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1 **“Meiotic genes” are constitutively expressed in an asexual amoeba**
2 **and are not necessarily involved in sexual reproduction**

3
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15
16 **Keywords**

17 *Acanthamoeba*, Muller’s ratchet, polyploidy, asexual reproduction, meiosis, RNA-seq

18

19 **Abstract**

20

21 The amoebae (and many other protists) have traditionally been considered as asexual organisms but
22 suspicion has been growing that these organisms are cryptically sexual or are at least related to
23 sexual lineages. This contention is mainly based on genome studies in which the presence of
24 “meiotic genes” has been discovered. Using RNA-seq (next generation shotgun sequencing,
25 identifying and quantifying the RNA species in a sample), we have found that the entire repertoire
26 of meiotic genes is expressed in exponentially growing *Acanthamoeba* and we argue that these so
27 called meiotic genes are involved in the related process of homologous recombination in this
28 amoeba. We contend that they are only involved in meiosis in other organisms that indulge in
29 sexual reproduction and that homologous recombination is important in asexual protists as a guard
30 against the accumulation of mutations. We also suggest that asexual reproduction is the ancestral
31 state.

32

33 **1. Introduction**

34

35 It is currently assumed that sexual reproduction is the ancestral state of eukaryotes and that
36 asexuality arose later [1-4]. Meiosis is necessary in sexual reproduction to produce haploid gamete
37 cells. These gametes then fuse to form a fertilized egg in which parental genomes rearrange to
38 produce a unique diploid nucleus. This being the case, an organism cannot reproduce sexually
39 without genes that facilitate meiosis, but some have inverted this argument and suggest that the
40 possession of meiotic genes indicates a facility for sex [5]. The list of protists in which these meiotic
41 genes have been discovered and for which sexual reproduction has therefore been inferred is
42 growing, and presently includes *Entamoeba*, *Leishmania* and *Giardia* [6], *Ostreococcus* [7],
43 *Trichomonas* [8], the choanoflagellate *Monosiga* [9], algae [10], mycorrhizal fungi [11], the
44 dinoflagellates [12,13], the freshwater amoeba *Cochliopodium* [14], and the soil amoeba,
45 *Acanthamoeba* [15]. Evidence does exist for sexual processes in a minority of these groups such
46 as *Leishmania* [16] but there is no evidence for this in others such as *Acanthamoeba*. It has also
47 been pointed out that these “meiosis genes” may have other functions such as homologous
48 recombination [17,12] in polyploid organisms including *Acanthamoeba* [18]. Here we report that
49 all genes previously classified as being meiosis specific are expressed constitutively in
50 exponentially growing *Acanthamoeba* cultures in which no cell fusion has been reported. We
51 therefore conclude that they are not likely to be involved primarily in meiosis and speculate that in
52 *Acanthamoeba* they have other functions such as homologous recombination (the exchange of
53 genetic information between two extensively homologous strands of DNA).

54

55 **2. Materials and Methods**

56 Two strains of *Acanthamoeba* (GS-336 and SB-53) were used in this study both of which are of the
57 T4 genotype and both are closely related to the Neff strain (ATCC 30010) for which a complete
58 genome is available [19]. *Acanthamoeba* strains were grown in axenic media (Bacto tryptone 14.3
59 g/L, yeast extract 7.15 g/L, glucose 15.4 g/L, Na₂HPO₄ 0.51 g/L and KH₂PO₄ 0.486 g/L pH6.5) in
60 which the doubling time was measured to be 8.5 hours at 20°C. RNA was extracted from
61 exponential *Acanthamoeba* cultures using an RNeasy Mini Kit (Qiagen). The quality of the RNA
62 was determined by agarose gels and by a QUBIT RNA BR (broad-range) Assay Kit (Thermo-Fisher
63 Scientific). cDNA Libraries were prepared for automated TruSeq stranded mRNA-seq from the
64 total RNA from single culture of the two *Acanthamoeba* strains. The sequencing data generation
65 was made with HiSeq-4000 75PE by Edinburgh Genomics. The reference genome (FASTA and
66 GTF files) from *Acanthamoeba castellanii* was obtained from ENSEMBL Protists [19,20]. Raw
67 data quality control was performed using the FASTQC program (Simon Andrews
68 <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The genome was indexed and reads

69 were aligned to the reference genome using STAR to obtain the required BAM files [21]. The
70 alignments and the BAM files were visualised using SAMtools and IGV to verify the quality of the
71 results [22,23]. GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and amoebaDB
72 (<http://amoebadb.org/amoeba/>) were searched for meiosis specific genes.

