Sexual Reproduction in Bdelloid Rotifers

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Data Availability

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

Difference matrices, phylograms, alignments (tic plots), and raw data are available at https://github.com/tsackton/rotifer-outcrossing

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Sample collection and 10x sequencing

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

Nanopore sequencing and assembly of Nanopore reads

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

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

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

Alignment of 10x megabubbles to Nanopore reads

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

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

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

Test for contamination

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

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

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

PREVIOUS STUDIES

Heterozygosity

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

Paucity of retrotransposons

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

Genome structure

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

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

Allele sharing

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

Meiosis-related genes

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

RESULTS

Alignments

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

Allele sharing in the alignments

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

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

Genealogy

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

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

Homozygosity

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

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

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

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

Generations since the MA-MM sharing event

The number of generations since the MA-MM sharing event may be estimated from the 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 $1.3X10^{-6}$ per bp. Substitution rates
377 measured in accumulation experiments with *Caenorhabditis elegans*, asexual *D. pulex*,
378 and *D. melanogaster range* from 2.3 to $5.5X10^{-9}$ per generation (Flynn *et al.* 2017).
379 Taking a substitution rate of $4X10^{-9}$ 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 $4X10^{-9}$ 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 $4X10^{-5}$ 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

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

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

Bdelloid life history - eluding the Red Queen

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

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

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

Literature Cited

Aguilera A., E. Zettler, F. Gomez, L. Amaral-Zettler, N. Rodriguez, *et al.*, 2007 Distribution of Eukaryotes in an extremely acidic, high-metal river. Mol. Biol. 1–41.

Arkhipova I. R., and M. Meselson, 2000 Transposable elements in sexual and ancient asexual taxa. Proc. Natl. Acad. Sci. 97: 14473–14477. https://doi.org/10.1073/pnas.97.26.14473

Arkhipova I., and M. Meselson, 2005a Deleterious transposable elements and the extinction of asexuals. BioEssays 27: 76–85.

Arkhipova I. R., and M. Meselson, 2005b Diverse DNA transposons in rotifers of the class Bdelloidea. Proc. Natl. Acad. Sci. U. S. A. 102: 11781–11786. https://doi.org/10.1073/pnas.0505333102

Birky C. W., 2010 Positively negative evidence for asexuality. J. Hered. 101: 42–45. https://doi.org/10.1093/jhered/esq014

Charles J. St., and T. D. Petes, 2013 High-Resolution Mapping of Spontaneous Mitotic Recombination Hotspots on the 1.1 Mb Arm of Yeast Chromosome IV. PLoS Genet. 9: e1003434. https://doi.org/10.1371/journal.pgen.1003434

Debortoli N., X. Li, I. Eyres, D. Fontaneto, B. Hespeels, *et al.*, 2016 Genetic Exchange among Bdelloid Rotifers Is More Likely Due to Horizontal Gene Transfer Than to Meiotic Sex. Curr. Biol. 26: 1–10. https://doi.org/10.1016/j.cub.2016.01.031

Dolgin E. S., and B. Charlesworth, 2006 The fate of transposable elements in asexual populations. Genetics 174: 817–827.

Ehrenberg C. G., 1838 *Die Infusionsthierchen als vollkommene Organismen. EinBlick in das tiefere organische Laben der Natur*. L. Voss, Leipzig.

Flot J.-F., B. Hespeels, X. Li, B. Noel, I. Arkhipova, *et al.*, 2013 Genomic evidence for ameiotic evolution in the bdelloid rotifer Adineta vaga. Nature 500: 453–457. https://doi.org/10.1038/nature12326

Flot J., N. Debortoli, B. Hallet, J. Narayan, and K. Van Doninck, 2018 Reply to Cross-Contamination Explains '"Inter and Intraspecific Horizontal Genetic Transfers'"' between Asexual Bdelloid Rotifers (Wilson, Nowell & Barraclough 2018)."' bioRxiv. https://doi.org/10.1101/368209

Flynn J. M., F. J. J. Chain, D. J. Schoen, and M. E. Cristescu, 2017 Spontaneous mutation accumulation in daphnia pulex in selection-free vs. competitive environments. Mol. Biol. Evol. 34: 160–173. https://doi.org/10.1093/molbev/msw234

Fontaneto D., T. G. Barraclough, K. Chen, C. Ricci, and E. A. Herniou, 2008 Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. Mol. Ecol. 17: 3136–3146. https://doi.org/10.1111/j.1365-294X.2008.03806.x

França M. B., a D. Panek, and E. C. a Eleutherio, 2007 Oxidative stress and its effects during dehydration. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 146: 621–631. https://doi.org/10.1016/j.cbpa.2006.02.030

Fredrickson J. K., S. W. Li, E. K. Gaidamakova, V. Y. Matrosova, M. Zhai, *et al.*, 2008 Protein oxidation: key to bacterial desiccation resistance? ISME J 2: 393–403.

