

1 **Sexual Reproduction in Bdelloid Rotifers**

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8 **Data Availability**

9 The sequencing reads (Illumina and Nanopore) and assemblies generated in this study
10 are available from NCBI BioProject XXXXX (accession pending).

11

12 Difference matrices, phylograms, alignments (tic plots), and raw data are available at

13 <https://github.com/tsackton/rotifer-outcrossing>

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17 **Running title: Bdelloid sexual reproduction**

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19 **Keywords** Allele sharing, bdelloid rotifers, life history, *Macrotrachella quadricornifera*,
20 population structure, Red Queen, sexual reproduction

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26 **ABSTRACT** Many hypotheses have been advanced to explain why, despite its
27 **substantial costs, sexual reproduction is nearly universal in eukaryotes and why**
28 **its loss generally leads to early extinction. Posing an exception to all such**
29 **hypotheses are a few groups thought to be entirely asexual that arose millions of**
30 **years ago. Of these, the most extensively studied are the rotifers of Class**
31 **Bdelloidea, common freshwater invertebrates of worldwide distribution. Here we**
32 **present genomic evidence showing that a bdelloid species, *Macrotrachella***
33 ***quadricornifera*, is facultatively sexual, removing a challenge to hypotheses for**
34 **the evolutionary benefit of sex and making it likely that sexual reproduction is**
35 **essential for long-term evolutionary success in all eukaryotes.**

36

37

INTRODUCTION

38 First described nearly 350 years ago (van Leeuwenhoek 1677, 1702), bdelloid
39 rotifers are minute freshwater invertebrates commonly found in lakes, ponds and
40 streams and in ephemerally aquatic habitats such as temporary pools and the water
41 films on lichens and mosses (Figure 1). Characterized by their ciliated head and
42 bilateral ovaries, bdelloids are classified in 4 families, 19 genera and some 500
43 morphospecies. The bdelloid radiation began tens of millions of years ago, as shown by
44 the synonymous site difference between families and the presence of bdelloid remains
45 in ancient amber. Although typically only several tenths of a millimeter in size and
46 containing only ca. 1,000 nuclei, mostly in syncytial tissues, bdelloids have ganglia,
47 muscles, digestive, excretory, reproductive and secretory systems; photosensitive and
48 tactile sensory organs; and structures for crawling, feeding and swimming. Bdelloids are

49 degenerate tetraploids, descended from an ancient tetraploid ancestor (Mark Welch *et*
50 *al.* 2008, 2009; Hur *et al.* 2009; Flot *et al.* 2013; Nowell *et al.* 2018).

51 The only observed means of bdelloid reproduction is from eggs produced in well-
52 differentiated ovaries, with no reduction in chromosome number (Hsu 1956a; b). A few
53 days after deposition a young bdelloid emerges and a few days later commences egg-
54 laying, producing up to 32 eggs over a period of up to a few weeks during which there is
55 little death, after which the death rate increases more or less exponentially (Meadow
56 and Barrows Jr. 1971; Ricci 1983). Depending on species and conditions, the time from
57 egg deposition to death is about a month (Ricci 1983; Ricci and Fascio 1995). Bdelloids
58 are eutelic, with no cell division after eclosion except in the germ line.

59 Bdelloids are extremophiles, being able to survive prolonged desiccation, some
60 species more than others, as well as starvation and extremes of temperature, and to
61 resume reproduction upon restoration of favorable conditions, highly unusual abilities
62 probably apomorphic to the Class (Ricci 1998; Ricci and Caprioli 2005; Ricci and
63 Perletti 2006; Nowell *et al.* 2018). Bdelloids have a highly effective system of anti-
64 oxidant protection, as manifested by their extreme resistance to ionizing radiation and to
65 IR-induced protein carbonylation (Gladyshev and Meselson 2008; Krisko *et al.* 2012),
66 apparently an adaptation to avoid the oxidative damage caused by desiccation in other
67 systems (França *et al.* 2007; Fredrickson *et al.* 2008).

68 Although bdelloids have been systematically studied ever since adequate
69 microscopes became available (Ehrenberg 1838; Hudson and Gosse 1886) there is no
70 confirmed observation of males and the prevailing view is that they are entirely asexual.
71 It has been estimated that some 500,000 bdelloids from a variety of habitats and from

72 laboratory culture have been examined by contemporary rotifer workers without ever
73 having seen males or hermaphrodites (Birky 2010). The only report to the contrary, in a
74 treatise otherwise devoted to observations of males and sexual periods of the
75 facultatively sexual rotifers of Class Monogononta, is a hesitant account of having twice
76 seen a single male among many bdelloids of species *Rotaria rotatoria* “present in
77 almost incredible numbers” beneath the ice of a frozen lake in Denmark in November
78 1923 (Wesenberger-Lund 1930). Sampling conducted there in January 2019 found few
79 bdelloids and no males but neither was there any ice, the winter of that year having
80 been among the warmest on record (Martin Sorensen, personal communication).

81 Despite the failure to document the existence of males, it may be that bdelloids
82 reproduce sexually only occasionally and under conditions not adequately
83 investigated—a possibility made less implausible by estimates that outcrossing of
84 *Saccharomyces cerevisiae* in the field may occur as seldom as once in 25-50,000
85 generations (Ruderfer *et al.* 2006; Magwene *et al.* 2011), owing to the repression of
86 meiosis which, however, can be relieved in the laboratory by growth in specific media.

87 In the following, we first review observations once interpreted as evidence for
88 bdelloid asexuality but now known to have other explanations. We also summarize
89 recent genomic findings suggestive of bdelloid sex. We then present an extensive study
90 of allele sharing in the bdelloid *Macrotrachella quadricornifera* showing it to be
91 facultatively sexual. We also discuss the relation between bdelloid life history and
92 population structure and implications for how bdelloid males and mating might be
93 discovered.