73 A complete set of “meiosis genes” has been identified in *Acanthamoeba* through GenBank searches
74 and by BLAST searches using known homologs from a variety of other organisms. The
75 identification of each candidate has been studied by phylogenetic analysis to ensure that the
76 *Acanthamoeba* homolog position was compatible with isoforms from other organisms. Where there
77 was more than one candidate gene, phylogenetic analysis and direct pairwise sequence comparisons
78 of better characterised orthologs from other species were made to ensure that the correct
79 *Acanthamoeba* ortholog had been selected. Sequences were compiled using Seaview [24] and
80 BioEdit [25] was used to edit alignments by eye and to determine levels of identity. Maximum
81 likelihood phylogenetic trees were created with PhyML [26] using the GTR model with 100
82 bootstrap pseudo-replicates.

83

84 **3. Results**

85 We have analysed a set of meiosis-specific genes by maximum likelihood phylogenetic analysis to
86 ensure that these genes are likely to be homologs of meiosis specific genes identified and
87 characterised in other organisms. An example (Hop2) is shown in Figure 1 where the identified
88 *Acanthamoeba* homolog branches in the expected position with good support among the
89 amoebzoa. All other meiosis-specific *Acanthamoeba* homolog genes have been similarly tested.
90 Only two meiosis-specific genes within the set studied here were found to have more than one
91 candidate in the *Acanthamoeba* genome. In both cases it was clear from the phylogenetic tree
92 analysis and by individual pairwise sequence comparisons which of these was the best candidate
93 for the *Acanthamoeba* homolog and these were selected for this study.

94 The specificity of the RNA-seq approach was tested by searching for cyst specific protein genes as
95 a negative control ACA1_075210, ACA1_075240, ACA1_327930, ACA1_399800 and none of
96 these appeared in our expressed protein data base. Cyst specific protein 1 is expressed in
97 *Acanthamoeba* as it differentiates into cysts [27]. As expected, actin (ACA1_361250,
98 ACA1_361250) and EF1 α (ACA1_138040) genes were heavily expressed (Figure 2). The lack of
99 cyst specific transcripts confirms that these particular cultures are in log phase as cysts form in post-
100 log phase.

101 We have discovered that all the identified meiosis genes are expressed in exponentially growing
102 amoebae indicating that the expression is not restricted to cells undergoing meiosis (Figure 2).
103 These genes include the core genes (Spo11, Hop1, Hop2, Mnd1, Mlh1, Mlh2, Pms1, Dmc1, Msh2,
104 Msh4, Msh5, Msh6, Rad50, Rad51, Rad52) that are “meiosis-specific” since they are known to
105 orchestrate meiosis only in organisms with a sexual ancestry [6, 8, 15]. Two other genes, HAP2 and
106 GEX1, have been included in the present study as they are involved in cell and nuclear fusion and
107 so have been used as markers for sexual reproduction [2].

108

109 **4. Discussion**

110 Current opinion tends to consider sexual reproduction as being ancestral and that asexual organisms
111 have subsequently lost this ability [1,2]. On theoretical grounds it has been concluded that asexual
112 reproduction can only be transient as such organisms would experience the accumulation of
113 deleterious mutations. This has become known as Muller’s ratchet [28]. However, a counter to this
114 argument is that Muller’s ratchet does not operate in organisms that are polyploid as the productive
115 mutation rate is limited by correction through homologous recombination [18]. It has been argued
116 that the bdelloid rotifers have adopted another way around the problem of Muller’s ratchet without
117 sexual reproduction through extensive horizontal gene transfer [29]. However, this idea has been

118 challenged by the observation that the genomic DNA used for this study was significantly
119 contaminated [30]. It is interesting to note that like *Acanthamoeba*, the genome of the bdelloid
120 rotifer *Adineta vaga* contains a set of core meiotic genes in the clear absence of meiosis [31].