Gladyshev E. A., M. Meselson, and I. R. Arkhipova, 2007 A deep-branching clade of retrovirus-like retrotransposons in bdelloid rotifers. Gene 390: 136–145. https://doi.org/10.1016/j.gene.2006.09.025

Gladyshev E., and M. Meselson, 2008 Extreme resistance of bdelloid rotifers to ionizing radiation. Proc. Natl. Acad. Sci. U. S. A. 105: 5139–5144. https://doi.org/10.1073/pnas.0800966105

Gladyshev E., and I. Arkhipova, 2010 A subtelomeric non-LTR retrotransposon Hebe in the bdelloid rotifer Adineta vaga is subject to inactivation by deletions but not 5' truncations. Mob. DNA 1: 12.

Hartfield M., S. I. Wright, and A. F. Agrawal, 2018 Coalescence and linkage disequilibrium in facultatively sexual diploids. Genetics 210: 683–701. https://doi.org/10.1534/genetics.118.301244

Hickey D. A., 1982 Selfish DNA: a sexually-transmitted nuclear parasite. Genetics 101: 519–531.

Holsinger K. E., and N. C. Ellstrand, 1984 The Evolution and Ecology of Permanent Translocation Heterozygotes. Am. Nat. 124: 48–71.

Hsu W. S., 1956a Oogenesis in the Bdelloidea rotifer Philodina roseola Ehrenberg. Cellule 57: 283–296.

Hsu W. S., 1956b Oogenesis in Habrotrocha tridens (Milne). Biol. Bull. 111: 364–374.

Hudson C. T., and P. H. Gosse, 1886 *The Rotifera or Wheel-Animalcules*. Longmans, Green, London.

Hur J. H., K. Van Doninck, M. L. Mandigo, and M. Meselson, 2009 Degenerate tetraploidy was established before bdelloid rotifer families diverged. Mol. Biol. Evol. 26: 375–383. https://doi.org/10.1093/molbev/msn260

Krisko A., M. Leroy, M. Radman, and M. Meselson, 2012 Extreme anti-oxidant protection against ionizing radiation in bdelloid rotifers. Proc. Natl. Acad. Sci. U. S.

A. 109: 2354–2357. https://doi.org/10.1073/pnas.1119762109

Ladle R. J., R. A. Johnstone, and O. P. Judson, 1993 Coevolutionary dynamics of sex in a metapopulation: escaping the Red Queen. Proc. R. Soc. Biol. Sci. Ser. B 253: 155–160.

Lasek-Nesselquist E., 2012 A Mitogenomic Re-Evaluation of the Bdelloid Phylogeny and Relationships among the Syndermata. PLoS One 7: e43554. https://doi.org/10.1371/journal.pone.0043554

Leewenhoeck A. van, 1677 Observations, Communicated to the Publisher by Mr. Antony van Leewenhoeck, in a Dutch Letter of the 9th of Octob. 1676. Here English'd: concerning Little Animals by Him Observed in Rain-Well-Sea- and Snow Water; as Also in Water Wherein Pepper Had Lain In. Philos. Trans. R. Soc. London 12: 821–831. https://doi.org/10.1098/rstl.1677.0003

Leewenhoeck A. van, 1702 Letter to the Royal Society December 25, 1702. Proc. R. Soc. London B 23: 1291–1311.

Magwene P. M., Ö. Kayıkçı, J. A. Granek, J. M. Reininga, Z. Scholl, *et al.*, 2011 Outcrossing, mitotic recombination, and life-history trade-offs shape genome evolution in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. 108: 1987–1992. https://doi.org/10.1073/pnas.1012544108

Mark Welch D. B., and M. Meselson, 2000 Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science. 288: 1211–1215.

Mark Welch D. B., and M. S. Meselson, 2001 Rates of nucleotide substitution in sexual and anciently asexual rotifers. Proc. Natl. Acad. Sci. 98: 6720–6724.

Mark Welch D. B., J. L. Mark Welch, and M. Meselson, 2008 Evidence for degenerate

tetraploidy in bdelloid rotifers. Proc. Natl. Acad. Sci. 105: 5145–5149. https://doi.org/10.1073/pnas.0800972105

Mark Welch D. B., C. Ricci, and M. Meselson, 2009 Bdelloid rotifers: Progress in understanding the success of an evolutionary scandal, pp. 259–279 in *Lost Sex: The Evolutionary Biology of Parthenogenesis*, edited by Schoen I., Martens K., Dijk P. van. Springer.

Meadow N. D., and C. H. Barrows Jr., 1971 Studies on aging in a bdelloid rotifer .I. The effect of various culture systems and longevity and fecundity. J. Exp. Zool. 176: 303–314.

