groups including the 'asexual' model organisms *Acanthamoeba* and *Amoeba proteus* which present most of proteins associated to sexual processes (**Figure 2; Suppl. Table 2; Suppl. Alignments and Trees**). All proteins surveyed were identified in the three major amoebozoan lineages Tubulinea, Evosea, and Discosea, except for REC8 (not detected in Evosea). The most parsimonious interpretation would be that all of these genes were present in the amoebozoan ancestor. On average, each MEP was detected in about 44% of the samples, while each non-MEP were detected in 75% of the samples. Considering that most of the data obtained is derived from transcriptomes, the occurrence of MEP was expected to be lower than other non-MEP which are involved in general DNA metabolism regardless of the life cycle stage and are continuously expressed. Nevertheless, the proteins HOP2 and MND1were each detected in 90% of the samples. The proteins used in the present study were grouped according to their function in functional groups: syngamy (HAP2 and GEX1), sister chromatid cohesion (SMC1, SMC3, RAD21, and REC8), introduction of double-stranded breaks (DSB) (SPO11, MRE11, and RAD50), pairing and synaptonemal complex (SC) (HOP1, PCH2, and ZIP4), homologous recombination (HR) (DMC1, RAD51A, HOP2, and MND1), crossing over and its resolution through pathway I (MER3, MSH4-5, MLH1, MLH3, and EXO1) and pathway II (MUS81 and MMS4), and gene conversion by mismatch repair of the resulting heteroduplexes (MSH2, MSH6, MLH1, PMS1-2/MLH2, and MLH4) (**Figure 2**).

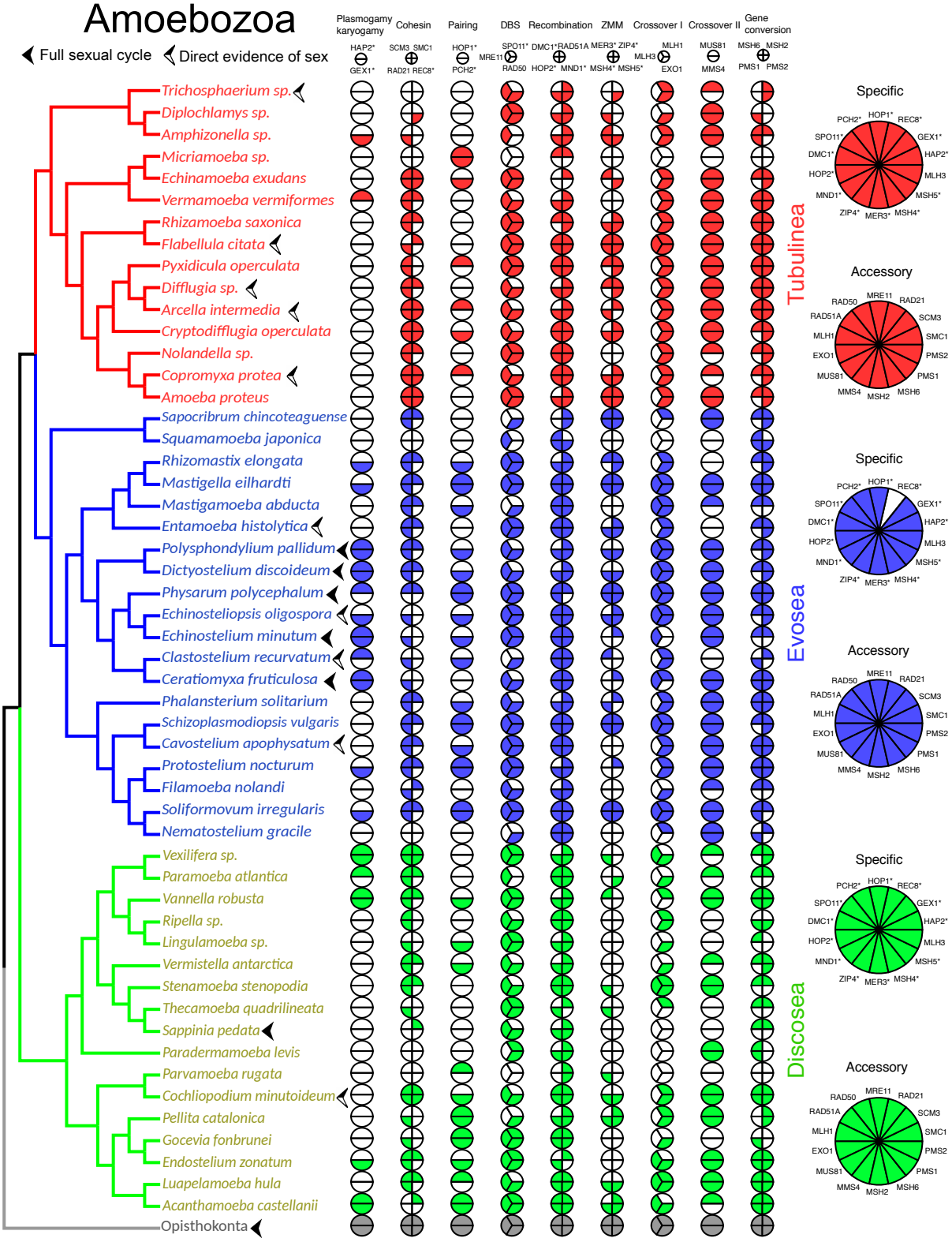
Amoebozoa
Full sexual cycle
Direct evidence of sex
Plasmogamy karyogamy
Cohesin
Pairing
DBS
Recombination
ZMM
Crossover I
Crossover II
Gene conversion
HAP2* GEX1*
SCM3 SMC1 RAD21 REC8*
HOP1* PCH2*
MRE11
SPO11* RAD50
DMC1* HOP2*
RAD51A MND1*
MER3* ZIP4* MSH4* MSH5*
MLH1 MLH3 EXO1
MUS81 MMS4
MSH6 MSH2 PMS1 PMS2
Trichosphaerium sp.
Diplochlamys sp.
Amphizonella sp.
Micriamoeba sp.
Echinamoeba exudans
Vermamoeba vermiformes
Rhizamoeba saxonica
Flabellula citata
Pyxidicula operculata
Difflugia sp.
Arcella intermedia
Cryptodifflugia operculata
Nolandella sp.
Copromyxa protea
Amoeba proteus
Sapocribrum chincoteaguense
Squamamoeba japonica
Rhizomastix elongata
Mastigella eilhardti
Mastigamoeba abducta
Entamoeba histolytica
Polysphondylium pallidum
Dictyostelium discoideum
Physarum polycephalum
Echinosteliopsis oligospora
Echinostelium minutum
Clastostelium recurvatum
Ceratiomyxa fruticulosa
Phalansterium solitarium
Schizoplasmodiopsis vulgaris
Cavostelium apophysatum
Protostelium nocturum
Filamoeba nolandi
Soliformovum irregularis
Nematostelium gracile
Vexilifera sp.
Paramoeba atlantica
Vannella robusta
Ripella sp.
Lingulamoeba sp.
Vermistella antarctica
Stenamoeba stenopodia
Thecamoeba quadrilineata
Sappinia pedata
Paradermamoeba levis
Parvamoeba rugata
Cochliopodium minutoideum
Pellita catalonica
Gocevia fonbrunei
Endostelium zonatum
Luapelamoeba hula
Acanthamoeba castellanii
Opisthokonta
Tubulinea
Evosea
Discosea
Specific
Accessory
Specific
Accessory
Specific
Accessory

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

We could assess the presence of the orthologs associated with syngamy in several lineages distributed among Tubulinea, Evosea and Discosea. Noteworthy, the fusogen HAP2 may not be easily detected in transcriptomic data due to its probable low expression levels in specific mating types only and due to the observation that GEX1 is broadly but only fairly conserved among eukaryotes (Ning et al. 2013). They could be detected in most genomic data (*Dictyostelium*, *Polysphondylium,* and *Acanthamoeba*). However, both forms are absent and seemingly lost in the *Entamoeba* lineage. The detection of both forms is a strong evidence of mixing dependent of an ancestral system of gamete recognition and fusion, implying the existence of different mating types.

Regarding the main meiotic steps, the proteins implied in sister chromatid cohesion, promoted by the cohesin complex subunits, are widely present in Amoebozoa. REC8 was lost in

Dictyostelium and *Entamoeba*, but it is present in Tubulinea and Discosea and, based on our results, it appears to have been lost in some ancestor of Evosea which concentrates most genomic datasets available. Previous studies failed to detect REC8 among protists and this protein is basically known only from plants, animals, and fungi (Malik et al. 2008; Schurko and Logsdon 2008). The proteins involved in the introduction of DSB are distributed among the whole diversity of the group. Strikingly, *Dictyostelium* and *Polysphondylium* have lost SPO11, previously reported by Bloomfield (Bloomfield 2018). These organisms are some of the few amoebozoans with well-known sexual cycles (Gregory W. Erdos, Raper, and Vogen 1973; Francis 1975). Distantly related *Acanthamoeba*, *Amoeba proteus, Physarum,* and *Entamoeba* present the whole set of DSB genes. Some datasets present more than one copy of SPO11, as in the case of *Pyxidicula*, *Entamoeba*, *Physarum*, *Acanthamoeba*, and others **(Suppl. Table 2)**, but it is not possible to determine with certainty whether such a duplication is ancestral or not **(Suppl. trees)**. Proteins associated with pairing of chromosomes and SC assembly, occur in most amoebozoan groups with losses of HOP1 and ZIP4 in *Dictyostelium* and *Polysphondylium* and HOP1 and PCH2 in *Entamoeba*. The presence of SC proteins in *Arcella* and *Physarum* corroborates ultrastructural data from literature (Aldrich 1967; Mignot and Raikov 1992). ZIP4 was identified in the mixogastrid *Echinostelium*, another genus whose SC has been demonstrated through electronic microscopy (Haskins, Hinchee, and Cloney 1971). Presence of both HOP1 and ZIP4 in *Protostelium* and *Acanthamoeba* among others is a strong evidence for occurrence of SC in these groups as well. The machinery for meiotic HR is ubiquitous among amoebozoan lineages. However, the meiosis specific DMC1 was lost in *Dictyostelium* and *Polysphondylium*. The double strand invasion is stabilized by another set of MEP, namely MER3, MSH4 and MSH5 (components of yeast ZMM complex). Members of the ZMM complex are widely present in representatives of most amoebozoan lineages indicating a possible maintenance of meiosis specific interference pathway I as the main pathway to resolve crossovers. Additionally, pathway I resolution proteins MutLγ (MLH1-MLH3) and EXO1 are

present as well. Class II crossover pathway proteins MUS81 and MMS4 could also be detected in most lineages (seemingly lost in *Entamoeba*). The machinery involved in nuclear DNA mismatch repair and gene conversion is also present basically in all lineages.

Discussion

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

most genetic exchange in bdelloids is probably due to lateral gene transfer rather than to meiotic sex.

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

A recent study detected the presence of meiotic genes in Amoebozoa concluding that amoebozoans are ancestrally and ‘secretly’ sexual (Tekle et al. 2017). As we already discussed, Amoebozoa could be proposed to be ancestrally sexual based solely on the position of fully sexual lineages nested inside Amoebozoa if we assume that the topology of the tree produced by Kang et al. (2017) is a good approximation of the real phylogenetic relationships of the lineages inside this

super group. As such, mere confirmation of presence of MEPs simply corroborates the hypothesis of amoebozoans being ancestrally sexual. However, the patterns of occurrence may shed light into evolution of sex in the group, while additionally indicating putative presence of sexual processes in lineages assumed to be asexual based on lack of observations of otherwise. However, some issues regarding the study of Tekle et al. (2017) are concerning as most positives results are not of MEP, but rather only meiosis *related* proteins. Additionally, plasmogamy and karyogamy were not surveyed, even though they are integral parts of any *bona-fide* sexual cycle. We demonstrated the presence of both plasmogamy and karyogamy proteins in several amoebozoans and focused directly on MEP, a result supporting the occurrence of sexual processes in the group. Tekle and collaborators also did not survey the highly conserved MEPs ZIP4 and PCH2 while searching for the non-conserved ZIP1 and RED1, which led to the expected negative results. They also failed to detect HOP1 (except for *Physarum*) in their datasets. Thus, results lacking the SC-associated proteins HOP1, ZIP4, PCH2, RED1 and ZIP1 are an artifact of their approach. The authors also dismissed the very occurrence of SC in Amoebozoa, despite previous reliable microscopic documentation for some groups, and proposed a putative 'novel crossover pathway' for amoebozoans without evidence. We demonstrated the presence of both HOP1 and ZIP4 in several lineages, what is consistent with occurrence of SC as revealed earlier by ultrastructural documentation. Another major issue with their results is their assumption that 'Mycetozoa' (we assume here that this taxon refers to Myxogastria sensu (Adl et al. 2012)) lost SPO11 and that they may have another mechanism to initiate meiotic recombination, seemingly in a SPO11-independent pathway, again without evidence and based on another artifact. While it is likely that dictyosteliids lack SPO11, this is not true for other groups as our results demonstrate clearly the presence of SPO11 in Myxogastria (e.g. in *Physarum*) and other related groups within Evosea. We also greatly expanded sampling with a total of 52 taxa here against 29 there (for more details, see **Suppl. Table 3**). Their poor taxon sampling issue is more pronounced in Tubulinea: Tekle and collaborators sampled only three

species with seemingly poor datasets leading to a complete absence of any positive results for MEP in Tubulinea, while we provided robust positive results for several MEP in 15 different species of Tubulinea, in a clear demonstration of another artifact resulting from their approach. Other problematic issues include their methods, as most MEP are results of gene duplication events, it is necessary to discriminate between different paralogues upon reconstruction of each MEP family. That is not what one can observe in Tekle et al. (2017), as paralogues of protein families were analyzed separately in different reconstructions (e.g. MND1-HOP2, RAD51A-DMC1 and MSH4-MSH5), which yielded poorly recovered trees that cannot be used to ascertain the presence of MEP in the surveyed lineages. Moreover, due to poor taxon sampling and limited methodological power, a key MEP, REC8, was not detected, while present in our analysis. In the present study we present many more positive results for cell fusion and meiotic machinery in the group and we have the opportunity to offer new perspectives to understand the biology of Amoebozoa by pointing out artifacts generated by Tekle et al. (2017).

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

The existence of a mating type system has direct implications for observing meiosis in amoebae, as clonal cultures (which is often the norm in protistological laboratories) will not exhibit any signs of sexual processes, leading to the observation that a given organism is asexual. The cell fusogen HAP2, as well as GEX1, were not detected in *Entamoeba* genomes, and seem to be lost in this genus. This loss does not imply these organisms have lost capacity of mixing (Fungi have lost the fusogen and have sexual cycles (Speijer, Lukeš, and Eliáš 2015)), but rather that they may have lost the mating type system and could be homothallic. Accordingly, selfing or unisexual mating happens in parasites, explaining their apparent clonal structure (Feretzaki and Heitman 2013). But mixing alone does not support a canonical sexual cycle, as parasexual processes may happen afterwards. *Candida albicans,* which grows as diploid cells of two different mating types, can fuse to form a tetraploid cell, which returns to a diploid state through loss of chromosomes (Bennett and Johnson 2003). Thus, the co-occurrence of fusogens and MEP provide stronger evidence for sexual processes in a given lineage. We have detected genes for the proteins required for syngamy and every meiotic step for the entirety of Amoebozoa, challenging the common conception that amoebae are 'asexual' organisms. Most groups present a full cohesin complex and its meiotic variant. The occurrence of pachytene check regulation PCH2 and SC are also conserved in the group. The machinery responsible for the introduction of DSB and re-section of the broken ends are present in most lineages with the exception of a very specific loss of SPO11 in dictyostelids. Such a loss is intriguing since losses of SPO11 are not known outside dictyostelids as this topoisomerase was detected in previous works with all candidate asexual protists surveyed (Ramesh, Malik, and Logsdon 2005; Carlton et al. 2007; Fučíková, Pažoutová, and Rindi 2015). Given that dictyostelids are known to have sexual cycles, they are probably relying on another pathway to introduce DBS onto chromosomes. Alternative mechanisms for DBS have been described for fission yeast *S. pombe* and *Caenorhabditis* (Farah et al. 2005; Pauklin et al. 2009), suggesting that there must be alternative processes in dictyostelids.