121 In some lineages that have been viewed as being asexual, evidence has been discovered for the
122 existence of sexual reproduction. The general trend is for members of the excavata, sexual
123 reproduction tends to dominate. This has been described in *Trypanosoma* where cell fusion is
124 reported [32], in *Naegleria lovaniensis* inferred from isoenzyme analysis [33] in microscopic
125 analysis of *Leishmania* amastigotes within macrophages [16], from population genetic analysis in
126 *Giardia* [34], and in *Trichomonas* [35]. The amoebozoa seem to be dominated by asexual members
127 such as *Entamoeba* and *Acanthamoeba*, but meiosis and sexual reproduction has been demonstrated
128 in others, for example by genetical analysis in *Dictyostelium* [36], and by morphological
129 examination in *Cochliopodium* [4, 37] and in the testate amoeba *Arcella* [38]. Many protists
130 including those assumed to be from the most primitive lineages show no indication of sexual
131 reproduction. A growing list of organisms that were assumed to be asexual but which are found to
132 possess meiosis specific genes are suspected to have a sexual reproductive capacity which may be
133 hidden by culture conditions. For example, Ramesh and co-workers contend that “The presence of
134 these genes indicates that: (1) *Giardia* is capable of meiosis and, thus, sexual reproduction” [6].
135 However, in our view, all that the presence of these genes indicates is that the lack of sexual
136 reproduction in these organisms cannot be blamed on a lack of these genes.

137 The fact that all the meiotic genes are expressed in logarithmically growing *Acanthamoeba* in
138 significant quantities means that they are unlikely to be primarily involved in meiosis since there is
139 no indication that these amoebae are fusing or any other sign of meiosis or sexual reproduction.
140 Although the difference between sexual and asexual reproduction is usually quite distinct, several
141 redefinitions of the processes have lessened the distinction. True sexual reproduction usually
142 includes meiosis to produce haploid gametes, cell fusion, then nuclear fusion, to form a diploid cell.
143 Within the context of *Giardia*, ‘sexual reproduction’ and ‘sex’ have been defined much more
144 broadly as “any process in which chromosomes from two cells, or two nuclei in the same cell, are
145 combined in the same nucleus and undergo recombination to produce new genotypes” [40]. If we
146 further broaden this definition to include the combination of two genes in the same nucleus, then
147 gene conversion or homologous recombination can also be defined as ‘sex’. This definition is
148 unlikely to attract support, but it can be argued that traditional sexual reproduction and homologous
149 recombination are at opposite ends of the same spectrum. It is our opinion however, that
150 *Acanthamoeba* and similar organisms are best described as reproducing asexually and that the
151 homologous recombination that is expected to operate between similar chromosomes in the
152 polyploid nucleus cannot be described as sexual or even parasexual.

153
154 In summary, we argue that the presence of meiotic genes does not necessarily mean that meiosis is
155 occurring as a prelude to sexual reproduction. We further argue that these genes are instead involved
156 in homologous recombination between multiple copies of genomic elements in the polyploid
157 nucleus of *Acanthamoeba* thus allowing this asexually reproducing amoebae to avoid the
158 deleterious accumulation of mutations. Others too have suggested that meiotic genes have other
159 functions [12] including homologous recombination [17, 40]. The same is likely to hold for some
160 of the many other protists such as *Acanthamoeba*, in which meiotic genes have been discovered
161 [15] but for which there is no other evidence for sexual reproduction. If this is the case then it
162 makes it more likely that the theoretical last common eukaryotic ancestor was asexual. This would
163 remove the awkward necessity of finding a compatible and compliant mate in the vast empty spaces
164 likely to have existed at the time that these early cells lived. Sex is a very expensive and complex

165 phenomenon that is expected to have arisen well after these initially asexual populations, using the
166 same set of genes used in homologous recombination.