94

95

MATERIALS AND METHODS

96 **Sample collection and 10x sequencing**

97 *M. quadrifornifera* isolates MA, MM and CR are from a group of 29 individuals
98 morphologically identified as belonging to Family Philodididae collected from ground
99 moss at widely separated sites in northeast United States in the autumn of 2011
100 (Signorovitch *et al.* 2015). Six of the isolates, including MA, MM and CR, belong to the
101 same mitochondrial clade. Cultures were established from single eggs, fed with *E. coli*
102 and maintained in 0.24u Millipore-filtered spring water at 20 °C in 100X20 mm plastic
103 Petri dishes with continuous gentle rotation. Washed rotifers were shipped under dry ice
104 to HudsonAlpha (Huntsville AL) for DNA extraction, library preparation, 10x Illumina
105 sequencing and provision to us of fastq files.

106

107 **Nanopore sequencing and assembly of Nanopore reads**

108 Washed flash-frozen rotifers of isolate MA were digested for 17 h at 52 °C in 100
109 mM EDTA, 50 mM tris pH 9.0, 1% sodium sarcosyl, 1 mg/ml freshly dissolved
110 proteinase K. DNA was isolated with a Qiagen MagAttract HMW DNA Kit and its size
111 distribution analyzed with an Agilent Technologies 4200 TapeStation. DNA purity was
112 verified by determining 260/280 and 260/230 ratios with a Nanodrop ND-1000 and its
113 concentration was determined with a Qubit 3.0 fluorometer. DNA was prepared for
114 sequencing with an Oxford Nanopore Ligation Sequencing Kit 1D without shearing and
115 1.1 µg (48µl) of the ligated DNA was loaded into the DNA repair end-end preparation
116 step. Flow cells were prepared following the protocol from the same kit. DNA libraries
117 were quantified with the Qubit and 420 ng of DNA was loaded for each sequencing run.

118 Base calling for Nanopore reads was done with Albacore 2.3.4 and the results
119 summarized with Nanoplot 1.20.0. Reads longer than 10kb were selected with fastp
120 0.19.5 and aligned to the NCBI-nt database with Blastn using an e-value cutoff of 1e-25,
121 removing reads with a best hit to a non-Animalia sequence. Statistics for the Nanopore
122 reads are given in Supporting Material Table 1.

123 Scaffolds assembled from the 10x reads of each of the three isolates were obtained
124 with Supernova 2.1.1 using default parameters. Genome size, needed as an input
125 parameter for Supernova, was estimated with Jellyfish 2.2.5, based on the distribution
126 of k-mers. Scaffolds were aligned to the NCBI nt database with Blastn with settings as
127 above and non-Animalia scaffolds were removed. Assembly statistics are presented in
128 Supporting Material Table 2. Phased sequences (megabubbles) were then obtained
129 from the 10x assemblies with Supernova mkoutput with style=megabubbles.

130 **Alignment of 10x megabubbles to Nanopore reads**

131 From the initial set of 979,864 Nanopore reads, 22,977 of those longer than 10 kb
132 that did not have a significant Blast hit to non-Animalia sequences in Genbank were
133 aligned to the megabubbles from the 10x assemblies with Minimap2 2.15-r905. From
134 the resulting alignments longer than 8kb, those in which megabubbles of at least two of
135 the three isolates aligned to the same Nanopore read were chosen for analysis. These
136 alignments of 10x megabubbles, comprising groups of either 6 or 4 homologous
137 sequences, were then realigned among themselves with Clustal Omega 1.2.3, and
138 trimmed with Gblocks 0.91b to remove indels and alignment disruptions caused by
139 repeats of unequal length, neither adding nor rearranging sequences. When two or
140 more alignments overlapped, only the longest was retained. Lastly, 1 kb was removed

141 from the ends of each alignment using EMBOSS seqret and alignments shorter than 1
142 kb were discarded. Pairwise SNP differences between homologs in each alignment
143 were obtained with snp-dists 0.6.3. Five alignments in which MM or CR differed by more
144 than 10 % from both homologs of MA were rejected. This may occur when, owing to a
145 deletion in MM or CR occurring since their divergence from MA, there is no MM or CR
146 sequence homologous to the Nanopore read, leaving only the homeologous sequence
147 to align with it. This left 1,117 separate genomic regions for analysis. The alignment
148 workflow is depicted in Supporting Information Figure 1. That trimming neither adds nor
149 rearranges sequences is confirmed by manual inspection of 5 randomly chosen regions
150 of MA-MM sharing (regions 10, 104,142, 179, 262, total length 62,172 bp) in untrimmed
151 MA-MM-CR alignments revealing only a single departure from perfect alignment, a one
152 base deletion in a homopolymer run of As.

153 The sequence accuracy of the assemblies was determined by comparison with
154 published sequences of the four regions of MA, MA, and CR of total length 116, 220 bp
155 sequenced by Signorovitch *et al.* 2015, revealing a near-perfect match (Supplemental
156 Material Table 3). Comparison of mitochondrial sequences in the 10x assemblies
157 identified by blast searches against published mitochondrial sequences of MA, MM and
158 CR (Lasek-Nesselquist 2012) revealed a perfect or nearly-perfect (>99%) match for
159 each isolate.