Nowell R. W., P. Almeida, C. G. Wilson, T. P. Smith, D. Fontaneto, *et al.*, 2018 Comparative genomics of bdelloid rotifers: Insights from desiccating and nondesiccating species, (C. Tyler-Smith, Ed.). PLOS Biol. 16: e2004830. https://doi.org/10.1371/journal.pbio.2004830

Omilian A. R., M. E. A. Cristescu, J. L. Dudycha, and M. Lynch, 2006 Ameiotic recombination in asexual lineages of Daphnia. PNAS 103: 18638–18643. https://doi.org/10.1073/pnas.0606435103

Ricci C., 1983 Life histories of some species of Rotifera Bdelloidea. Hydrobiolgia 180: 175–180.

Ricci C., and U. Fascio, 1995 Life-history consequences of resource allocation of two bdelloid rotifer species. Hydrobiologia 299: 231–239. https://doi.org/10.1007/BF00767330

Ricci C., 1998 Anhydrobiotic capabilities of bdelloid rotifers. Hydrobiologia 387/388: 321–326.

573 Ricci C., and M. Caprioli, 2005 Anhydrobiosis in bdelloid species, populations, and
574 individuals. Integr. Comp. Biol. 45: 759–763.
575 Ricci C., and F. Perletti, 2006 Starve and survive: stress tolerance and life-history traits
576 of a bdelloid rotifer. Funct. Ecol. 20: 340–346.
577 Rodriguez F., and I. R. Arkhipova, 2016 Multitasking of the pirna silencing machinery:
578 Targeting transposable elements and foreign genes in the Bdelloid Rotifer Adineta
579 vaga. Genetics 203: 255–268. https://doi.org/10.1534/genetics.116.186734
580 Ruderfer D. M., S. C. Pratt, H. S. Seidel, and L. Kruglyak, 2006 Population genomic
581 analysis of outcrossing and recombination in yeast. Nat. Genet. 38: 1077–1081.
582 https://doi.org/10.1038/ng1859
583 Schaefer I., K. Domes, M. Heethoff, K. Schneider, I. Schön, *et al.*, 2006 No evidence for
584 the “Meselson effect” in parthenogenetic oribatid mites (Oribatida, Acari). J. Evol.
585 Biol. 19: 184–193. https://doi.org/10.1111/j.1420-9101.2005.00975.x
586 Schon I., K. Martens, and P. van Dijk (Eds.), 2009 *Lost Sex: The evolutionary biology*
587 *of parthenogenesis.* Springer, Dordrecht.
588 Signorovitch A., J. Hur, E. Gladyshev, and M. Meselson, 2015 Allele Sharing and
589 Evidence for Sexuality in a Mitochondrial Clade of Bdelloid Rotifers. Genetics 200:
590 581–590. https://doi.org/10.1534/genetics.115.176719
591 Signorovitch A., J. Hur, E. Gladyshev, and M. Meselson, 2016 Evidence for meiotic sex
592 in bdelloid rotifers. Curr. Biol. 26: R754–R755.
593 https://doi.org/10.1016/j.cub.2016.06.051
594 Simion P., J. Narayan, A. Houtain, A. Derzelle, L. Baudry, *et al.*, 2020 Homologous
595 chromosomes in asexual rotifer Adineta vaga suggest automixis. 10–12.

https://doi.org/10.1101/2020.06.16.155473

Tucker A. E., M. S. Ackerman, B. D. Eads, S. Xu, and M. Lynch, 2013 Population-genomic insights into the evolutionary origin and fate of obligately asexual Daphnia pulex. Proc. Natl. Acad. Sci. U. S. A. 110: 15740–15745. https://doi.org/10.1073/pnas.1313388110

Vakhrusheva O. A., E. A. Mnatsakanova, Y. R. Galimov, T. V. Neretina, E. S. Gerasimov, *et al.*, 2020 Genomic signatures of recombination in a natural population of the bdelloid rotifer Adineta vaga. Nat. Commun. 11. https://doi.org/10.1038/s41467-020-19614-y

Wesenberger-Lund C., 1930 Contributions to the biology of the rotifera, part II: The periodicity and sexual periods. Kgl. Danske Vidensk. Selsk. Skr. Naturv. Og Math. Afd. 9. II: 1–230.

Wilson C. G., and P. W. Sherman, 2010 Anciently Asexual Bdelloid Rotifers Escape Lethal Fungal Parasites by Drying Up and Blowing Away. Science. 574–577.