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

In the case of Amoebozoa, as the ancestor of all lineages was clearly sexual, our positive results support to the assumption that the whole lineage is sexual, many of these taxa with unknown sexual or meiotic processes. This permits us to make overarching conclusions that will need to be further investigated: i) meiotic sex is cryptic, ii) current laboratory conditions are not suitable for sexual cycles and, iii) perhaps in some cases meiotic events are mistakenly reported as mitosis. The latter might well be the case, as meiotic divisions could be interpreted as two sequential mitotic divisions. In many cases we hypothesize that haploid and diploid forms have roughly the same

morphological appearance. But the assumption that most or all amoebozoan would perform meiosis and sex in the same manner seems to be rather simplistic given the high diversity of forms in the group. Alternative processes may exist in some lineages, hypothetically among polyploid forms such as *Acanthamoeba*, *Polychaos*, and *Entamoeba*. Maciver (Maciver 2016) proposed that polyploid amoebae and other organisms presenting high numbers of genome copies may have the possibility to recombine their chromosomes rather frequently and revert deleterious mutations through the process of recombination and gene conversion. For this process they would employ their conserved meiotic machinery. This would allow for genome stability without the necessity of spending energy undergoing meiosis or fusing with other individuals. In this framework, a parasexual process would maintain genome stability in polyploids. Although an interesting idea, this hypothesis is not supported with evidence.

One can entertain the idea that a given protein involved in meiosis could be coopted for another function (pending functional demonstration). Even if it was the case, it is unlikely that several of them would assume new functions in the same lineage. Traditionally seen as an assemblage of asexual mitotically reproducing organisms, amoebozoans (especially Tubulinea and Discosea) should be understood as putative sexual organisms, with direct implications to different fields. Regarding public health, the results presented here changes the way we approach pathogenic species, their response to drugs used in their control, as well as dynamics and evolution of drug resistance. Some instances can be observed in pathogenic fungi and apicomplexans parasites: crossings between different plant pathogens *Tapesia yallundae* strains yielded progeny with higher level of fungicide resistance (P. S. Dyer et al. 2000); sex and recombination were also associated to spread of drug resistance and virulence in human pathogens (Heitman et al. 2014); a highly virulent *Toxoplasma gondii* strain was demonstrated to be produced by out-crossing and that clonal population structure and expansion of an epidemic clone was maintained by selfing (Wendte et al. 2010). Thus, clonal population structures are not an evidence of asexuality, but rather a consequence

of repeated unisexual reproduction in a self-compatible strain (Feretzaki and Heitman 2013). Multi-drug resistance in *E. histolytica* (Orozco et al. 2002) may be explained by sexual recombination among different strains. Regarding taxonomy, some corrections may issue from molecular data in the same way it happened with some fungi where some species may be synonymized because haploid and diploid forms vary morphologically. *Aspergillus fumigatus*, an ascomycete implicated in human disease was thought to be asexual and recently discovered to have a fully functional sexual cycle (O'Gorman, Fuller, and Dyer 2009). The sexual part of the cycle (teleomorph) known as *Neosartorya* is now synonymized with *Aspergillus*.

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

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Supplementary Table 1: Sources of datasets employed in the present study.

Supplementary Table 2: General results of the present study. Occurrence of meiosis related and meiosis-specific proteins in diverse Amoebozoa. Number insides parentheses indicate that more than one paralogue was observed.

Supplementary Table 3: A comparison between the sampling of the present study and Tekle et al., 2017. We sampled twice as many Evosea and fivefold more Tubulineans.

Supplementary alignments and trees: All alignments and their resulting trees generated by this project.

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Capítulo 4: All eukaryotes are sexual, unless proven otherwise

Many so-called asexuals present meiotic machinery and might be able to have sex

(*Publicado na Bioessays, 2019*).

Abstract

Here we show a wide distribution of meiotic machinery indicating occurrence of sexual processes in all major eukaryotic groups, without exceptions, including the putative 'asexuals'. Meiotic machinery evolved from archaeal DNA repair machinery by means of ancestral gene duplications. Sex is very conserved and widespread in eukaryotes, even though its evolutionary importance is still a matter of debate. The main processes in sex are plasmogamy, followed by karyogamy and meiosis. Meiosis is fundamentally a chromosomal process which implies recombination and ploidy reduction. Several eukaryotic lineages were proposed to be asexual because their sexual processes were never observed, but presumed asexuality correlates with lack of study. We stress the complete lack of meiotic proteins in nucleomorphs and their almost complete loss in the fungus *Malassezia*. Inversely, complete sets of meiotic proteins are present in fungal groups Glomeromycotina, *Trichophyton* and *Cryptococcus.* Endosymbiont *Perkinsela* and endoparasitic Microsporidia also present meiotic proteins.

Keywords

Adineta, asexual, *Candida*, Glomeromycotina, *Malassezia*, plasmogamy, meiosis, sex.

1. Introduction

Sexual processes are currently interpreted as ancestral in eukaryotes. Even the most basal and distantly related lineages show evidence of sexual processes, conversely the existence of truly asexual lineages is difficult to demonstrate (Speijer, Lukeš, and Eliáš 2015). Here we define eukaryotic sex by a combination of fundamental processes such as plasmogamy (cell fusion), karyogamy (nuclear fusion) and meiotic reduction with recombination (Kondrashov 1997; Carr, Leadbeater, and Baldauf 2010). Generally speaking, upon plasmogamy two haploid cells working as gametes fuse and proceed to karyogamy, i.e. nuclei fusion, resulting in a diploid cell nucleus. Eventually diploid nuclei perform meiosis with crossing-over. Some processes are meiosis-specific, such as: bouquet formation, synaptonemal complex (SC) assembly, inter-homologue crossing-over and reductional division of the cell leading to production of haploid cells, normally four (Denise Zickler and Kleckner 2015). Fusion of cells with subsequent recombination is not exclusively eukaryotic, as this process has been observed in archaeal organisms (Naor and Gophna 2013). The eukaryotic novelty resides in the fact that eukaryotic sexual processes are performed in a highly orchestrated manner, innovations such as the assembly of SC as a way of precisely pairing homologous chromosomes, facilitating recombination and correct segregation and assortment of chromosomes upon cell division (D. Zickler and Kleckner 1999). In eukaryotes, meiosis is stimulated by environmental or cellular stress and is associated to the formation of resistance cysts in many single-celled organisms (Thomas Cavalier-Smith 2010).

Many evolutionary hypotheses have been proposed to explain the persistence and pervasiveness of sexual processes in eukaryotes. The occurrence of such an elaborate and costly process in most eukaryotic lineages is an indication of its importance. Earlier hypotheses emphasized the maintenance of genetic variability and the possibility of recombination, increasing the ability of organisms to face an ever-changing environment (Maynard Smith 1971). In this framework, parasites would represent a constant challenge to the survival and fitness of their hosts;

conversely, host defenses would also pose obstacles to the survival of its parasites and both would necessarily need frequent recombination events to keep in pace with each other and make possible the long-term survival of both, leading to co-evolution of hosts and parasites, the 'Red Queen Hypothesis' (Hamilton, Axelrod, and Tanese 1990). Different lines of reasoning propose that sexual processes would be important for large scale maintenance and repair of the nuclear genome in order to avoid aging as telomeres are continuously being reduced after many rounds of cell division (Siderakis and Tarsounas 2007). Others yet present evidence that meiosis is important for controlling the selfish elements inside the eukaryotic genome by meiotic silencing of unpaired DNA (Shiu et al. 2001). Transposable elements are ubiquitous in eukaryotes and pose a threat to the host genome because they translocate randomly, possibly interrupting coding regions, breaking chromosomes, and producing inversions and translocations. During meiosis, homologous chromosomes are aligned allowing for recombination and silencing of newly translocated selfish elements (Shiu et al. 2001). As meiosis promotes a large scale repair of the nuclear genome, it was suggested that meiosis evolved and was maintained as a way to repair the genome from continuous DNA damage caused by increased amounts of reactive oxygen species generated by mitochondrial metabolism (Speijer 2016; Hörandl and Speijer 2018).

2. Meiosis toolkit can reveal the nature of asexual scandals

Meiotic processes are performed by a meiosis-specific machinery (the "meiosis toolkit"), and this set of proteins may be used for indirect detection of meiosis based on genomic information. This method was proposed as an approach to provide molecular evidence for the occurrence of sex in putative asexual lineages (Schurko and Logsdon 2008). The meiotic toolkit includes proteins implied in the introduction of double-stranded breaks onto DNA, SC assembly, homologous recombination and crossing-over resolution. A complementary approach is the detection of plasmogamy and karyogamy-specific proteins in order to provide a similar kind of evidence

(Speijer, Lukeš, and Eliáš 2015). The existence of cryptic sexual processes in putative asexuals such as *Giardia* and *Trichomonas* was proposed based on testing for the presence of the meiosis-specific machinery (Ramesh, Malik, and Logsdon 2005; Malik et al. 2008). The same approach was later applied to other putative asexual groups with similar results (Carr, Leadbeater, and Baldauf 2010; Chi, Parrow, and Dunthorn 2014; Kraus et al. 2019), but many seemingly asexual lineages remain to be investigated.

Considering the phylogenetic distribution of sexual lineages and the distribution of plasmogamy and karyogamy associated proteins in eukaryotes, the ancestor of all eukaryotes was capable of sexual processes in a very similar way to modern eukaryotes, i.e., it presented a mating type system and performed meiosis (Dacks and Roger 1999; Speijer, Lukeš, and Eliáš 2015). Thus, an asexual lineage must be a result of a secondary loss of sex. However, presumed lack of sexual processes correlates directly with the lack of study, i.e., the less studied the group is, more likely it will be considered asexual in the literature. A good example of this is the description of sexual cycles for Myxogastria among 'asexual' amoebozoans, which has been attributed to the simple fact that they were more intensively studied when they were still considered to be a group of Fungi (Spiegel 2011). The classification of Myxogastria inside Amoebozoa is fairly recent (T. Cavalier-Smith 1998). Another problem is the heavy bias towards parasites, which represent around 90% of all protist genomes available (Sibbald and Archibald 2017). Parasites are normally highly specialized and present a trend to have reduced genomes, bearing characteristics that cannot be extrapolated to free-living groups. Finally many new lineages are being currently described and very little is known about their biology, life cycles and sexual processes: *Pygsuia, Capsaspora*, *Palpitomonas* (Brown et al. 2013; Hertel, Bayne, and Loker 2002; Yabuki, Inagaki, and Ishida 2010). Apparently, the sole evolutionary stable asexual lineage supported by convincing evidence is a group of tiny metazoans, the bdelloid rotifers, all other asexual groups being recent triploid populations of sexual species (Judson and Normark 1996; van Dijk and Bakx-Schotman 2004; Gutekunst et al. 2018). Besides the fact that males or meiosis have never been observed in this

group, the absence of certain ubiquitous selfish genetic elements in their genomes was considered incompatible with sexual processes (Arkhipova and Meselson 2000). The sequencing of the genome of the bdelloid rotifer *Adineta vaga* revealed an ancestrally degenerate tetraploid genome incompatible with meiotic recombination (Flot et al. 2013). The transition in some ancestor to a tetraploid state must have impaired meiosis in the group that was already performing asexual processes in the same way their sister group (Monogonont rotifers) does, normally reproducing by parthenogenesis interspersed with occasional sexual processes, sometimes shifting to obligate parthenogenesis (Stelzer et al. 2010).

3. Is there sex for eukaryotes trapped inside cells?

Even though sexual processes are widespread and conserved in eukaryotes in general, some specific situations seems to prevent any possibility of mating and, consequently, of performing meiotic reduction. Highly reduced intracellular parasites, as in the case of Microsporidia, are metabolically active only inside the cells of their hosts inside structures called "parasitophorous vacuoles". As such, finding a pair to mate seems to be a big challenge for these parasites. However, fusion of parasitophorous vacuoles of the microsporidian *Encephalitozoon* were reported (Lee and Heitman 2017). Some plant and animal populations are triploid and propagate purely by asexual means, for a reduction of the chromosome number by half is not possible (van Baarlen et al. 2000; Gutekunst et al. 2018). Finally, plasmogamy seems difficult in a group of apparently non-motile, modified eukaryotes dwelling inside the cytoplasm of other eukaryotes in symbioses of unknown nature as in the case of the flagellate *Perkinsela* inhabiting the cytoplasm of *Paramoeba* (Tanifuji et al. 2017). Would the endosymbionts mate upon mating of the host, in a case of 'double-mating'? In extreme cases, red and green algal cells are trapped inside other eukaryotic cells as secondary endosymbionts and present a highly reduced but still functional nucleus as a result of an ongoing process of reduction, as in the cases observed in Chlorarachniophytes and Cryptophytes (Archibald

2007; Suzuki et al. 2015). Other symbionts, parasites or not, face similar challenges and, possibly, some groups may have modified sexual processes or may even have lost sex altogether. However, a clonal genetic structure of a given population, as observed in some parasitic species populations, is not necessarily an evidence for loss of sex because some lineages are capable of selfing and several repeated rounds of self-mating lead to a clonal structure in the population (Wendte et al. 2010).

As such, because the literature alone is not a good parameter to determine true asexuality, we use a combined bioinformatics approach to identify the occurrence and distribution of the most conserved plasmogamy and meiosis-specific proteins based on a genomic scale of a broad sampling of eukaryotic lineages. We specifically focused on putative asexual organisms and several lineages that have not been included in previous studies or have been only recently described.

Inferring the presence of genes responsible for plasmogamy and meiosis in a broad eukaryotic sample

Our strategy was homology assessment via phylogenetic trees. As most meiosis specific proteins are a result of ancient gene duplication events, the only way to assure that a given sequence is a meiotic paralogue is through the reconstruction and interpretation of trees including model organisms, as a simple search for similarity is not sufficient to establish deep homology (Fitch 1970). As some protein families are very large, reconstructions were performed by means of smaller trees, each major eukaryotic group separately. Due to the large number of trees necessary to assess the occurrence of several protein families in all eukaryotic major groups and selected lineages, a pipeline was written to perform the search, alignments, and reconstructions of trees.