160 **Test for contamination**

161 It might be thought that contamination of MM DNA with MA DNA or the reverse in the
162 DNA sent to HudsonAlpha for sequencing or occurring there, could mimic allele sharing.
163 Although it is most unlikely that contamination could be so massive and of the particular

164 frequency required to mimic the MA-MM sharing we observe in half of the 622 MA-MM
165 alignments, a test was conducted to directly rule out the possibility. All 10x Illumina
166 reads sequenced from MA, MM, and CR were aligned to each of the 1,177 alignments.
167 Using each haplotype in turn as a reference, bwa mem and GATK were used to
168 produce variant calls, implemented in a Snakemake pipeline developed by the Harvard
169 Informatics group
170 (https://github.com/harvardinformatics/shortRead_mapping_variantCalling). The variant
171 calls were then processed with vcftools to generate counts of reads at each variable
172 nucleotide position supporting each allele. The fraction of Illumina reads supporting the
173 alternate allele (the nucleotide that differs from the reference haplotype) shows a
174 characteristic tri-modal pattern for all three isolates, with peaks at 0 (reference
175 homozygote), 0.5 (heterozygote), and 1 (alternate homozygote), as shown for MA and
176 MM in Supplemental Figure 2. Such a pattern is expected for true variable positions in a
177 diploid. Contamination would instead produce a pattern in which heterozygous positions are
178 supported by a fraction of reads determined by the proportion of the total DNA that was from a
179 contaminating source, for which no evidence is seen in any of the three assemblies.

180 Further, if contaminating DNA had displaced one homolog often enough to account for the
181 observation of MA-MM sharing in half of the alignments containing both isolates, it would be
182 expected at least occasionally to displace both homologs, producing MA-MM alignments of the
183 form a/b a/b. Contamination is therefore also ruled out by the entire absence of such regions
184 among the 622 alignments containing MA and MM.

185

186

PREVIOUS STUDIES

187 **Heterozygosity**

188 In sexuals, heterozygosity caused by mutation is limited by haploid drift. The finding of
189 much greater synonymous difference between gene copies in bdelloids than in
190 monogononts was therefore initially interpreted as evidence for asexuality (Mark Welch
191 and Meselson 2000, 2001). Continued investigation, however, showed that bdelloids
192 are degenerate tetraploids, and that the highly diverged gene copies are homeologs,
193 not homologs (Mark Welch *et al.* 2008; Hur *et al.* 2009). Bdelloid heterozygosity, the
194 difference between homologs, lies within the range known for sexuals, providing no
195 evidence for asexuality. Moreover, in asexual *Daphnia pulex* and *Saccharomyces*
196 *cerevisiae* the frequency with which a nucleotide site is covered by a tract of
197 homozygosity, as may result from germline mitotic crossing-over at the four-strand
198 stage or from certain processes of DNA damage repair, is much greater than the
199 frequency of nucleotide substitution (Omilian *et al.* 2006; Xu *et al.* 2011; St. Charles and
200 Petes 2013; Flynn *et al.* 2017). In sexuals, heterozygosity lost by such processes may
201 be regained by outcrossing. But if bdelloids are ancient asexuals and if loss of
202 heterozygosity is more frequent than substitution, the absence of outcrossing should be
203 manifested as unusually *low* heterozygosity, the opposite of what had been thought
204 (Magwene *et al.* 2011; Hartfield *et al.* 2018). The observation that bdelloid
205 heterozygosity is within the range known for sexual taxa therefore suggests that
206 bdelloids may be sexual, with lost heterozygosity regained by occasional outcrossing.
207 The moderate levels of heterozygosity seen in other putative ancient asexuals
208 (Schaefer *et al.* 2006; Schon *et al.* 2009) would then suggest that they too engage in
209 outcrossing.

211 **Paucity of retrotransposons**

212 Sexual reproduction allows vertically transmitted deleterious transposable elements to
213 proliferate in populations, whereas the loss of sex, by preventing their replenishment
214 may eventually free a population of such elements (Hickey 1982; Dolgin and
215 Charlesworth 2006). As a test for asexuality, bdelloids, monogonont rotifers and
216 sexually-reproducing animals of numerous other phyla for were examined for genomic
217 sequences coding for reverse transcriptases of LINE-like retrotransposons. These were
218 found to be abundant in all the sexually-reproducing taxa but were not detected in
219 bdelloids, as expected for asexuality (Arkhipova and Meselson 2000, 2005a; b).
220 Nevertheless, although bdelloids are nearly devoid of LINE-like retrotransposons, later
221 work showed that they are not entirely absent (Gladyshev *et al.* 2007; Gladyshev and
222 Arkhipova 2010) and that bdelloids have particularly effective retrotransposon silencing
223 systems (Rodriguez and Arkhipova 2016). The paucity of LINE-like retrotransposons is
224 therefore non-evidentiary as regards bdelloid sexuality.

225

226 **Genome structure**

227 A draft genome sequence of the bdelloid, *Adineta vaga*, with numerous breaks in the
228 colinearity of homologous regions and individual scaffolds containing genes in direct or
229 palindromic repeats but no copy elsewhere in the genome was initially taken as
230 evidence that bdelloids had evolved ameiotically (Flot *et al.* 2013). But subsequent
231 genomic sequencing of three other bdelloid species, including *Adineta ricciae*, a close
232 relative of *A. vaga*, found that the unusual genomic features that had been interpreted
233 as evidence for ameiotic evolution are largely absent, suggesting that their apparent

234 presence in *A. vaga* resulted from mis-assembly (Nowell *et al.* 2018) as later shown to
235 be the case by the demonstration in *A. vaga* of homologous chromosome pairs (Simion
236 *et al.* 2020).