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170 **Research ethics**

171 No ethical consent was sought from the local ethics committee since it was clear that this was not
172 necessary in this case.

173

174 **Animal ethics**

175 Only amoebae were used in this study and as these are neither sentient or conscious, ethical
176 considerations are not applicable.

177

178 **Permission to carry out fieldwork**

179 No fieldwork was involved in this study.

180

181 **Data accessibility**

182 All sequence data involved in this study are accessible either through GenBank
183 (<https://www.ncbi.nlm.nih.gov/genbank/>) or the amoebaDB data base
184 (<http://amoebadb.org/amoeba/>), and in most cases both. Sequence alignment data for figure 1 are
185 available from the Dryad Digital Repository [41].

186

187 **Author's contributions.** S.K.M. conceived the study analysed the data and wrote the paper.
188 A.O.F.V. and Z.K. isolated strains, performed the RNA-seq experiments, analysed the data,
189 contributed intellectually to the paper's content and edited the manuscript. All authors have read
190 and approved the final published version of this manuscript. All authors agree to be accountable for
191 all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of
192 the work are appropriately investigated and resolved

193

194

195 **Competing interests.** All authors declare that there are no competing interests.

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199

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204 final approval for publication and agree to be held accountable for the work performed therein.

205 **Figure/table legends**

206

207 **Table 1.** Meiosis/recombination associated genes in *Acanthamoeba* and their expression level as
208 determined by RNA-seq. *Acanthamoeba* homologs were identified by BLAST and confirmed by
209 phylogenetic analysis. *LogCPM values reflect the level of expression of these transcripts in
210 exponentially growing axenic *Acanthamoeba* cultures. The two values are derived from two separate
211 measurements from two different *Acanthamoeba* strains upper value from SB-53, lower GS-336.

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Figure 1. An unrooted phylogenetic analysis of Hop2 showing that the *Acanthamoeba* gene groups with the amoebzoa (orange group) as expected. Maximum likelihood analysis of the protein sequences showing branch support. The tree was created with PhyML [26] using the GTR model with 100 bootstrap pseudo-replicates.

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Figure 2. The approximately 13,000 RNA transcripts are displayed in order of their relative abundance (blue bars) present in exponential (GS-336) *Acanthamoeba* cultures (SB-53 gave similar results). Most abundant transcripts left, least right. The “meiosis specific” transcripts are highlighted in red. The actin genes (ACA1_361250, ACA1_361250) and EF1 α (ACA1_138040) show the highest expression.

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	Rad50	Rad51	Rad52	Spo11	Hop1	Hop2	Mre11	Mnd1	Dmc1
GenBank	XP_004339639	ELR18834	XP_004337923	ELR12359	XP_004340201	XP_004334651	ELR17651	XP_004340260	XP_004353078
AmoebDB	ACA1_063900	ACA1_166930	ACA1_188580	ACA1_374260	ACA1_369130	ACA1_091480	ACA1_064360	ACA_36980	ACA1_071720
LogCP M*	5.80 6.08	4.05 4.17	4.62 4.76	1.72 1.77	0.82 1.06	5.68 4.83	5.08 5.18	4.45 4.59	-0.05 0.11

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	Pms1	Mlh1	Mlh2/Mlh3	Msh2	Msh4	Msh5	Msh6	HAP2	GEX1
GenBank	XP_004342239	XP_004351570	XP_004367469	XP_004337972	XP_004352766	ACA13171(part)	ELR15471	XP_004341525	XP_004341936
AmoebDB	ACA1_115690	ACA1_149810	ACA1_195260	ACA1_031570	ACA1_068220	ACA_094390	ACA1_340910	ACA1_266960	ACA1_133490
LogCP M*	4.70 4.48	3.96 4.57	2.12 2.94	6.04 5.54	1.75 2.66	3.52 4.63	6.57 6.70	3.88 4.15	1.58 2.89

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Table 1