Complete proteoms were downloaded from public databases: GenBank, 1000 Plants, Ensembl Protists. Streptophyte data was kindly provided by (Vries et al. 2018). The sampling included the broadest possible eukaryotic taxonomic spectrum based on translated available

genomes. In cases where no genome was available, transcriptomes were used instead. Transcriptomes were assembled with Trinity (Grabherr et al. 2011) and translated with TransDecoder. Some groups were specially aimed because of their poor evidence regarding sexual processes, eg., Microsporidia and other fungi, Cryptophytes, Rhizaria, Haptophytes, small flagellates, and amoeboflagellates such as *Capsaspora*, *Malawimonas*, *Monocercomonoides*, 'Excavates' and others. Nucleomorphs of cryptophytes and chlorarachniophytes, the endosymbiont of *Paramoeba* (*Perkinsela*) and the putative asexual bdelloid rotifer *Adineta vaga* and relatives were also included. For all selected groups, proteins involved directly in plasmogamy and meiotic processes, or considered specific to it were employed for searches and reconstruction. The reconstruction of protein families is important because most meiosis specific genes are a result from ancestral events of gene duplications and the best way to differentiate the meiotic homologs from their respective non-meiotic paralogs is through reconstruction of protein trees. The proteins used for this are HAP2 (implicated in plasmogamy (Liu et al. 2015)), REC8 (chromosome cohesion (Revenkova and Jessberger 2005)), HOP1, PCH2, and ZIP4 (implicated in the assembly of synaptonemal complex (Anuradha and Muniyappa 2004; Lynn, Soucek, and Börner 2007; Farmer et al. 2012)), SPO11, DMC1, HOP2, MND1 (implicated in recombination (Petukhova et al. 2005; Malik et al. 2007)), MER3, MSH4, MSH5, MLH3 (resolution of meiosis-specific crossover pathway I (T. F. Wang, Kleckner, and Hunter 1999; Nakagawa and Kolodner 2002; Snowden et al. 2004)), and MUS81, which is not meiosis specific (alternative crossover resolution pathway II (de los Santos et al. 2003)).

Alignments of the above proteins were produced with MAFFT (Katoh and Standley 2013), after they were obtained from model organisms (**Suppl. Table 1**) using BLASTP (Altschul et al. 1990) on Genbank. Resulting alignments were used for construction of a profile with HMMER *hmmbuild* tool (Eddy 2011). Profiles were employed to search local databases for candidate homologs with *hmmsearch* tool. Candidate sequences were extract from databases through the use of HMMER *esl-sfetch* tool and aligned with MAFFT. Resulting alignments were trimmed with

Trimal (Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009) and used as input for Iqtree reconstructions (Nguyen et al. 2015). Reconstructions were performed with Iqtree setting a simple mixed substitution model (LG+C10+F+G) arbitrarily selected and 1000 Ultrafast bootstraps (Hoang et al. 2018). The resulting trees were employed for the discrimination of possible meiotic paralogues for each eukaryotic groupings or arbitrary selections of putative asexuals that received more attention. Proteins from eukaryotic model organisms were used to identify their homologues in other groups of eukaryotes on the trees. Presence/absence of meiotic proteins is summarized in tables used as input for Coulson Plots (Field, Coulson, and Field 2013), so that the patterns can be easily visualized and identified. If a representative of some group presents a given protein, the whole group is marked as presenting it.

4. Proteins associated with sexual processes are widespread in eukaryotes

Systematic reconstructions of protein families involved in DNA repair and meiosis clearly demonstrate their archaeal origin (**Figure 1**). Proteins involved in double-strand break, homologous recombination, sister-chromatid cohesion and crossover resolution arose through gene duplication from proteins that have important roles in DNA repair processes in Archaea, while a few could not be traced to either bacteria or archaea (RAD21-REC8 and HOP2-MND1). These patterns are evidence of vertical inheritance of the meiotic machinery directly from Archaea without bacterial contributions. Several gene duplication events in the transition from Archaea to Eukarya explain the extensive paralogy observed in the trees. Fusogen HAP2 has neither traceable paralogues in eukaryotes nor homologs in archaea and was proposed to bear structural homology to viral proteins (Fédry et al. 2017).

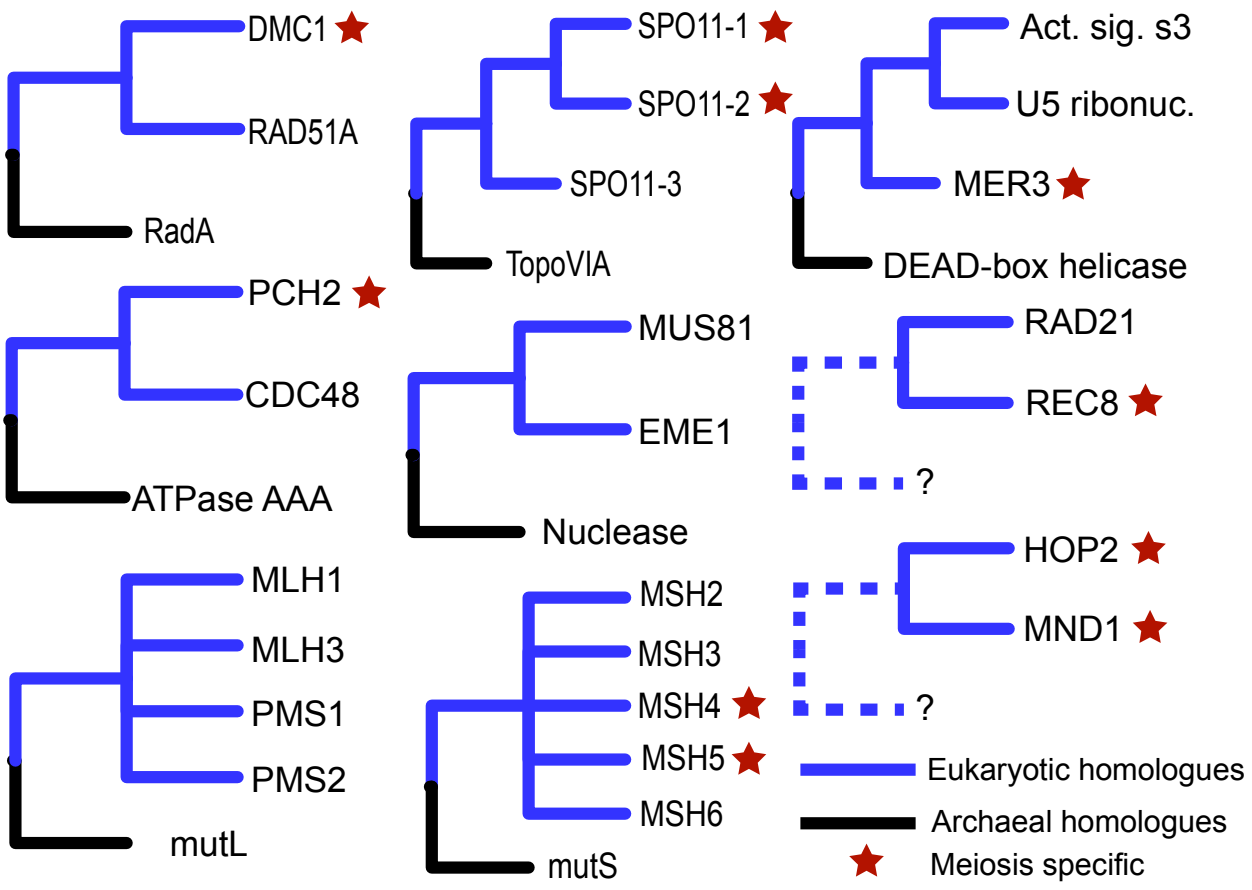

Figure 1. Simplified phylogenies of most meiotic proteins occurring in eukaryotes. Different meiosis-specific protein families present similar evolutionary patterns: they share an archaeal origin and evolved by extensive gene duplication events of proteins involved in general DNA repair processes.

Proteins associated with sexual processes are widespread in all major eukaryotic groups surveyed and also in many unaffiliated lineages (**Figure 2**). Eukaryotic super-groups known to be fully sexual (Metazoa, Fungi, SAR, Viridiplantae, and Rhodophyta) present full sets of the meiotic machinery. Similarly, several lineages of eukaryotes for which sexual processes and meiosis have but scant observational evidence, also present most or all of the plasmogamy and meiotic proteins: Cryptista, Haptista, Amoebozoa, Euglenozoa, Heterolobosea, Metanomada, Choanoflagellata, Glaucophyta, Jakobida, Centrohelida, Rhizaria, Dinoflagellata. Isolated and recently described groups for which sexual cycles are unknown also present sets of meiotic proteins compatible with the occurrence of sex: *Capsaspora*, *Pygsuia*, *Thecamonas*, *Amoebidium*, *Sphaeroforma*,

Corallochytrium, Gefionella, Ancyromonas, Fonticula, Ministeria. As some datasets are derived from transcriptomic data, negative results for some proteins do not necessarily mean losses in the group, except for groups with several genomes.

The patterns of occurrence of the fusogen HAP2 and of karyogamy protein GEX1 revealed for the majority of the eukaryotic diversity supports not only the ancestral sexual character of eukaryotes but also the existence of an ancestral mating type system (Speijer, Lukeš, and Eliáš 2015). This also corroborated an earlier proposition, based on phylogenetic patterns, of sexual processes as an ancestral character (Dacks and Roger 1999), even though the literature does not mention sex for several groups. Our results also corroborate the hypothesis of sex being ancestral and pervasive in eukaryotes. Additionally, the sexual eukaryotic common ancestor was likely self-incompatible. Departing from this premise, the absence of this system in some groups, e.g. Fungi and Vertebrates, is a secondary loss. Despite the presence of self-compatible parasitic lineages, e.g. apicomplexan genera *Toxoplasma* and *Sarcocystis* (Wendte et al. 2010) and, apparently, amoebozoan genus *Entamoeba* (Hofstatter, Brown, and Lahr 2018), the loss of the ancestral mating system does not necessarily mean a transition to homothallism (self-compatibility) as other systems of sex determination may evolve independently in several groups (Heitman 2015).

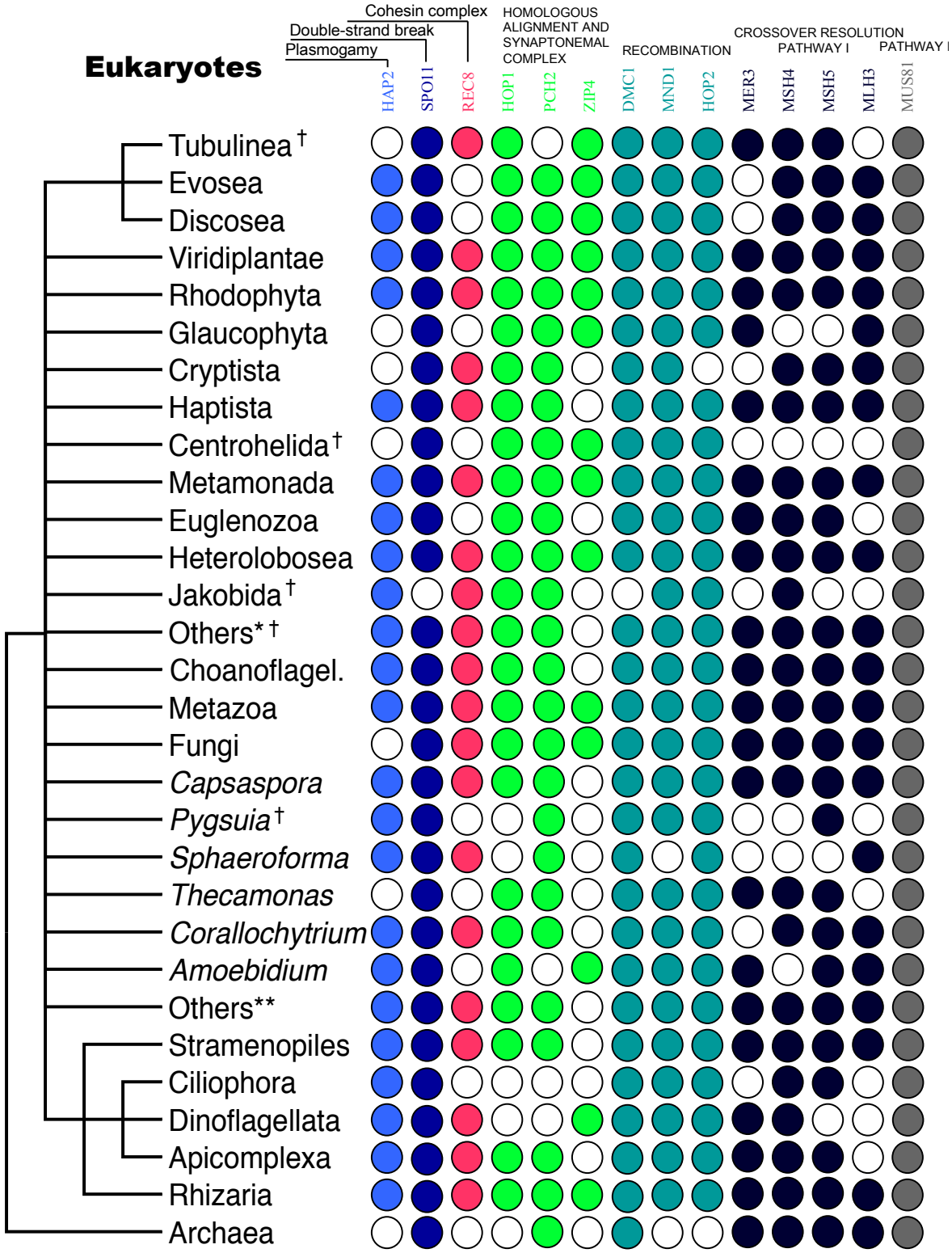

Figure 2. Distribution of plasmogamy (HAP2) and meiotic proteins in eukaryotes. Representatives were chosen for supergroups based on available genomes and transcriptomes. The big picture here represents the minimum occurrence of the surveyed proteins in those lineages because some groups have only transcriptomic data available and some apparent absence may not be real. The presence of Archaea as a sister group to all eukaryotes does not mean that they

perform meiosis but that several meiosis-specific genes can be traced back to archaeal organisms. All eukaryotic super-groups and smaller lineages have a pattern of occurrence of meiotic proteins compatible with sexual life cycles and are likely sexual. Another implication of the patterns observed here support the view that the ancestor of all eukaryotes was sexual as well and sexual cycles were maintained. Tubulinea, Evosea and Discosea form the super-group Amoebozoa; Stramenopiles, Rhizaria, Ciliophora, Dinoflagellata and Apicomplexa form the super-group SAR. **Gefionella*, *Malawimonas*, *Tsukubomonas*; ***Amastigomonas*, *Ancyromonas*, *Breviata*, *Diphylleia*, *Fonticula*, *Ichthyophonus*, *Ministeria*, *Parvularia*. †Results based solely on transcriptomic data.

5. Sexuals and 'asexuals' share the same meiotic machinery

Some large eukaryotic lineages lack overall evidence for sexual cycles and are therefore considered "asexuals" in the literature. According to (Adl et al. 2018) Archaeplastida is composed by three major lineages, two fully sexual and one with unknown sexual activities: Rhodophyceae, Chloroplastida and Glaucophyta, the last one with complete life cycles missing for all species (Kies and Kremer 1986; Jackson, Clayden, and Reyes-Prieto 2015). Despite the poor molecular data available for Glaucophyta, most meiotic proteins may be detected for representatives of this group (**Figure 2**). Combined evidence, i.e. the close relationship with fully sexual groups, presence of flagellated cells and the presence of an almost complete set of meiotic proteins, supports the existence of sexual cycles in glaucophytes. Some red algae are also considered asexual: unicellular *Cyanidioschyzon* and *Galdieria* (Albertano et al. 2000; Ciniglia et al. 2004). Both present a set of proteins compatible with sexual life cycles including a mating type system and meiosis (**Suppl. trees**). The green algae *Ostreoccoccus*, *Helicosporidium* and *Chlorella*, for which no sexual cycle is known, present the machinery for meiosis and the last two (Trebouxiophyceae) also present plasmogamy/mating types protein HAP2 (**Suppl. trees**). (Fučíková, Pažoutová, and Rindi 2015) have previously reported similar results for the meiotic machinery for several unicellular green algae. We add to it the confirmation of HAP2 in Trebouxiophyceae; a mating type system may occur in this group.