237

238 **Allele sharing**

239 A finding of two individuals closely related with respect to a given genomic region but
240 more distantly related with respect to its homolog, a form of phylogenetic
241 noncongruence known as allele sharing, would mean that recently in their ancestry the
242 region had undergone some form of genetic exchange between individuals, as in sexual
243 reproduction, homologous horizontal transfer, or parasexuality. A striking example of
244 allele sharing was found by (Signorovitch *et al.* 2015) in each of four genomic regions
245 2.4 to 9.7 kb in length among isolates MA, MM and CR. At each region, MA identically
246 shared a homolog with MM while the other homolog of MA was identical to a homolog of
247 CR in two regions and nearly so in the other two. That these observations were
248 evidence for sexual reproduction was disputed and attributed instead to horizontal
249 genetic transfer (Debortoli *et al.* 2016; Signorovitch *et al.* 2016) but the evidence for
250 HGT was cast into doubt by an analysis showing that it could be explained as the result
251 of cross-contamination among isolates (Flot *et al.* 2018; Wilson *et al.* 2018). It should
252 also be noted that although HGT clearly occurs in bdelloids, it is not known if it is ever
253 homologous.

254

255 **Meiosis-related genes**

256 A survey of the genomes of four bdelloid species belonging to two bdelloid families for
257 the proteomes of 12 genes considered to be meiosis-specific found all but one, *red1*, to
258 be present in each species (Nowell *et al.* 2018). But neither was *red1* found in
259 *Drosophila melanogaster*, a sexual species known to lack it. Although five of these
260 genes had not been found in the draft assembly of the *A. vaga* genome (Flot *et al.*
261 2013) their detection in the four other bdelloid species suggests that they are present in
262 *A. vaga* as well and that bdelloids engage in meiosis.

263

264 RESULTS

265 Alignments

266 As described in METHODS, we obtained alignments of phased sequences from 1,177
267 separate genomic regions of *M. quadricornifera* isolates MA, MM and CR. Of these, 331
268 are with all three isolates, 291 with MA and MM, 110 with MA and CR and 445 with MM
269 and CR, having a mean length of 12,490 bp (range 2,051 - 32,937 bp) and altogether
270 covering 14.7 Mb, approximately 4% of the *ca.* 360 Mb genome (Table 2). Matrices
271 giving pair-wise differences between homologous sequences, phylograms and plots of
272 the spatial distribution of differences between homologs (“tic” plots) for four
273 representative regions are given in Table 1 and, for all 1,177 regions, in Supplemental
274 Material Tables 4-6.

275

276 Allele sharing in the alignments

277 Half of the MA-MM and MA-MM-CR alignments, 315 of 622, comprise a discrete class
278 in which a homolog of MA is identical to a homolog of MM (Table 2; Fig. 2B). The

279 frequencies of identical MA-MM sharing in the MA-MM and MA-MM-CR alignments
280 considered separately are 0.519 and 0.495, respectively or 0.506 overall (S.E. = 0.02).
281 MA and MM also share identical homologs with CR, but in a much smaller proportion of
282 the alignments (Fig. 2A, C). CR shares identical homologs with MA in 12 of 441
283 alignments, and with MM in 18 of 776 alignments, or 2.7% and 2.3% respectively. In
284 alignments without identical sharing the differences between the homolog of MA most
285 similar to a homolog of MM form a broad distribution with a mean of 0.96 SNPs per 100
286 bp (S.E. = 0.61), Fig. 2B. Most or all of the regions identically shared between MA and
287 MM must be considerably longer than the alignments in which we find them, as shown
288 in a plot of the frequency of identical MA-MM sharing against alignment length in
289 consecutive intervals each comprising 76-79 alignments (Fig. 3). The frequency of
290 identical sharing is not significantly different from 50 percent in even the
291 longest alignments (18-33 kb).

292

293 **Genealogy**

294 MA and MM identically share a homolog in a discrete class amounting to half of the MA-
295 MM and MA-MM-CR alignments while their other homologs, within and between MA
296 and MM, are substantially diverged, as expected for diploids related as grandchild and
297 grandparent, half siblings, aunt and uncle or nephew and niece in a genetically diverse
298 panmictic population. The near equality of MA-CR and MM-CR sharing frequencies
299 indicates that CR is equidistant from MA and MM and therefore that MA and MM are not
300 grandparent and grandchild or aunt/uncle-nephew/niece but rather half siblings or
301 double first cousins.

302 Inspection of tic plots for the triple alignments reveals a few with long interior
303 regions of MA-MM identity covering most but not all of the alignment (Supplemental
304 Table 4). In these regions there is substantial divergence from CR, showing that such
305 identity is not the result of extreme conservation but instead reflects more remote
306 relationships between MA and MM in addition to their relation as half siblings or double
307 first cousins. Similarly, alignments in which CR is identical to MA or MM over much but
308 not all the alignment are likely to reflect remote relationships between CR and MA and
309 between CR and MM. In general, more distant relations will be manifested as shorter
310 regions of identity by descent, owing to the recombination that occurs at each meiosis.
311 For individuals related as half-sibs or double first cousins the regions of identity by
312 descent from their common grandparents, assuming one cross-over per meiosis in each
313 arm of the 10 chromosomes of *M. quadricornifera*, will average several Mbp in length,
314 far longer than our longest alignments, consistent with the observation that the
315 frequency of identically shared regions does not fall off with their length (Fig. 3).

316

317 **Homozygosity**

318 In each of the three isolates there are a few regions that are entirely homozygous
319 (Table 2). No more frequent in the shorter half of the alignments than in the longer half,
320 they must generally be longer than the alignments in which they occur. These regions
321 may be identical by descent or may have arisen by conversion or by germ-line mitotic
322 crossing-over at the four-strand stage. Their infrequency shows, for each isolate, that its
323 parents cannot have been closely related. The more frequent homozygosity and lower
324 heterozygosity of isolate MM may reflect more frequent occurrence of such

325 homozygosing events along its clonal lineage. The few alignments in which conversion
326 may have erased evidence of sharing are not included in the totals given above or in
327 Table 2.

328

329 **Recombination**

330 The finding that a particular isolate, MA, was the double sharer in every one of the four
331 regions examined by Signorovitch *et al.* 2015, sharing one of its homologs with MM and
332 its other homolog with CR, suggested that the regions may not have recombined and
333 therefore that entire haplotypes may have passed through meiosis intact, as in certain
334 plants (Holsinger and Ellstrand 1984). In contrast, we find this pattern in only 4 of our
335 331 much longer MM-MA-CR alignments. It may be that the pattern encountered in the
336 earlier study resulted from identity by descent from remote ancestors, in which case
337 such regions would generally be shorter than our alignments and therefore not counted
338 here as allele sharers. That entire haplotypes are not conserved and that recombination
339 definitely occurs is seen in the observation that MA and MM share only a quarter of their
340 genomes and share even less with CR and in the existence of alignments in which
341 sharing extends over only part of the region.

342

343 **DISCUSSION**

344 **Horizontal transfer, parasexuality and automixis**

345 That half of the 622 genomic regions we examined in MA and MM constitute a discrete
346 class in which a homolog is identically shared between isolates is expected for half-sibs
347 or double first cousins from a large genetically diverse population of sexually

348 reproducing diploids and cannot be explained as the result of horizontal genetic transfer
349 or parasexuality. For it to have occurred by HGT would require massive transfer of long
350 DNA segments between MA and MM coincidentally at half the 622 alignments in which
351 MA and of MM are included without ever transferring both homologs, there being no
352 alignments in which MA and MM are of the form a/b and a/b.

353 Recent observations in *A. vaga* of homologous chromosome pairs attributed to
354 automixis (Simion *et al.* 2020), and of Hardy-Weinberg equilibrium and a fall-off of
355 linkage with distance, attributed to HGT (Vakhrusheva *et al.* 2020), are entirely
356 consistent with sexual reproduction and, as neither automixis nor HGT can account for
357 the allele sharing we find in *M. quadricornifera*, may be regarded as further evidence for
358 sexual reproduction.

359 Neither can the allele sharing we observe be explained as the result of
360 parasexuality. In the parasexual cycle, known only in certain fungi and protozoans,
361 nuclei from two individuals fuse, forming nuclei of doubled ploidy that during subsequent
362 generations undergo occasional mis-division, only rarely yielding viable diploids. In
363 bdelloids, this would require nuclei from different individuals, sequestered in ovaries
364 within the body of the animal, somehow to undergo fusion, followed by a series of
365 random chromosome losses to give viable segregants, all having ten chromosomes and
366 with MA-MM identical sharing in half of the genomic regions we examined.

367

368 **Generations since the MA-MM sharing event**

369 The number of generations since the MA-MM sharing event may be estimated from the
370 frequency of substitution differences between shared homologs; from the number of

371 generations that would cause mutational reduction of the frequency of identical sharers
372 to fall significantly below the observed value of 0.5; from the frequency of homozygosity;
373 and from the mitochondrial difference between MA and MM.

374 In addition to the 315 alignments in which MA and MM share identical homologs
375 there are 6 in which they share homologs that differ by a single substitution. For a mean
376 alignment length of 15 kb this is a frequency of 1.3×10^{-6} per bp. Substitution rates
377 measured in accumulation experiments with *Caenorhabditis elegans*, asexual *D. pulex*,
378 and *D. melanogaster* range from 2.3 to 5.5×10^{-9} per generation (Flynn *et al.* 2017).
379 Taking a substitution rate of 4×10^{-9} and assuming a Poisson distribution of the small
380 differences between shared homologs, as expected for mutation, this suggests that the
381 shared homologs may be separated by 100-200 generations.

382 If there were as many as 2,000 generations separating the shared homologs of
383 MA and MM and again assuming a substitution rate of 4×10^{-9} per generation, the
384 expected number of substitutions in regions 18-33 kb in length, the longest interval in
385 Figure 3, would be 0.14 – 0.26, reducing the proportion of identical sharers to 0.43 –
386 0.39, substantially less than the observed value of 0.5, suggesting that the number of
387 generations between the shared homologs is no more than about 1,000.

388 As tracts of homozygosity arising in a genetically diverse population are generally
389 made heterozygous by outcrossing, the age of such tracts and the frequency of
390 substitutions in them will increase with the number of generations since the last
391 outcross. Assuming the likelihood of a site being covered by a tract of homozygosity to
392 be 4×10^{-5} per generation (Omilian *et al.* 2006; Xu *et al.* 2011; St. Charles and Petes
393 2013; Flynn *et al.* 2017) and considering that the total length of MA regions is 13.4 Mbp

394 of which perfectly homozygous regions comprise some 151 kbp, or about 1.1%, it
395 appears that there have been some 340-500 generations from when the sharing event
396 occurred to MA and 550-800 generations to MM.

397 A fourth estimate of the number of generations since homologs of MA and MM
398 separated may be obtained by assuming that their mitochondria descend from a
399 common mother or maternal grandmother. Taking the difference of 5 substitutions or
400 2.5×10^{-5} between their 14 kb mitochondria (Lasek-Nesselquist 2012) and a
401 mitochondrial mutation rate of 1.5×10^{-7} (Xu *et al.* 2012; Flynn *et al.* 2017), would
402 suggest that the shared homologs separated some 170 generations ago. These various
403 estimates agree in suggesting that the shared homologs of MA and MM are separated
404 by no more than about a thousand generations.