The presence of a wide inventory of meiotic and plasmogamy proteins in Haptophyta (**Figure 2**) is compatible with their sexual life cycles, already recognized as having heteromorphic alternating haploid and diploid stages (Paasche 2001). Similar results, an inventory of proteins mainly derived from *Guillardia theta*, point to the existence of sexual processes in Cryptista, for which only scant indirect evidence for sex is currently available (Kugrens and Lee 1988; Speijer, Lukeš, and Eliáš 2015). The smaller sex inventory of Centrohelida is preliminary as it is basically derived from transcriptomes and the sequencing of one or more genomes for this group could provide more information on the subject. Anyway, a handful of meiotic proteins in some representatives of Centrohelida combined with dimorphism of life cycles stages in some species (Zlatogursky et al. 2018) suggest the possibility of sexual life cycles for this group as well. Meiosis has never been observed for any of these three large groups of eukaryotes and all kinds of evidence supporting it are indirect.

Sex and meiosis are abundantly registered for members of SAR (Stramenopiles, Alveolata, and Rhizaria). Apicomplexan parasites, ciliates, oomycetes, diatoms and brown algae have well-known sexual processes and meiosis extensively documented and present many, if not all, plasmogamy and meiotic proteins (**Figure 2**). However, some groups still lack observations of meiosis and other processes, among them some dinoflagellates and several rhizarian groups. Regarding dinoflagellates, *Symbiodinium* lacks cytological signs of sex but molecular evidence supports its occurrence (Chi, Parrow, and Dunthorn 2014). It was hypothesized to occur in the rarely observed free living stage of this endosymbiont. Additionally, mating types may exist in dinoflagellates as evidenced by the presence of HAP2 in some available datasets. Plasmogamy and meiotic proteins for *Oxyrrhis* and *Noctiluca* were detected (**Suppl. Trees**). Regarding Foraminifera, sexual cycles are well known for some species but not for *Reticulomyxa,* whose genome provided evidence for the existence not only of sex, but for a yet not observed flagellar apparatus (Glöckner et al. 2014). Flagellated cells are unknown for *Reticulomyxa filosa* and the combined inventories of meiotic proteins and flagellar apparatus point to the formation of flagellated gametes in the species

when certain conditions are met. Another rhizarian, *Paulinella chromatophora*, presents several proteins compatible with sex (**Suppl. Trees**). This amoeba had drawn attention because of its chromatophore in an ongoing process of primary endosymbiosis (Marin, Nowack, and Melkonian 2005); the description of a sexual cycle in such an organism would provide insights for the role played by sex in the establishment of endosymbionts and the acquisition of genes by endosymbiotic gene transfer or how the presence of an endosymbiont could interfere or even disrupt sexual processes. In general, rhizarian lineages are poorly understood and deserve more attention. Finally, the early diverging stramenopile *Blastocystis*, inhabitant of animal guts, is cause of much confusion in the literature regarding both its life cycle and pathogenicity, as discussed by (Tan 2008). Characterized as pleomorphic due to its several different observed cell morphologies, reconstruction of its actual life cycle is difficult. The genus exhibits a full set of conserved meiotic proteins (**Suppl. trees**), but apparently lacks the ancestral mating type system. These results support a sexual cycle with meiosis, maybe with self-compatibility (homothallism).

Several lineages of flagellates are unaffiliated or have a dubious monophyly of their groupings. Most of them have unknown sexual life cycles and, normally, only one stage of their cycles is described. Among them Metamonada and Heterolobosea call attention because of the completeness of their inventories for sex, despite the fact of being considered asexuals (**Figure 2**). (Adl et al. 2018) do not mention anything regarding sex for Metanomada, Euglenozoa, Heterolobosea, Jakobida and others. The issue of sex was previously examined for *Giardia* (Ramesh, Malik, and Logsdon 2005) and *Trichomonas* (Malik et al. 2008) confirming the presence of most meiotic proteins for both. The sequencing of the genome of *Naegleria gruberi* revealed a conserved machinery as well (Fritz-Laylin et al. 2010). Similarly, our results confirm the presence of conserved machineries for *Monocercomonoides*, *Dientamoeba*, *Trimastix*, *Chilomastix*, *Carpediemonas*, *Jakoba, Leishmania, Bodo* and others in a clear pattern of maintenance of most or all parts of the machinery (**Suppl. trees**). Additionally, several groups related to Opisthokonta also present conserved machineries and are probably fully sexual: *Capsaspora*, *Sphaeroforma*, *Pygsuia*,

Thecamonas, *Corallochytrium*, *Amoebidium*, *Ministeria*, *Monosiga*, *Salpingoeca*, etc. Among the last ones, preliminary sexual processes have been already described only for the choanoflagellate *Salpingoeca* (Woznica et al. 2017). Finally, the presence of conserved machineries in all these groups justify the hypothesis of a sexual ancestor for all eukaryotes and support full sexual cycles in all these groups with mating types system and canonical or modified forms of meiosis, yet to be observed and described.

6. Are there ancient asexuals?

We highlight below a few eukaryotic groups that present unique cases when analyzed under the paradigm described above: nucleomorphs of Cryptophytes and Chlorarachniophytes (trapped relicts of nuclei from red and green algae respectively, incorporated long ago and under ongoing reduction processes coupled to endosymbiotic gene transfer); *Perkinsela* (an intracellular symbiont of *Paramoeba sp*.); *Oenothera biennis*, a functionally asexual angiosperm; Bdelloid rotifers (Metazoa) *Adineta vaga, A. ricciae*, *Rotaria macrura*, *R. magnacalcarata* considered to be an 'asexual scandal' (convincing evidence supports the notion that the group is asexual) and representatives of their sexual sister-group, *Brachionus* (Monogononta); Microsporidia (intracellular parasites that are inactive outside hosts, in a similar way to viruses); and several lineages of Fungi (**Figure 3**).

Nucleomorphs completely lack plasmogamy and meiotic proteins (**Figure 3**) and seemingly lost all of their meiotic machinery and even non-meiotic paralogs of them indicating that sex was lost long ago in these relicts with extremely reduced genomes. Descendants of fully sexual groups (Viridiplantae and Rhodophyta), nucleomorphs are but a shadow of the nuclei from which they descend. They are in a highly advanced ongoing process of genome size reduction and are expected to disappear completely by the end of the process of secondary endosymbiosis (Archibald 2007). Nucleomorphs can be used as a eukaryotic negative control for occurrence of meiotic proteins as

well. Another endosymbiont, *Perkinsela*, still keeps important plasmogamy and meiotic proteins and maybe performs sexual processes even though it lives inside the cytoplasm of another eukaryote (*Paramoeba*). *Perkinsela* might have the chance of sex when the host itself has sex, assuming its host is sexual.

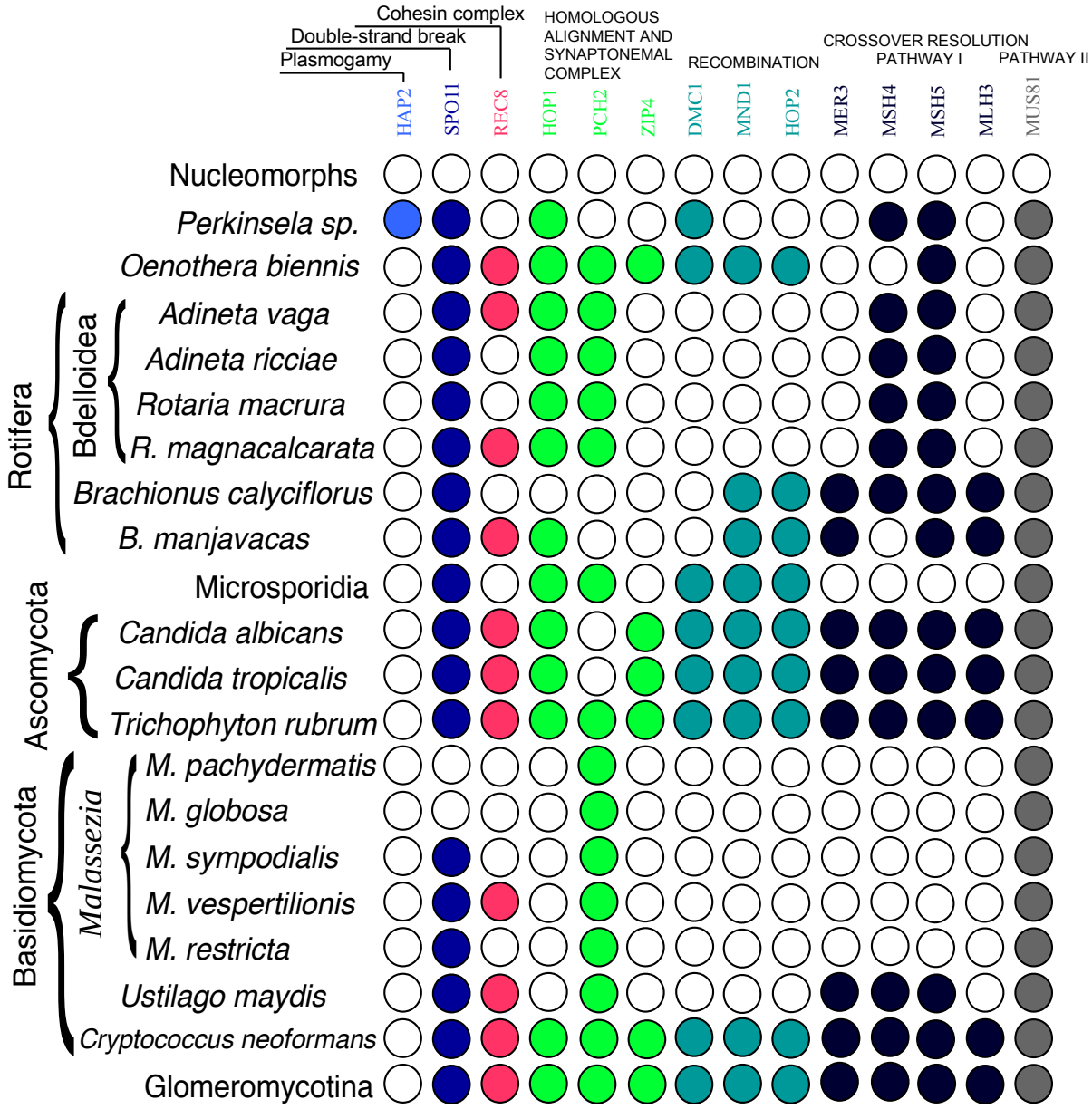

Figure 3. Occurrence of plasmogamy and meiotic genes in selected groups of intracellular eukaryotes or groups traditionally considered to be asexual. Nucleomorphs are nuclear relicts of green and red algae that can be found inside of Chlorarachniophytes and and Cryptophytes respectively. Nucleomorphs have lost their plasmogamy and meiotic proteins completely. *Perkinsela* is an endosymbiotic flagellate living inside *Paramoeba sp*. *Oenothera biennis* is an

angiosperm considered to be functionally asexual. *Adineta* and *Rotaria* are bdelloid rotifers. The whole group is considered to be a truly asexual order of metazoans and were once called 'evolutionary scandals'. *Brachionus* is a genus of monogonont rotifers, sister group of bdelloids but with records of life cycles involving parthenogenesis and episodic sexual reproduction; some populations seem to be asexual in the same Bdelloids are. Microsporidia are highly reduced intracellular parasites of eukaryotes. Microsporidia have a reduced set of meiotic proteins, but it is still compatible with sexual processes. *Candida albicans*, an ascomycete yeast, occurs on animals and may be associated with disease as well; *C. albicans* is considered parasexual, but this genus presents both sexual and parasexual species and the loss of meiosis should be quite recent. *Trichophyton* is a dermatophyte associated with skin diseases in humans and other mammals. *Malassezia* is a basidiomycete genus of fungi living on mammal skin and associated with dandruff. *Malassezia* species have lost basically all their meiotic genes, including SPO11 in two species, a very unusual finding. *Malassezia* is a strong candidate for an asexual eukaryote. *Ustilago* is a sexual parasite of plants and sister group to *Malassezia* and keeps a simplified set of meiotic proteins. *Cryptococcus* is another basidiomycete associated with disease in humans and other animals; it presents a full set of proteins involved in meiosis and its sexual cycle was described recently. Glomeromycotina is a very important group of fungi from the ecological perspective for they associate with plants forming arbuscular mycorrhizae. Sexual processes have never been observed for this group until recently, when recombination of nuclei was assessed in dikaryotic syncytia (Chen et al. 2018). Glomeromycotina present a complete set of proteins specific to meiosis, are probably fully sexual and may perform meiosis with all of its characteristic steps.

Oenothera biennis, a member of primrose family Onagraceae, evolved permanent translocation heterozygosity independently several times in many species, what rendered them functionally asexual (Johnson, Smith, and Rausher 2009). *O. biennis* kept the meiotic machinery even though recombination is rare and alignment of homologous chromosomes basically does not happen. As the close relatives of *O. biennis* are fully sexual, there was no reason to believe that it would lose is meiotic machinery yet and a residual rate of recombination may still occur upon meiosis.

Bdelloid and monogonont rotifers share some meiotic proteins, namely SPO11, REC8, HOP1, MSH4, and MSH5 but only monogononts have MND1, HOP2, and MER3, seemingly lost in bdelloids. The presence of some (highly divergent) meiotic proteins in the genome of the seemingly asexual bdelloids suggests that the meiotic machinery in this group assumed new

functions and might be employed for a very efficient DNA repair of double strand breaks on chromosomes after periods of extreme desiccation; they might be deployed for ameiotic egg production as well. Bdelloids have a very efficient DNA repair and can survive intense ionizing radiation and extreme desiccation (Nowell et al. 2018). The possible acquisition of new functions by meiotic proteins can weaken the current approach, but, considering only metazoans, this phenomenon seem to have occurred only once among a very large amount of lineages. Thus, even though meiotic proteins can evolve to assume new functions in theory, this is still a matter of debate and experimentation.

Microsporidia is another group for which little is known about sexual cycles. They are heavily reduced intracellular parasites and present some of the smallest known eukaryotic genomes (Corradi et al. 2010). This extreme reduction of their genome must have impacted their meiotic machineries. Although greatly reduced, they still present important meiotic proteins like SPO11 and DMC1. Meiosis was described in some microsporidian groups (Hazard and Brookbank 1984) and seems to happen in a simplified way, probably lacking the interference bearing crossover pathway I (absence of MER3, MSH4, and MSH5) and they likely resolve their crossing-overs with MUS81, in the same way of *Schizosaccharomyces* and *Tetrahymena* (Hollingsworth and Brill 2004; Shodhan et al. 2014).