405

406 **Abundance of close relatives in the sampled population**

407 Isolates MA, MM and CR were collected at widely separated sites as part of a
408 collection of only 29 individuals (Signorovitch *et al.* 2015). What aspects of bdelloid life
409 history could make finding relatives as close as MA and MM in so small and widely
410 dispersed a sample of what must be an enormous population? It must be that the
411 sampled population is largely made up of relatively few, very large, widely dispersed
412 clones descended from recent crossing. Such an unusual population structure would
413 result if sexual periods occur only rarely, during a population bloom, with multiple
414 rounds of mating among two or more founding types, producing large numbers of
415 closely related individuals. It may be that males are produced and mating occurs only
416 when particular mating types are present together, causing one or both to produce

417 haploid eggs and haploid males. At some stage, from fertilization to zygote
418 development, selfing may be prevented and heterozygosity thereby maintained. This,
419 followed by wide dispersion and extensive clonal reproduction would give rise to very
420 large, widely dispersed clones of the products of recent crossing. Meanwhile, lines that
421 fail to outcross would suffer loss of heterozygosity and rapid clonal erosion, as seen in
422 asexual *Daphnia pulex* (Tucker *et al.* 2013), driving them to extinction unless revived by
423 timely outcrossing.

424 On this picture, field observations intended to detect males and mating should be
425 made during population blooms, as may require specific external stimuli, and in sizeable
426 bodies of water should it be that different but compatible types must be present in order
427 to initiate mixis. Further, by analogy with monogononts, the appearance of bdelloid
428 males may be confined to only a short interval during a population bloom, therefor
429 requiring frequent sampling for their detection (Wesenberger-Lund 1930).

430

431 **Bdelloid life history - eluding the Red Queen**

432 It may be asked if there is a relation between the infrequency of bdelloid outcrossing
433 and bdelloid life history. A distinctive feature of the latter is the ability of bdelloids to
434 withstand desiccation and resume reproduction upon rehydration, an ability not present
435 in most of the fungi and other organisms that infect, parasitize, prey on or compete with
436 bdelloids (Wilson and Sherman 2010). In habitats that undergo desiccation and
437 rehydration the population of desiccation-intolerant antagonists will be greatly reduced
438 at each episode of desiccation while bdelloids will resume reproduction upon
439 rehydration. Bdelloids gain additional freedom from having to co-evolve with biological

440 antagonists by their ability to survive prolonged starvation, extremes of temperature and
441 exposure to toxic conditions lethal to other taxa (Ricci and Perletti 2006; Aguilera *et al.*
442 2007). Further, owing to their small mass when desiccated, about 10ug, once airborne,
443 even if associated with a small amount of adhering material, bdelloids may be
444 transported by wind or vectors over considerable distances (Fontaneto *et al.* 2008),
445 transferring to an environment where antagonists may be less abundant or less
446 antagonistic. The combination of anhydrobiosis and resistance to conditions inimical to
447 other taxa and dispersibility by wind, birds or other vectors therefore affords substantial
448 protection from what would otherwise be co-evolving biotic antagonists, reducing the
449 need for frequent recombination by largely eluding the “Red Queen” (Ladle *et al.* 1993;
450 Wilson and Sherman 2010; Wilson 2011). Although largely freed of the need for sexual
451 reproduction to keep up with biological antagonists, other benefits of sexual
452 reproduction, despite its costs, apparently maintain it in bdelloids.

453 The present evidence for sexual reproduction in a species of bdelloid rotifers, the
454 group once considered the best example of ancient asexuality, makes it likely that there
455 are no ancient asexuals and that sexual reproduction is universally essential for long-
456 term evolutionary success in eukaryotes.

457

458

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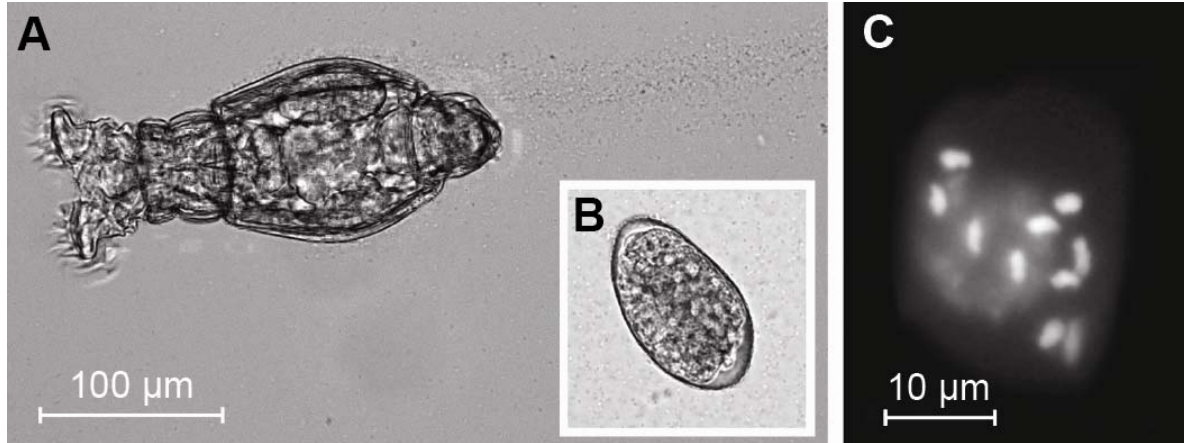
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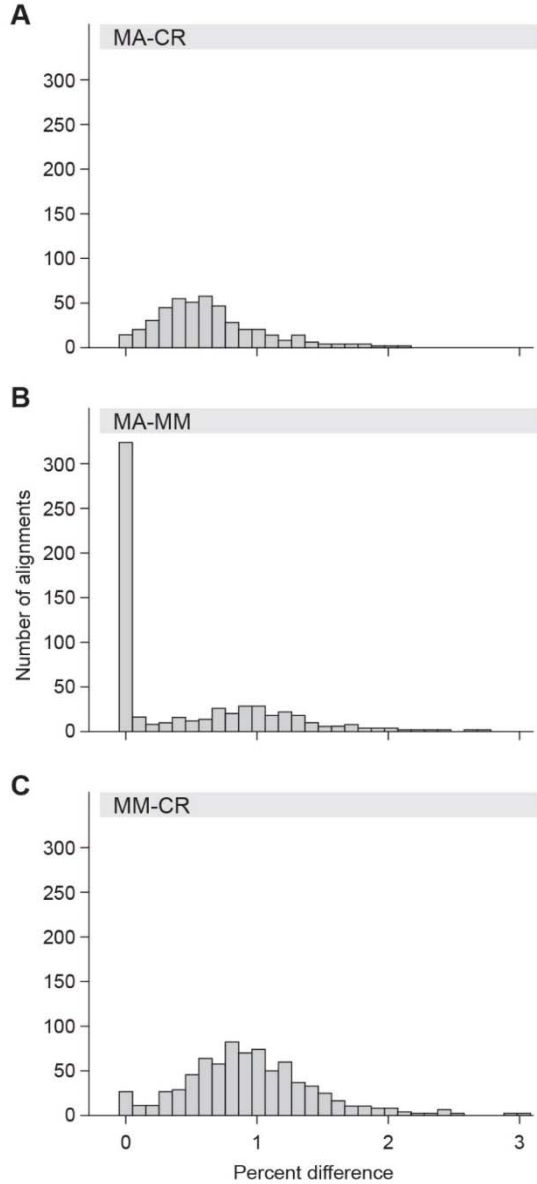