The ascomycete fungus *Candida albicans*, a pathogen and model organism is considered an ameiotic parasexual (Bennett and Johnson 2003). They live as diploid cells and perform fusion to produce tetraploid cells; the tetraploids then transition to a diploid state through random loss of chromosomes and not through a canonical meiosis. Recombination between homologous chromosomes mediated by SPO11 is frequent in this species (Forche et al. 2008). The evolution of a parasexual cycle in this group must be quite recent in evolutionary terms, for it still keeps several meiosis-specific proteins and other species in the same genus undergo meiosis (Lee et al. 2010). Moreover, the meiotic machinery may be used for this parasexual cycle and is thus kept. Another ascomycete, *Trichophyton rubrum,* the most important dermatophyte of its genus in humans has no

known sexual cycle and evidence suggest that all isolates of this species belong to the same mating type (Metin and Heitman 2017). The presence of a complete meiotic protein set and mating types strongly support the occurrence of an undescribed sexual cycle in this species. The genus *Trichophyton* presents other species whose sexual cycles are unknown (L. Wang et al. 2006).

Strikingly, the almost complete absence of meiotic proteins (except for PCH2) in basidiomycete fungus *Malassezia* makes this group a strong candidate for a truly asexual or ameiotic eukaryote. Such an absence of meiotic proteins is only comparable to that of nucleomorphs and sexual processes or meiosis have never been observed in this fungal group. However, the presence of a fungal mating system in their genomes is indicative that a parasexual process is still happening in this group (Xu et al. 2007). Some species still keep SPO11 and this is an indicative that they may have evolved a parasexual cycle similar to that of *Candida albicans*. Both groups live on mammals or inside them and may be subject to similar selective pressures. This slow growing group of basidiomycetes, associated with dandruff in the literature, fills a very specific niche living on fatty acids secreted by the skin of mammals (Xu et al. 2007). They are considered asexual even though related groups (*Ustilago*) are fully sexual. It would be surprising if this fungus can perform meiosis while lacking almost all proteins implicated in the process. All five *Malassezia* species sampled here present a highly reduced set of meiotic proteins, a trend in the group. *Ustilago*, a genus related to *Malassezia*, presents an already reduced but functional set of meiotic proteins. The absence of basically all meiotic proteins indicates not only loss of meiosis, but that this happened a long time ago in evolutionary terms, for the loss of genes is hypothesized to be random and such a process would require millions of years to happen (Li, Gojobori, and Nei 1981). *Cryptococcus* is also a fungal pathogen living on man and other mammals and occupies very similar niches compared to *Malassezia*, however, it possesses a complete set of meiotic genes. Although considered asexual for a long time, *Cryptococcus* was demonstrated to perform a full sexual cycle (Nielsen et al. 2003). Glomeromycotina (here represented by *Gigaspora* and *Rhizophagus*, both are fungi involved in arbuscular mycorrhiza) presents a full meiotic set, even though the whole group is

considered to be asexual. The results strongly support the existence of canonical meiosis in this group. A conserved meiotic machinery was described for *Glomus* (Glomeromycotina) previously (Halary et al. 2011). Additionally, evidence for sexual processes in Glomeromycotina were presented recently (Chen et al. 2018). Frequent recombination of nuclei inside of dikaryotic coenocytic mycelium (a mycelium containing two different kinds of nuclei) was revealed by single nucleus sequencing in *Rhizophagus*.

7. Conclusions and outlook

There is no perceivable difference in the distribution of meiotic proteins between groups known to be fully sexual and putative 'asexuals'. The assessment that many protist lineages for which no sexual processes are described present sets of proteins compatible with full sexual cycles implies that they should be capable of performing sex and meiosis. A sexual ancestor for all eukaryotes was previously proposed (Dacks and Roger 1999; Speijer, Lukeš, and Eliáš 2015); our data not only corroborate this assumption, but indicates the maintenance of sexual processes by all major groups. Considering that the ancestor or all eukaryotes was sexual and the widespread maintenance of the meiotic machinery in basically all groups, there is a natural tendency to assume that all of them may be sexual and that an asexual lineage is a result of a specific and secondary loss of sex. On the other hand, assuming that large and diverse eukaryotic lineages are asexual would require strong evidence. The lack of observations for sexual cycles in most of those groups are probably a result of the scarcity of studies in some territories of the eukaryotic tree. Such organisms deserve more attention. Accumulating evidence suggests that all eukaryotes must be considered sexual *a priori,* and not the other way around.

Approaches for testing the occurrence of sexual cycles in many groups could include: non-monoclonal cultures of organisms to be tested; subjecting individuals to different kinds of stress to stimulate encystment or meiosis; karyotype counting of individual cells in distinct life-cycle stages;

verification of differential expression of meiosis-specific proteins in stages suspected to involve meiosis (specially cysts); ultrastructural studies of cysts for verification of synaptonemal complexes, reductional divisions or karyogamy. As sex in many lineages are being robustly predicted systematically by bioinformatics, descriptions of new sexual life cycles should be just a matter of time.

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Supplementary table 1: Accession numbers and description of plasmogamy and meiosis-specific proteins used for this study.

Supplementary trees: Phylogenies of plasmogamy and meiotic protein families reconstructed for each major eukaryotic group.

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Capítulo 5: Complex evolution of the mismatch-repair system in eukaryotes

Abstract

Repairing DNA damage is one of the most important functions of the 'housekeeping' proteins. DNA molecules are subject to different kinds of damage, and repair systems are specific to each kind of damage: double-strand breaks can be repaired by recombinases (recA/RAD51A); mismatchs are repaired by mutS-mutL system and so on. Both processes are highly conserved in eukaryotes and involve the works of several ancestral paralogues, which are a result of an extensive process of gene duplication and further specialization upon the evolution of the first eukaryotes, including an important part of the meiotic machinery. However, analyses performed some years ago provided different histories for the evolution of each system: the recombinases would be inherited from Archaea, but the mismatch-repair (MMR) system would be inherited from Bacteria (mitochondria). While eukaryotic recombinases present a clear archaeal signature, the eukaryotic MMR system presents a bacterial signature. Recently, the discovery and sequencing of a new group of Archaea, Asgard, allowed a revisiting of the MMR system evolution with the addition of new data from a group closely related to the eukaryotic ancestor. This new analysis provided for a complex evolutionary history of eukaryotic MMR: an archaeal origin for the nuclear MMR system in eukaryotes, with subsequent acquisitions of other MMR systems from mitochondria and chloroplasts. Theses results suggest no mitochondrial contribution to meiosis in eukaryotes and demonstrates that revisiting seemingly settled evolutionary matters may be a worthy idea.

Introduction

The DNA is relatively unstable and subject to frequent breaks and several kinds of damage. DNA damage may be a result of different physical and chemical agents namely UV-radiation, gamma-rays, free oxygen radicals, replication errors, among others (Lindahl 1993). The consequences of DNA damage include from mutations to impairment of DNA function, compromising RNA transcription and protein synthesis, sometimes leading to cellular failure and death (Jackson and Bartek 2009). The evolution of DNA repair machineries allowed for efficient DNA repair and rescue of its functions and became a fundamental part of cellular housekeeping genes. For each kind of damage there is a specific repair system. Well characterized DNA repair systems include homologous recombination (Seitz et al. 1998), photolyases (Essen and Klar 2006), base excision repair (Krokan and Bjørås 2013), nucleotide excision repair (Schärer 2013), non-homologous end joining (Chang et al. 2017), mismatch repair (Li 2008), polymerases (Wood and Shivji 1997) and so on. Some of them are widely distributed in all cellular domains and bear a high level of primary sequence conservation, as in the case of the recombinases implied in homologous recombination and mutS-mutL system implied in mismatch-repair (Lin et al. 2006; Lin, Nei, and Ma 2007).

Efforts have been made to understand the origin and evolution of both DNA repair systems in Bacteria, Archaea and Eukarya (Lin et al. 2006; Lin, Nei, and Ma 2007). Eukaryotic recombinases have been proposed to be related to Archaeal recombinases, which underwent ancestral events of duplication in order to provide the different eukaryotic paralogues, one of them a component of the meiotic machinery (Lin et al. 2006). There is a second kind of recombinases in eukaryotes, this one with a bacterial signature. In this case, their origin can be traced back to both Alpha-proteobacteria and Cyanobacteria. They are a result of endosymbiotic gene transfers from mitochondria and chloroplasts and are active inside those organelles (Hofstatter et al. 2016). Similarly, the mutS homologues that can be observed in eukaryotes evolved by means of ancestral

gene duplications yielding several paralogues, some of with present meiotic functions as well (Lin, Nei, and Ma 2007). However, differently from recombinases, the mutS system implied in mismatch-repair (MMR) would be a contribution from the mitochondrial ancestor to early eukaryotes. Interestingly, bacterial mutS and mutL occur in some Archaeal groups, probably as a result of lateral gene transfers from bacteria to archaea (Lin, Nei, and Ma 2007).

Evidence suggests that the MMR process evolved independently twice after the split of Bacteria and Archaea (Castañeda-García et al. 2017). In Bacteria, the mutS proteins evolved early in bacteria evolution and became an important part of bacterial basic DNA maintenance machinery. The mutS dimer works together with mutL proteins in order to perform DNA repair. A dimer of mutS proteins identifies and is attracted to a site of mismatch on a DNA molecule and recruits a dimer of mutL, which cuts and removes the mismatch, allowing the action of DNA-polymerases and DNA-ligases to fill the gap and finish the process (Modrich and Lahue 1996). Some bacterial groups also present mutH, which bias the nicking and removing the mismatched site towards the unmethylated strand, i.e., the newly synthesized one (Smith and Modrich 1996). This bacterial MMR system, composed basically by mutS-mutL, was transferred laterally to archaeal groups and became part of their DNA maintenance systems as well (Lin, Nei, and Ma 2007). The other MMR system, in this case of apparent archaeal origin, is composed of NucS proteins that occur in several archaeal groups and in Actinobacteria (Takemoto et al. 2018). Although archaeal in nature, NucS homologues do not occur in eukaryotes.

The bacterial MMR system composed of mutS and mutL was acquired by eukaryotes and ancestrally duplicated several times in order to provide the eukaryotic paralogues that occur in most eukaryotic lineages (Modrich and Lahue 1996; Lin, Nei, and Ma 2007). Six mutS eukaryotic paralogues are widespread in eukaryotes, namely MHS1-6; additionally, there are four mutL paralogues, namely MLH1-4. All of them interact in specific ways with each other. Paralogues MHS4 and MSH5 are meiosis specific and do not realize MMR anymore, but participate of crossing-over resolution during meiosis. The evolution of MMR in eukaryotes is linked to the

evolution of meiosis itself. All the eukaryotic mutS homologues are highly conserved and operate MMR not only inside the nucleus, but also inside organelles with their own genome, namely mitochondria and chloroplasts. The occurrence of mutS homologues inside organelles, the bacterial signature of this DNA repair system, and phylogenetic patterns observed by Lin and colleagues (Lin, Nei, and Ma 2007) led to the assumption that the whole eukaryotic MMR system was acquired by eukaryotes laterally from Alpha-Proteobacteria upon the mitochondrial endosymbiosis event. However, the discovery of a new group of Archaea closely related to eukaryotes, the Asgard Archaea, offered the opportunity to revisit this matter (Zaremba-Niedzwiedzka et al. 2017). In this study, we revisit this subject with more data and more advanced techniques in order to verify this supposed mitochondrial contribution to meiosis in eukaryotes.

Material and methods

A strategic sampling of mutS and mutL representatives was performed in order to maximize the number of bacterial and archaeal phyla, while only a few eukaryotic representatives were selected. Special attention was given to Asgard Archaea group because of its close relationship to eukaryotes (Zaremba-Niedzwiedzka et al. 2017). Sequences obtained and characterized from model organisms, such as *Saccharomyces*, *Arabidopsis* or *Homo*, were used as a starting point. Different eukaryotic paralogues were aligned with 100 iterations of MAFFT (Katoh and Standley 2013) aligner for precision. The aligned set of proteins was given as input for HMMER tool *hmmbuild* (Eddy 2011), which was employed to build both mutS and mutL profiles used in the searches. Profiles were employed by HMMER tool *hmmsearch* (Eddy 2011) to search databases (built with strategic sampling) for mutS and mutL homologues. Best hits were extracted from databases with HMMER tool *esl-sfetch* (Eddy 2011) for further processing. Again, resulting sequences were aligned with 100 iterations of MAFFT (Katoh and Standley 2013); the resulting matrix trimmed with Trimal (Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009) where the sites had more than

50% indels/unaligned positions. IQ-Tree (Nguyen et al. 2015) was chosen as a state-of-art algorithm for reconstructions. Heavy mixture models (LG+C60+F+G) were set for reconstructions for both mutS and mutL trimmed matrices. An additional concatenated mutS-mutL matrix was produced for a third tree. Additionally, Asgard data were manually checked for the amount and the relative position of mutS and mutL homologues with each other in the available contigs.

Results

Systematic reconstructions of both mutS and mutL proteins exhibit similar patterns. In the general picture, eukaryotic MMR proteins were acquired three times independently by eukaryotes: one vertically from archaeal ancestors, again laterally from the mitochondrial ancestor, and once again from the primary chloroplast ancestor in photosynthesizing lineages (**Figure 1**). Contrary to the conclusions drawn by an earlier publication (Lin, Nei, and Ma 2007), the main group of eukaryotic mutS paralogues working inside the nucleus (paralogues MSH2-6) are not a mitochondrial contribution, but are a result of gene duplications of a mutS gene present in the archaeal ancestor (**Figure 1**). The archaeal ancestor, by its turn, acquired the mutS laterally from some bacterial group very long ago, before the evolution of the first eukaryotes. The same applies to mutL: mutS and mutL were probably acquired together by the archaeal group that gave origin to eukaryotes and then both underwent gene duplications that are an important step in early eukaryotic evolution. In Argard Archaea, mutS and mutL occur *in tandem* in the same way they occur in Firmicutes. This fact makes Firmicutes a good candidate for a donor of bacterial MMR to Asgard group. The same reconstructions reveal the mitochondrial origin of eukaryotic MSH1, which has an Alpha-Proteobacterial signature. MSH1 exhibits typical mitochondrial signal transit peptides and is imported by mitochondria, where it is active. Still in the same reconstructions, there is a third group of eukaryotic mutS with cyanobacterial origin, acquired from the chloroplast ancestor in the

photosynthesizing groups. Similarly to the mitochondrial mutS, the chloroplastic form exhibits a transit peptide with a chloroplast signal.