625

626 **Figure 1.** The bdelloid *M. quadricornifera*. (A) Adult. The animal has attached itself to
627 the glass slide by an adhesive secreted by its pedal gland, somewhat obscuring the tail.

628 (B) an unhatched egg shown at the same scale. (C) A metaphase nucleus, showing the
629 10 chromosomes characteristic of the species.

630



631

632 **Figure 2.** Histograms showing the distribution of divergence between the most similar

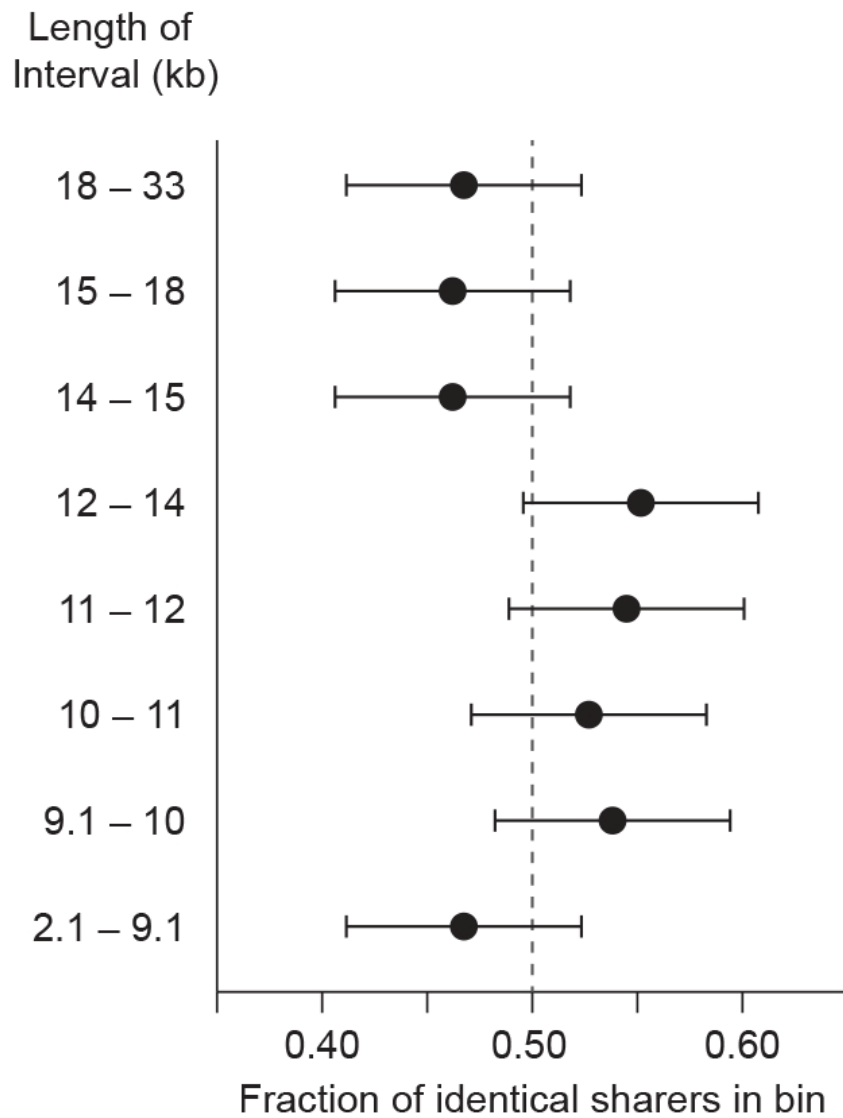
633 homologs in each of the three pairs of isolates. (A) MA-CR, (B) MA-MM, (C) MM-CR.

634 Alignments with identical or very nearly identical MA-MM sharing form a discrete class

635 constituting half of the regions. Bin size = 0.05 percent difference for the first bar in

636 panel B. Otherwise 0.1 percent.

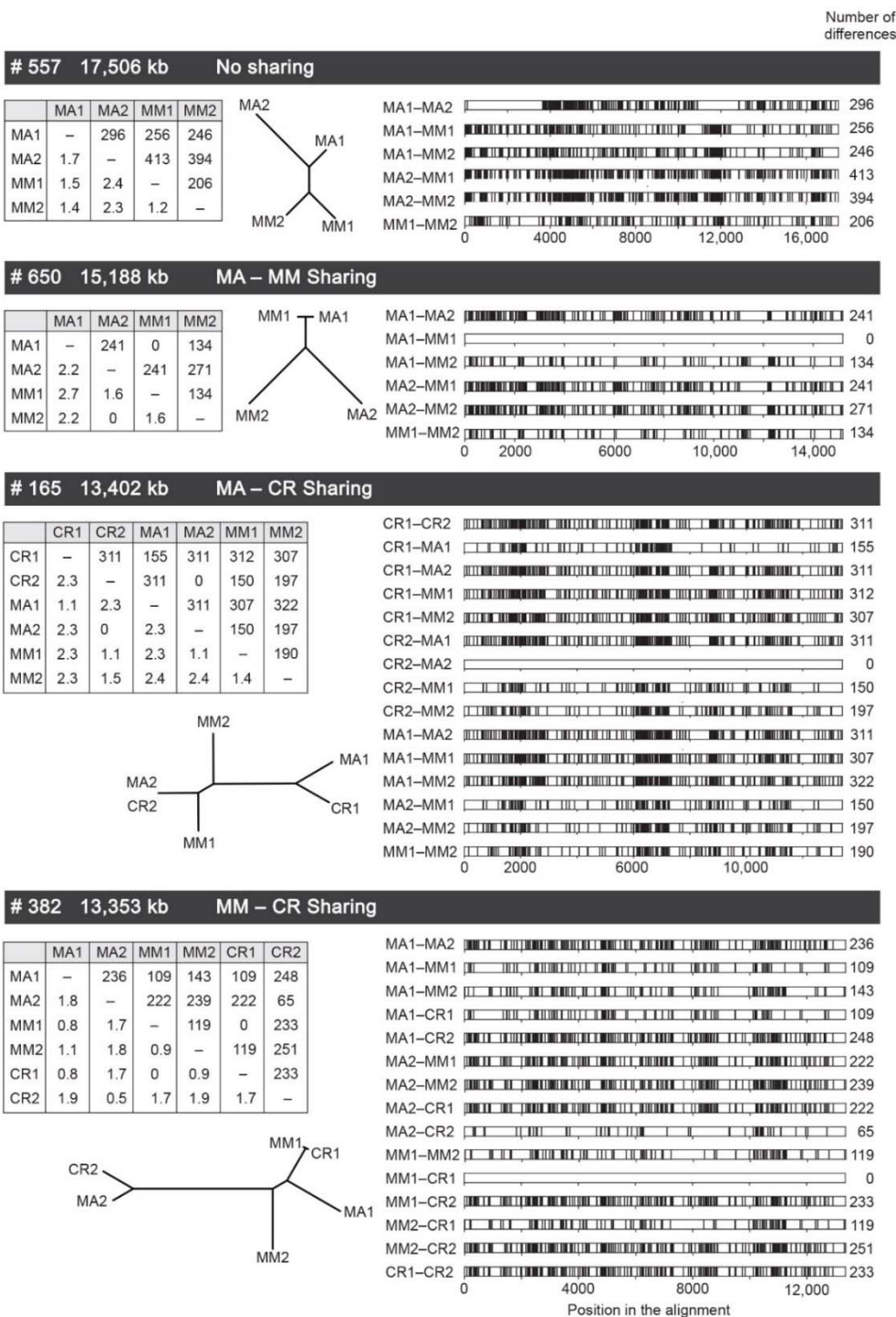
637



638

639 **Figure 3.** The mean frequency of identical MM-MA sharing in each of 8 bins of
640 alignments of increasing length, each containing 76-79 alignments. Error bars
641 represent standard errors.

642



643

644 **Table 1.** Difference matrices, phylograms and tic plots for four representative

645 alignments. Allele numbers in each alignment are arbitrary.

646

647

Summary Statistics										
		Identical Sharing Number / Proportion			Homozygous Number / Proportion			Average Heterozygosity		
Alignment	Size Range (kb) # of Alignments	MA-MM	MA-CR	MM-CR	MA	MM	CR	MA	MM	CR
MA-MM	2,149-32,937	151			12	8		0.019	0.010	
	291	0.519			0.041	0.028				
MA-CR	2,051-29,791		1		1		0	0.018		0.018
	110		0.009		0.009		0			
MM-CR	4,511-31,052			7		18	12		0.010	0.020
	445			0.016		0.04	0.027			
MA-MM-CR	2,705-29,510	164	11	11	1	4	3	0.018	0.009	0.018
	331	0.495	0.033	0.033	0.003	0.012	0.009			
All alignments	2,051-32,937	315	12	18	14	30	15	0.018	0.010	0.019
	1,177	0.506	0.027	0.023	0.019	0.028	0.017			

648

649 **Table 2.** Summary statistics.

650

651 **Acknowledgements.** We thank Nicole El-Ali and Claire Hartmann for Nanopore
652 sequencing, Jae Hur for the script used in generating tic plots, Janet Montgomery for
653 overall editing, and Irina Arkhipova, Timothy Barraclough, Brian and Deborah
654 Charlesworth, Antoine Hout, Paul Simion and Karine van Doninck for critical reading of
655 the manuscript. This work was supported by Oxford Nanopore, the Harvard Faculty of
656 Arts and Sciences and by an anonymous donor.

657

658 **Supplemental Material** is available for this paper.

659 **Correspondence** and requests for materials should be addressed to Matthew
660 Meselson.

661 **Author Contributions.** VL and TS: Genome assembly and data analysis. MM: Rotifer
662 culturing DNA preparation, data analysis and manuscript preparation.

663 **Competing Interest Declaration** The authors declare no competing interests.

664