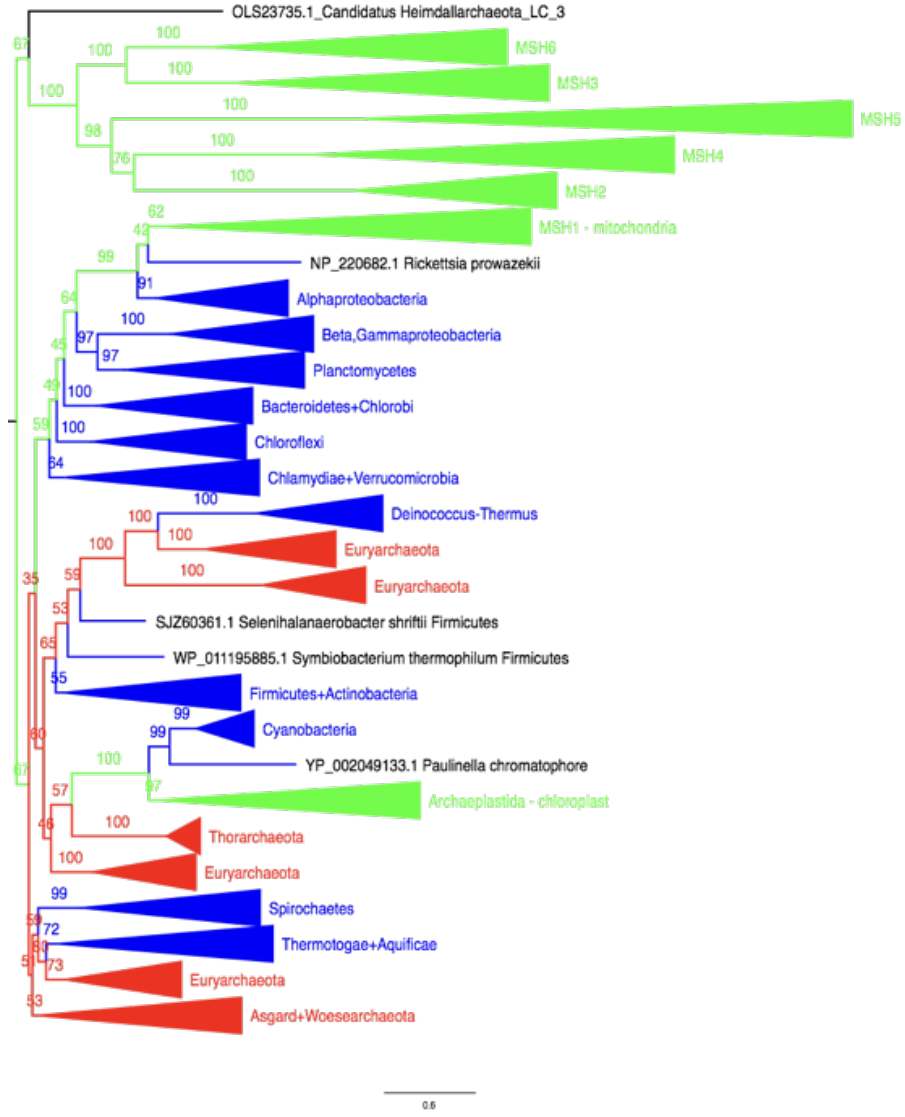

Figure 1. Distribution of eukaryotic mutS protein groups. Eukaryotic mutS was acquired three times: one ancestrally from Archaea and further duplicated to produce the nuclear paralogues; one acquisition from the mitochondrial ancestor, this one active inside the organelle; and again from the chloroplast in the photosynthesizing eukaryotes, active inside de organelle. Heimdallarchaeota is sister-group to all eukaryotic nuclear paralogues. Archaeal groups themselves seem to have laterally acquired the mutS from some bacterial donor once or a few times.

Patterns observed in the mutL reconstructions are similar to the ones observed in the case of mutS (**Figure 2**). The nuclear paralogues MLH1-4 exhibit the same patterns of the nuclear MHS2-6

and must have been acquired together. The mitochondrial mutL seems to have been lost and probably replaced by some nuclear paralogue. The chloroplastic mutL was seemingly lost in the green group, but kept by some red algae.

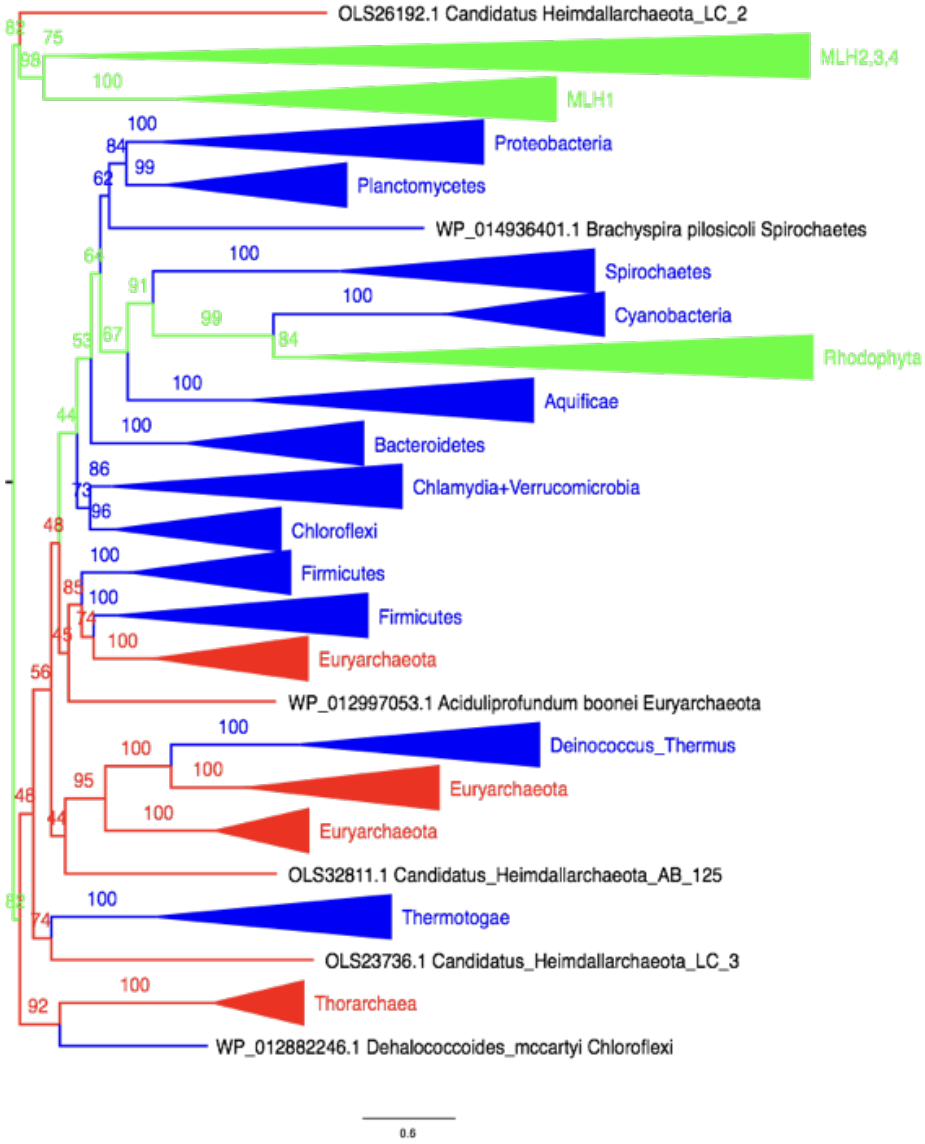

Figure 2. Distribution of eukaryotic mutL proteins. Two distinct groups can be seen: a group of nuclear paralogues, which has Heimdallarchaeota as sister-group; another group, from Rhodophyta, has Cyanobacteria as sister-group. No mitochondrial group could be detected for mutL.

A concatenation of mutS and mutL provides a similar result regarding the evolution of both proteins alone, but this time all Heimdallarchaea are attracted towards the nuclear eukaryotic group (**Figure 3**). This may be interpreted as an increase in the signal of the reconstruction, assuming both genes evolved in concerted evolution.

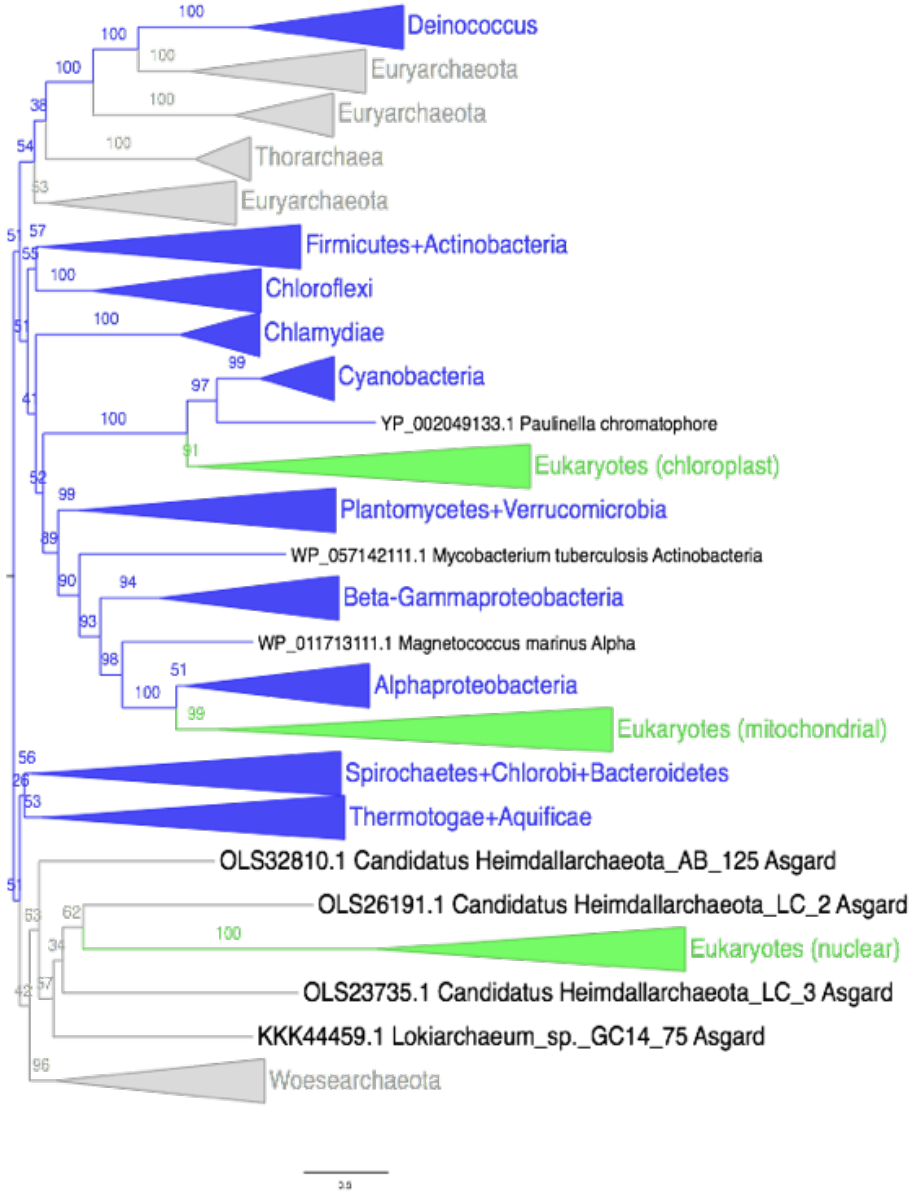

Figure 3. Concatenated mutS-mutL. The main effect of concatenation here is the attraction of all Heimdallarchaeota towards the nuclear eukaryotic group. The nature of the bacterial donor to archaeal groups could not be assessed.

Discussion

The importance of the MMR repair for maintenance of the integrity of the DNA and the high level of conservation of its components across all cellular domains have raised questions about the evolution of the whole MMR system, specially in eukaryotes (Lin, Nei, and Ma 2007). However, by revisiting this matter, we have found a very different and new history concerning mutS and mutL

evolution (**Figure 4**). Back then, a mitochondrial origin for all eukaryotic paralogues was proposed, including both nuclear and mitochondrial forms. The chloroplastic form could not be distinguished then. The introduction of Asgard data was crucial for retelling this history. Asgard is an archaeal group closely related to eukaryotes, or at least, is the closest known archaeal group to eukaryotes that was sampled to this day (Zaremba-Niedzwiedzka et al. 2017).

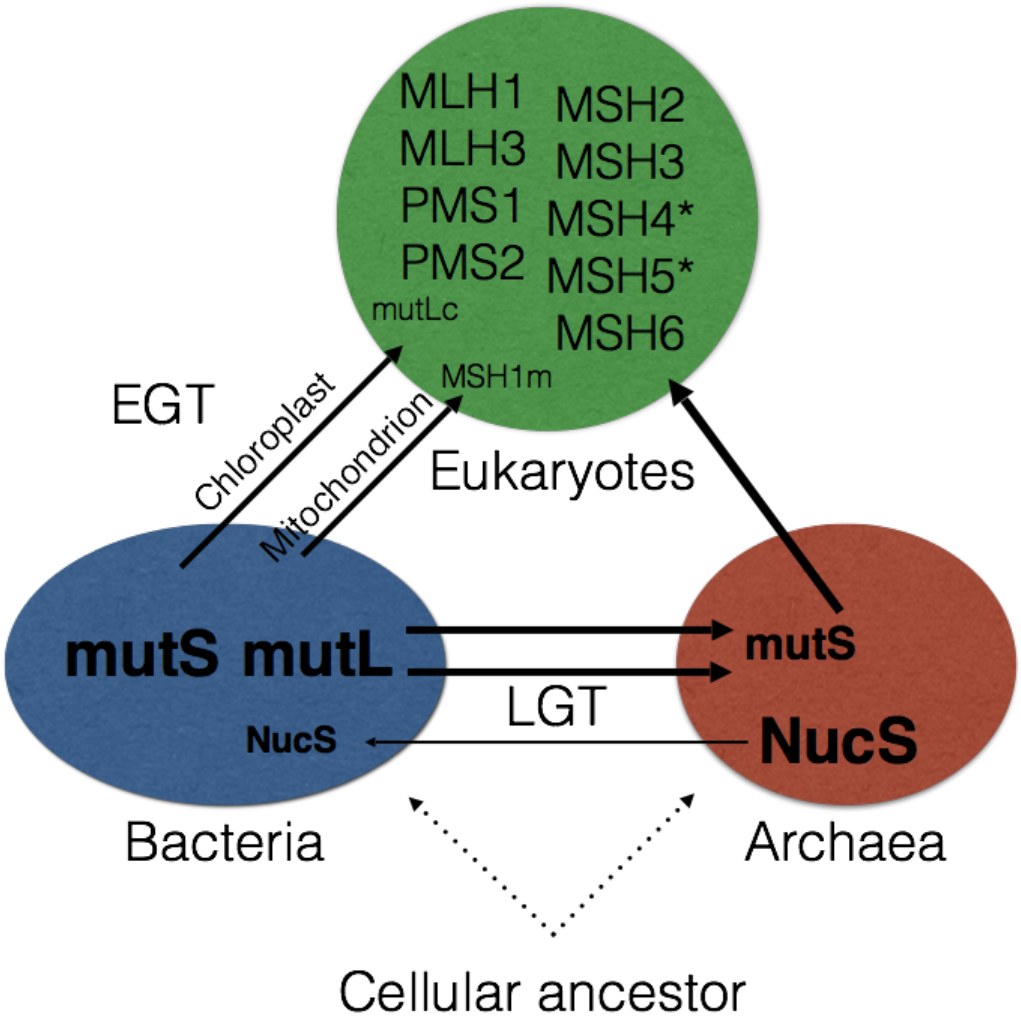

Figure 4. Interpretation of the most probable evolutionary history of the eukaryotic mismatch-repair system based on the patterns observed in the reconstructions of mutS and mutL. The nuclear eukaryotic paralogues are a result of duplications of archaeal mutS and mutL, which remained unduplicated in Archaea and Bacteria. Archaea have another system of mismatch-repair proteins, which are not homologous to mutS-mutL system and must have evolved independently, the NucS. The NucS system does not occur in eukaryotes.

The discovery that the nuclear MMR duties are carried out in eukaryotes by proteins of archaeal origin is more plausible than a mitochondrial origin for this process because other kinds of DNA repair systems that occur in eukaryotes are also of archaeal origin (Lin et al. 2006; Malik et al. 2007). Not only DNA repair systems, but also eukaryotic DNA replication and transcription machineries are of archaeal origin (Kwapisz, Beckouët, and Thuriaux 2008; Makarova, Krupovic, and Koonin 2014). Most nuclear genes that can be traced to organelles act in way similar to 'outsourcing'. Mitochondria and chloroplast import the products of genes that were endosymbiotically transferred to nuclear genome by means of a system of transit peptides that precede active sites of the mature active proteins implied in the process (Neupert and Herrmann 2007). Earlier analyses suggest a mitochondria contribution of Alpha-Proteobacteria to eukaryotic DNA repair system and meiosis (Lin, Nei, and Ma 2007); the new results support a very different history, which implies no mitochondrial contribution known so far to eukaryotic nuclear processes. Additionally, the importance of the mitochondrial endosymbiont to meiosis was overestimated.

As the mitochondrial variable seems to have been isolated here, the understanding of the evolution of sex may be developed independently from the mitochondrial symbiosis event. In this scenario, sex itself may be older than the mitochondria in eukaryotes. The discovery of the Asgard Archaea group played a fundamental role at establishing a new version for the evolutionary history of the MMR in eukaryotes. The introduction of new, strategic data allowed for a reconstruction of a very different model for the evolution of MMR in eukaryotes, a history worth revisiting, like many others.

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Capítulo 6: O sexo e a evolução dos eucariontes

Resumo

Durante muito tempo prevaleceu a classificação de cinco reinos para os seres vivos (organismos celulares). No entanto, o advento de técnicas de sequenciamento e reconstruções de filogenias moleculares levaram à classificação dos seres vivos em três domínios: Bacteria, Archaea e Eukarya. No que diz respeito aos eucariontes, alguns componentes são praticamente universais e ocorrem em quase todos os grupos, entre eles o núcleo, mitocôndrias, flagelos e sexo. Questões envolvendo a evolução do sexo são difíceis de se responder, porque o sexo é muito dispendioso do ponto de vista energético e, aparentemente, não oferece nenhuma vantagem seletiva imediata que possa ser selecionada. Memo assim, o sexo pode ser observado nos mais diversos grupos de eucariontes: animais, plantas, fungos, diversos grupos de protozoários e algas. Mesmo para os grupos de eucariontes para os quais o sexo nunca foi observado, a maquinaria de meiose pode ser encontrada. Sendo assim, o sexo parece ser um processo inerente à vida eucariótica, tendo evoluído no ancestral de todos os eucariontes e mantido em todos os grandes grupos, com uma maquinaria molecular especializada básica compartilhada por todos eles. Neste caso, a grande maioria dos eucariontes exibe um sistema de gametas complementares que se fundem e um processo de meiose que é responsável por forçar a recombinação entre cromossomos homólogos e reduzir a ploidia da célula ao mesmo tempo.

O sexo e a evolução dos eucariontes

A classificação dos organismos em cinco reinos, proposta por Robert Whittaker em 1969, continua sendo a mais disseminada dentre as hipóteses de classificação após cinco décadas (Whittaker 1969). Fundamentalmente, a proposta divide os seres vivos em cinco reinos: Monera,

Protista, Plantae, Fungi e Animalia. Tal classificação era baseada na presença de núcleo celular, ocorrência de multicelularidade e modo de nutrição de cada linhagem. Ainda popular atualmente, a antiga classificação de Whittaker foi profundamente alterada pelas descobertas de Woese e colaboradores ainda na década de 1970 (Woese and Fox 1977; Woese, Kandler, and Wheelis 1990). As descobertas, ligadas ligadas a uma descrição mais apropriada de microrganismos, abriram caminho para o estabelecimento dos assim chamados três domínios, definidos molecularmente e baseados em reconstruções de RNA ribossomal: Archaea, Bacteria e Eukarya. Neste esquema, os eucariontes teriam Archaea como grupo-irmão; a linhagem Archaea-Eukarya, por sua vez, teria Bacteria (Eubacteria) como seu grupo-irmão e todos os grupos seriam descendentes de um único ancestral celular universal. No entanto, reconstruções mais recentes, refeitas com um conjunto maior de dados e técnicas mais modernas, têm colocado Eukarya dentro de Archaea, deixando, assim, o domínio Archaea parafilético (Williams et al. 2013). Eukarya pode, adicionalmente, ser entendido como um grupo resultante de um processo de endosimbiose entre uma Archaea e uma Bacteria, os quais se encontraram há bilhões de anos e estabeleceram uma linhagem fagocítica extremamente bem-sucedida e diversa.

No que diz respeito a Eukarya e à classificação de sua imensa diversidade de formas e linhagens, esforços têm sido feitos no sentido de se organizar os diversos os grupos conhecidos a partir de prováveis relações entre si (T. Cavalier-Smith 1998). Um dos critérios mais importantes para a definição de grupos num primeiro momento foi o padrão de ocorrência de eventos de endossimbioses primárias e secundárias ao longo da evolução dos eucariontes. Por exemplo, uma suposta endossimbiose entre uma alga vermelha e um grupo de protozoários fagocíticos teria dado origem ao reino Chromista, o qual incluiria Heterokonta, Cryptophyta e, às vezes, Alveolata, entre outros. O reino Chromista não encontra respaldo em evidências e tende a ser descartado completamente pela comunidade científica (Hackett et al. 2007; Adl et al. 2019). Grandes rearranjos foram realizados e mesmo grupos aparentemente bem-estabelecidos, como os Fungi, passaram por modificações intensas com a perda de Myxogastria e Oomycota e a aquisição de Microsporidia (Adl

et al. 2019). A posição de Microsporidia dentro de Fungi, porém, têm sido questionada recentemente (Richards, Leonard, and Wideman 2017). Classificações mais modernas são baseadas em técnicas de filogenômica, ou seja, árvores construídas a partir de alinhamentos de dezenas ou centenas de proteínas concatenadas numa única matriz de dados. Destas análises resultam grandes grupos: 'Excavata', Opisthokonta, Amoebozoa, Archaeplastida, S.A.R., Cryptista, Haptista e outros (**Figura 1**).

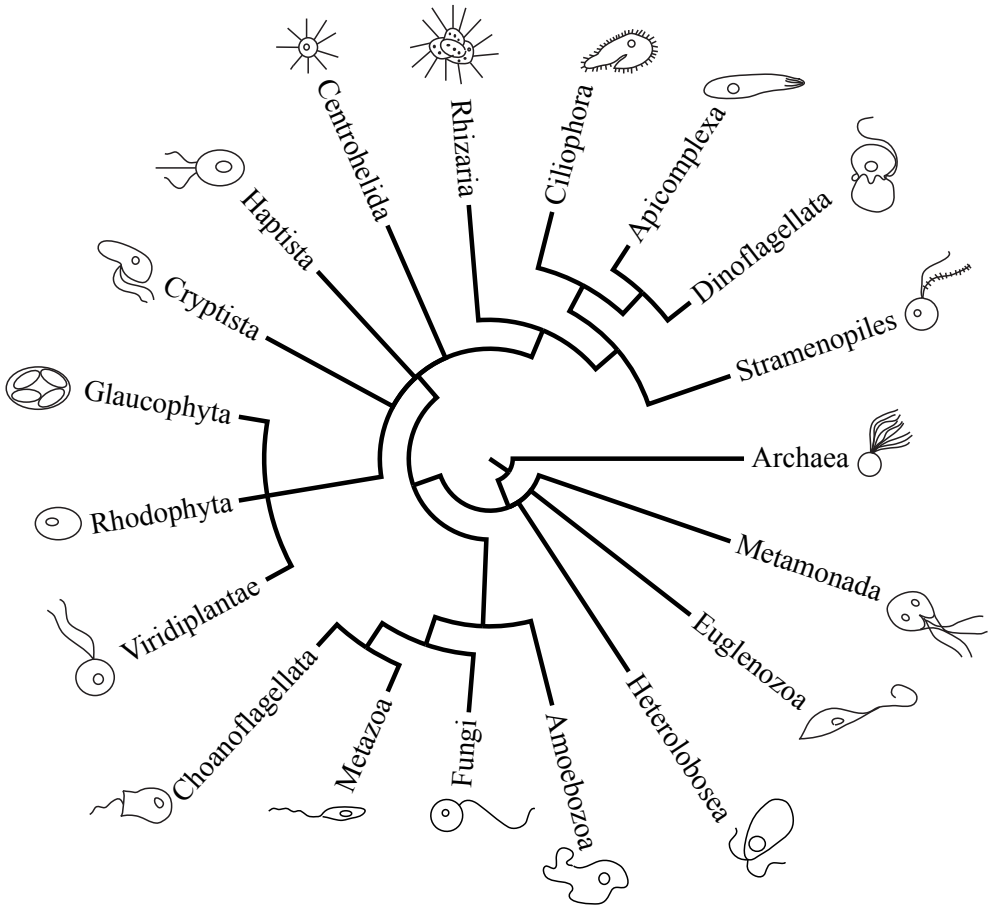

Figura 1: Distribuição dos grandes grupos de eucariontes.

De qualquer forma, alguns elementos básicos permeiam toda a diversidade da vida eucariótica: presença de núcleo, processos de fagocitose, presença de mitocôndrias, flagelos e ocorrência de sexo. Sendo assim, o ancestral hipotético de todos os eucariontes seria um organismo mitocondriado, provavelmente biflagelado e sexual, além de apresentar uma membrana nuclear e

realizar fagocitose como forma de nutrição. No entanto, diversas linhagens tiveram suas mitocôndrias reduzidas a mitossomos e hidrogenossomos (*Giardia*, *Entamoeba*, *Cryptosporidium*) ou até mesmo perdidas completamente (*Monocercomonoides*) (Roger, Muñoz-Gómez, and Kamikawa 2017); alguns grupos perderam um flagelo (Opisthokonta) ou os dois (Tubulinea, Rhodophyta, Dikarya, Angiospermae), assumindo hábitos diversos (Adl et al. 2019); e até mesmo o sexo parece ter sido perdido em alguns casos (Bdelloidea, *Malassezia*) (Hofstatter and Lahr 2019). Ao contrário de mitocôndrias e flagelos, os quais foram perdidos diversas vezes ao longo da evolução dos eucariontes, o sexo (ou pelo menos sua maquinaria molecular) parece ter sido amplamente conservado. A ocorrência de processos sexuais em particular sempre foi tratada na literatura sobre evolução do sexo como uma questão de difícil solução: como poderia um processo que aparentemente não oferece nenhuma vantagem evolutiva imediata e que se mostra extremamente dispendioso do ponto de vista energético ter sido favorecido pela seleção natural e ser tão persistente ao longo da evolução dos eucariontes?

A questão do sexo já foi abordada oportunamente por alguns autores, mas geralmente dentro de uma perspectiva zoocêntrica (Maynard Smith 1971). Em animais, sexo e reprodução são processos intimamente relacionados e coincidentes; no entanto, na maioria dos eucariontes, sexo e reprodução não estão relacionados. O viés zoocêntrico de análise levou ao surgimento de expressões como ‘reprodução assexuada’ em oposição a ‘reprodução sexuada’. Fora do contexto da zoologia (ou botânica), essas expressões não fazem sentido. Adicionalmente, grupos de protozoários têm sido usados como exemplos de organismos assexuados, ou simplesmente, têm sido apresentados como seres assexuados indiscriminadamente (Adl et al. 2012). Entre os principais exemplos de organismos assexuados pode-se notar a presença de amebas como modelo de ciclo de vida que não inclui qualquer processo sexual, criando um senso-comum, inclusive entre especialistas, de que estes organismos são assexuados (Smirnov et al. 2011). Isto se dá, em grande parte, pela dificuldade de se observar processos sexuais (como a meiose) em amebas. Inversamente, processos sexuais podem ser facilmente observados em outros grupos de protistas, como ciliados e

Apicomplexa. Porém, os processos sexuais de representantes de Apicomplexa são muitas vezes omitidos em representações do ciclo de vida de seus representantes (muitas vezes, refere-se à meiose como 'esporogonia') e, curiosamente, os processos sexuais de ciliados recebem o nome de 'conjugação' (Loidl et al. 2012), termo também empregado para processos observados em bactérias, estas sim seres assexuados.

A meiose eucariótica

A meiose é uma processo exclusivo dos eucariontes. As principais etapas do processo são altamente conservadas nas mais diversas linhagens eucarióticas: a formação do bouquet facilita o alinhamento dos cromossomos homólogos; uma vez alinhados, os cromossomos homólogos são ligados por uma estrutura proteica semelhante a um zíper e tal estrutura mantém os homólogos coesos; quebras de dupla-fita de DNA são produzidas por uma maquinaria que contém topoisomerases específicas da meiose; este dano provocado no DNA recruta uma maquinaria de reparo por homologia e força a recombinação dos cromossomos homólogos de forma coordenada; durante a recombinação, junções de Holliday se formam, as quais são resolvidas normalmente por uma maquinaria também específica da meiose; os cromossomos homólogos são separados com resultante redução da ploidia da célula, sendo que a ocorrência dos pontos de recombinação ajuda a organizar a correta distribuição de cromossomos para as células resultantes; a coesão entre as cromátides-irmãs é desfeita para que sejam separadas também, resultando em quatro células ou núcleos; um processo de conversão gênica pode ocorrer após a meiose.

Mesmo nos meios de protistologia, prevalecem visões de que alguns grupos de eucariontes seriam sexuados (ciliados, Apicomplexa, algumas algas verdes, as plantas terrestres, algumas algas vermelhas, algas pardas, oomicetos, muitos fungos e animais) e que outros grupos seriam assexuados (Amoebozoa, Rhizaria, Cryptophyta, Euglenozoa, Metamonada, Microsporidia, Glaucophyta, diversas algas verdes e vermelhas unicelulares, Glomeromycotina, *Capsaspora* e outras 'linhagens-órfãs'). Grupos mais estudados tendem a ser representados como grupos sexuados, ao passo que grupos menos estudados e unicelulares tendem a ser representados como assexuados (Spiegel 2011). Tendo em vista as prováveis relações entre as diferente linhagens de

eucariontes entre si, a visão de que grupos sexuados e assexuados estariam espalhados pela árvore dos eucariontes sem nenhum padrão claro exigiria a perda do sexo independentemente muitas vezes. Tal cenário não pode ser de maneira alguma um cenário parcimonioso.

Um ancestral sexual para todos os eucariontes já foi proposto a partir dos padrões de distribuição de processos sexuais nas diferentes linhagens (Dacks and Roger 1999). Nesta ocasião, a expressão 'sexuado facultativo' foi empregada para se referir a linhagens que apresentam seus processos sexuais desatrelados da reprodução, como seria o caso da maioria dos eucariontes, à exceção de animais, plantas terrestres e algas vermelhas. Tal expressão é vazia, uma vez que sexo e reprodução são processos não relacionados no ciclo de vida da maioria dos eucariontes, e quando o são, isso se deve a limitações impostas aos processos sexuais pela ocorrência de multicelularidade em determinados grupos. Novamente aqui, nota-se a influência do paradigma zoocêntrico na construção do conhecimento acerca do sexo em eucariontes. Sendo assim, pode-se sugerir que expressões como 'reprodução sexuada', 'reprodução assexuada' e 'sexuado facultativo' sejam evitadas ou deixem de ser usadas fora do contexto específico da zoologia; na realidade, a expressão 'sexuado facultativo' não faz sentido algum. Além de vazias e não informativas, tais expressões reforçam a ideia incorreta de uma associação obrigatória entre sexo e reprodução. O sexo pode ser entendido como um processo de recombinação de cromossomos e reparo de DNA; a reprodução, como um processo de multiplicação, aumento do número de indivíduos de uma população ou espécie.

Os processos sexuais em eucariontes parecem obedecer a algumas linhas gerais. Geralmente, processos sexuais são desencadeados por estímulos ambientais que provoquem algum tipo de estresse nos organismos implicados. A ocorrência de sexo em períodos de estresse e incerteza reforça a noção de que o sexo é importante adaptativamente por possibilitar novas combinações de caracteres genéticos; tais combinações novas favoreceriam alguns indivíduos permitindo-lhes um maior poder de adaptação a novas condições do ambiente ou mudanças nas pressões de seleção sobre os organismos. Nota-se, adicionalmente, uma associação entre meiose e a

formação de cistos ou esporos de resistência em muitos grupos (Thomas Cavalier-Smith 2010). Para isso, pode-se apontar inúmeros exemplos, nos quais a meiose ocorre dentro de cistos de resistência: Apicomplexa, Amoebozoa, Fungi, dinoflagelados, algas verdes etc. Tal padrão é sugestivo de que o cisto é uma característica ancestral e sempre esteve associado à meiose. Outro fator associado a processos sexuais é a presença de flagelos; alguns grupos apresentam flagelos somente em gametas – produzidos por meiose – e em nenhuma outra parte de seus ciclos de vida. Exemplos desta associação podem ser vistos em: Metazoa, plantas terrestres (briófitas, pteriófitas, cicadáceas), Foraminifera, Myxogastria, algas pardas, Apicomplexa (neste caso, não por meiose) etc.

Outra característica inerente ao sexo é a presença de gametas complementares. Numa perspectiva zoocêntrica, os tipos complementares são convencionalmente chamados de 'feminino' e 'masculino'. Por extensão, a mesma terminologia é empregada pra outros grupos, como plantas. O sistema de gametas complementares impede a autofecundação e força a recombinação entre os tipos diferentes, mesmo que o custo disso seja, muitas vezes, o não-encontro e a perda das células. O sistema de determinação de 'gêneros' (*mating types*) ou tipos complementares pode ser observado na maior parte dos eucariontes e deve ser ancestral (Speijer, Lukeš, and Eliáš 2015). Tal sistema envolve a participação da proteína de membrana HAP2, a qual é expressa em apenas um dos tipos de gametas independentemente da ocorrência de isogamia ou anisogamia. HAP2 medeia o processo de plasmogamia em praticamente todos os grandes grupos de eucariontes. Perdas notáveis de tal sistema podem ser observadas em Fungos e em Bilateria. Apesar das perdas, novos sistemas de diferenciação de gametas e 'gêneros' evoluíram nestes grupos, assumindo o lugar do sistema ancestral que foi perdido, muitas vezes levando a sistemas ainda mais complexos em fungos, por exemplo (Lee et al. 2010). Embora onipresente em eucariontes, a proteina HAP2 não pôde ser traçada a Archaea e uma suposta origem viral foi atribuída para a proteína (Fédry et al. 2017). Como evidência, semelhanças estruturais entre HAP2 e proteínas virais foram observadas.

Assim como o mecanismo de plasmogamia mediado por HAP2, a meiose conta com uma maquinaria específica. Proteínas-chave são empregadas pelas células em diferentes etapas da

meiose. Constituem passos importantes para a meiose: compactação dos cromossomos pela ação de cohesinas envolvendo a participação de REC8; quebra de dupla-fita de DNA cromossômico mediado pela ação de SPO11; emparelhamento e alinhamento de cromossomos homólogos para recombinação, processo mediado por HOP1 e PCH2; formação do complexo sinaptonemal entre os homólogos com participação de diversas proteínas estruturais que não apresentam homologia traçável da sequência primária entre grandes grupos de eucariontes, com exceção de ZIP4, a qual é conservada; recombinação (*crossing-over*) entre homólogos mediada por um complexo altamente conservado e que requer a participação de DMC1, HOP2, MND1; resolução das junções cromossômicas (*Double Holliday Junctions*) por meio do complexo de interferência, composto por MER3, MSH4, MSH5; resolução das mesmas junções cromossômicas por um complexo sem interferência, composto por MUS81 e MMS4; secção de fita simples por MLH1 e MLH3 ao final do processo, o que culmina com a separação física dos cromossomos homólogos e posterior segregação dos mesmos (Hofstatter, Brown, and Lahr 2018). Após a segregação dos homólogos, o processo de reparo de DNA corrige bases mal-pareadas (*mismatches*) nos trechos que foram recombinados, culminando num processo de conversão gênica. Alelos diferentes podem ser convertidos em um único alelo neste processo de conversão gênica, ou seja, um dos alelos desaparece no processo.

A maioria das proteínas citadas acima é específica da meiose e altamente conservada ao longo de toda a diversidade eucariótica. Por causa disso, a presença desta maquinaria pode ser usada para a detecção de processos sexuais 'ocultos' em linhagens de organismos tidos como assexuados e cujo genoma esteja disponível (Schurko and Logsdon 2008). A abordagem foi inicialmente aplicada a alguns organismos tradicionalmente tidos como assexuados, entre eles *Giardia* (Ramesh, Malik, and Logsdon 2005) e *Trichomonas* (Malik et al. 2008), com resultados positivos, ou seja, diversas proteínas da maquinaria da meiose foram detectadas. Resultados positivos como estes abrem a possibilidade de que organismos tidos como assexuados possam ser encarados como potencialmente sexuados, o que tem implicações evolutivas e para a biologia destes

grupos. Adicionalmente, pode haver implicações práticas decorrentes do sexo, como no caso de patógenos, já que diferentes cepas do mesmo parasita resistentes a um ou mais medicamentos podem passar por processos sexuais e algumas células podem se tornar duplo-resistentes num único evento de recombinação. De qualquer forma, a mera existência de processos sexuais em protozoários parasitas pode representar um desafio para o sistema imune do hospedeiro. De qualquer forma, *Giardia* e *Trichomonas* são linhagens muito basais e a presença da maquinaria de meioise nestes organismos reforça a noção de um ancestral sexuado para todos os eucariontes.

A possibilidade de um ancestral eucariótico comum a todas as linhagens ser sexuado não significa necessariamente que todos os seus descendentes sejam igualmente sexuados, dada a imensa diversidade dos eucariontes em todos os âmbitos de sua morfologia e biologia. Algumas linhagens poderiam ter perdido o sexo secundariamente e independentemente ao longo da história evolutiva dos eucariontes, a qual se estende seguramente por mais de um bilhão de anos. Se diversas linhagens puderam ter perdido a mitocôndria ou o flagelo, por que seria o sexo poupado em todos os casos? Umas maneiras de se responder preliminarmente esta questão seria a detecção (ou não) da maquinaria meiótica nas mais diversas linhagens conhecidas e amostradas de eucariontes. Dados recentes sugerem que basicamente todos os grandes grupos de eucariontes, Metazoa, Fungi, Chlorophyta, Rhizaria, Glaucophyta, Rhodophyta, Amoebozoa, Stramenopiles, Cryptophyta, Haptophyta, Metamonada, Euglenozoa, Alveolata, e mesmo linhagens isoladas (‘linhagens-órfãs’), *Capsaspora*, *Ministeria*, *Thecamonas* e outros, são plenamente sexuados por causa da onipresença da maquinaria meiótica (Hofstatter and Lahr 2019). A ocorrência da maquinaria meiótica completa nas diferentes linhagens indica, adicionalmente, que os possíveis processos meióticos são muito conservados e, portanto, potencialmente similares entre si. Em suma, estes resultados reforçam a noção de um eucarionte ancestral sexuado e sugerem a manutenção do sexo em todos os grandes grupos de Eukarya com poucas modificações. No entanto, uma pequena linhagem parece ter perdido a maquinaria meiótica totalmente e, com ela, os processos sexuais como um todo: o gênero de fungos basidiomicetos *Malassezia*, causador de caspa em humanos e

outros animais. Este pequeno gênero de fungos parece ser um forte candidato para um eucarionte verdadeiramente assexuado (Hofstatter and Lahr 2019). Além de *Malassezia*, um grupo de rotíferos, os Bdelloidea, parece carecer de processos sexuais há milhões de anos. Representantes desde grupo perderam diversas proteínas da maquinaria meiótica e os componentes restantes parecem ter assumido novas funções, provavelmente no reparo de DNA. Adicionalmente, machos nunca foram observados em séculos de estudo sobre este grupo e análises indicam que sua estrutura genômica é incompatível com processos sexuais, neste caso por causa de uma aparente tetraploidia degenerada (Flot et al. 2013). Alguns elementos de transposição onipresentes em outros grupos de animais estão ausentes em Bdelloidea, uma evidência adicional para sua putativa assexualidade (Arkhipova and Meselson 2000).

As mesmas análises também revelam a origem e a evolução da maquinaria da meiose na transição de Archaea para os primeiros eucariontes. As proteínas específicas da meiose e que atuam nas diversas etapas do processo são resultado de um amplo processo de duplicação gênica no ancestral eucariótico, com subsequente aquisição de novas funções por parte de parálogos (Hofstatter and Lahr 2019). No entanto, nem todos os componentes da maquinaria apresentam homólogos traçáveis em Archaea e, portanto, têm origem desconhecida. Aparentemente, não houve qualquer contribuição mitocôndrial para a maquinaria da meiose, já que nenhum dos componentes desta maquinaria pode ser traçado a Alpha-Proteobacteria. A origem da maquinaria da meiose pode ser vista como um belo exemplo de como os processos de duplicação gênica podem ser importantes para a evolução de novas características em seres vivos em geral. Parálogos produzidos por duplicação gênica estariam, teoricamente, livres das amarras da seleção (natural) purificadora e poderiam então adquirir novas funções/subfunções ou se especializar em processos específicos, como a meiose, a qual cooptou diversas maquinarias de reparo e manutenção de DNA para si. Tais processos poderiam ter acontecido antes ou durante a eucariogênese como um aprimoramento dos processos de fusão celular e recombinação que podem ser observados em Archaea atualmente, mas que já estariam acontecendo desde uma era que precede a própria existência dos eucariontes.

Mecanismos de duplicação gênica e sua importância

Em sua obra de 1970, *Evolution by gene duplication*, Susumo Ohno descreveu em detalhes processos de duplicação gênica e sua importância para a evolução dos eucariontes. Em tese, duplicações gênicas ocorrem como resultado de 'falhas' em processos celulares básicos, como a replicação do DNA, ocorrência de poliploidização ou distribuição incorreta de cromosomos na meiose. As cópias dos genes duplicados estariam livres da pressão da seleção natural e poderiam adquirir novas funções ou simplesmente perder sua função por decaimento mutacional. A ocorrência de duplicações gênicas é relativamente frequente e forneceu um rico material para novidades evolutivas nos mais diversos grupos de organismos. Uma destas inovações foi o surgimento de uma maquinaria específica para a realização da meiose no eucariontes.

Uma vez que as maquinarias conservadas de meiose e plasmogamia podem ser encontradas em todos os grandes grupos, resta a dúvida: se todos os eucariontes são ancestralmente sexuados e conservaram a maquinaria para tal, por que a meiose não é observada em todos os grupos, mas somente em alguns? E mesmo organismos-modelo extensivamente estudados, como *Cyanidioschyzon merolae*, *Galdieria sulphuraria*, *Amoeba proteus*, *Giardia intestinalis*, *Euglena gracilis* e outros, permanecem em ciclos de vida aparentemente mitóticos de forma indefinida em cultura. Uma vez que suas maquinarias meióticas conservadas são reveladas, deveria ser somente uma questão de tempo até que seus ciclos de vida fossem completamente descritos. Amebas (Amoebozoa) constituem um interessante exemplo, pois são tipicamente estudadas e entendidas como organismos assexuados, com exceção de um de seus grupos: Myxogastria. Este grupo foi por muito tempo considerado parte de Fungi, sob a denominação de Myxomycota, e alvo de mais estudos que outros grupos de Amoebozoa como consequência. Como resultado disso, Myxogastria é basicamente o único grupo dentro de Amoebozoa com ciclos sexuais conhecidos e razoavelmente descritos (Aldrich 1967). Tal fato indica que a qualificação de um grupo como 'assexuado' pode ser

um artefato da falta de estudos, já que há uma correlação entre falta de estudo e 'assexualidade' (Spiegel 2011). Mesmo em Myxogastria, a demonstração de processos sexuais é difícil por sua natureza secretiva. No artigo da publicação do genoma preliminar de *Physarum*, seu ciclo de vida aparece de forma equivocada (Schaap et al. 2015), com a meiose sendo apontada nos corpos de frutificação do organismo e não no interior do esporo já liberado, como seria o correto (Aldrich 1967). Novamente, como discutido anteriormente, a meiose em *Physarum*, um representante bem estudado do grupo, ocorre dentro do esporo/cisto e não dentro do corpo de frutificação como comumente retratado. Outros grupos de Amoebozoa carecem de informações sobre seus processos sexuais por falta de estudos mais detalhados sobre sua biologia. Apesar da aparência de assexuadas, todas as linhagens amostradas de Amoebozoa até o momento apresentam a maquinaria meiótica praticamente completa (Hofstatter, Brown, and Lahr 2018). Recentemente, demonstrou-se que *Entamoeba* expressa a maquinaria da meiose algumas horas após a formação do cisto de resistência; o cisto maduro termina com a formação de quatro núcleos, o que indica a ocorrência de meiose no interior do cisto (Ehrenkaufer et al. 2013).

Assim, como Amoebozoa, diversos outros grupos carecem de informações sobre seus ciclos de vida (sexuais), apesar da presença de maquinaria meiótica, em grande parte por falta de estudos. Enquadram-se neste caso Glaucophyta, Rhizaria, Cryptophyta, Euglenozoa, Metamonada, Glomeromycotina e outros. Mas como poderiam eles terem seus processos sexuais revelados? Diversas abordagens experimentais podem ser empregadas para revelar mais informações acerca de seus processos sexuais. Uma abordagem possível consiste em expor os organismos a diferentes tipos de estresse, já que processos sexuais e, especialmente a meiose, são desencadeados por estresse. Alternativamente, já que prevalece em eucariontes um sistema de gametas complementares, culturas monoclonais devem ser evitadas, porque a maioria das linhagens de eucariontes aparentemente não realiza auto-fecundação; diferentes culturas da mesma espécie podem também ser misturadas para se obter plasmogamia em cultura. Assumindo que a meiose está associada a cistos de resistência, atenção especial deveria ser dedicada aos cistos. Dada a imensa

diversidade de formas e processos em eucariontes, é possível que a meiose ocorra de formas muito diferentes daquelas observadas em animais, plantas e fungos; neste caso, a meiose poderia ser confundida com duas mitoses sequenciais. Para se demonstrar a meiose neste caso específico, seria necessário o emprego de técnicas de cariótipo: a contagem do número de cromossomos em diferentes momentos do ciclo de vida pode expor processos de redução do número de cromossomos e uma transição de um estado diploide para um estado haploide, com subsequente plasmogamia.

Em suma, um aprofundamento dos estudos sobre sexo em diferentes grupos de organismos pode revelar histórias naturais ainda desconhecidas. É importante um distanciamento do paradigma zoocêntrico, já que o conhecimento sobre ciclos de vida de animais pode contribuir muito pouco para o avanço do conhecimento da biologia dos processos sexuais e ciclos de vida em eucariontes em geral. Animais representam somente um pequeno ramo dentro da diversidade eucariótica e muita atenção tem historicamente sido dada a este grupo em detrimento de outros. Somente um entendimento mais detalhado da biologia dos diversos grupos de protozoários poderá fornecer subsídios para um entendimento mais profundo da evolução dos próprios animais e plantas e promover avanços científicos mais significativos nos diversos campos da biologia.

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