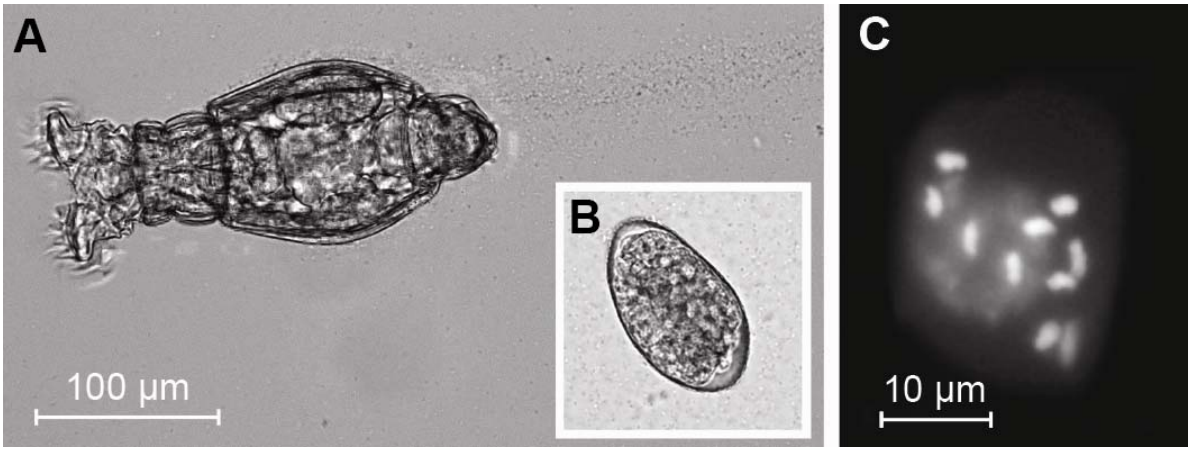
Wilson C. G., 2011 Desiccation-tolerance in bdelloid rotifers facilitates spatiotemporal escape from multiple species of parasitic fungi. Biol. J. Linn. Soc. 104: 564–574. https://doi.org/10.1111/j.1095-8312.2011.01737.x

Wilson C. G., R. W. Nowell, and T. G. Barraclough, 2018 Cross-Contamination Explains "Inter and Intraspecific Horizontal Genetic Transfers" between Asexual Bdelloid Rotifers. Curr. Biol. 28: 2436-2443.e15. https://doi.org/10.1016/j.cub.2018.05.070

Xu S., A. R. Omilian, and M. E. Cristescu, 2011 High rate of large-scale hemizygous deletions in asexually propagating Daphnia: Implications for the evolution of sex. Mol. Biol. Evol. 28: 335–342. https://doi.org/10.1093/molbev/msq199

Xu S., S. Schaack, A. Seyfert, E. Choi, M. Lynch, *et al.*, 2012 High mutation rates in the mitochondrial genomes of Daphnia pulex. Mol. Biol. Evol. 29: 763–769. https://doi.org/10.1093/molbev/msr243

624



625

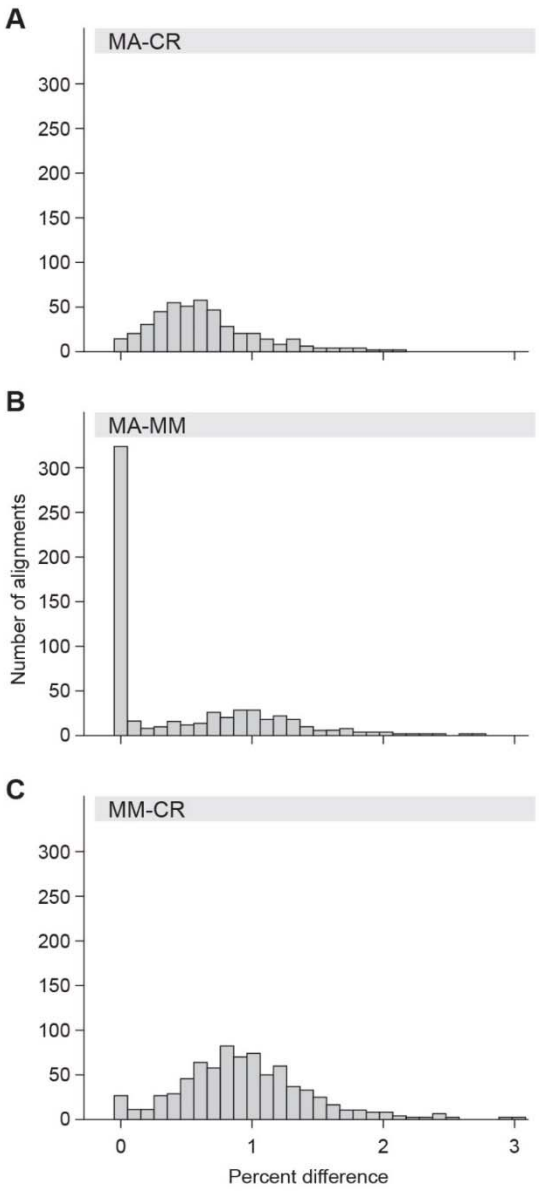
626 **Figure 1.** The bdelloid *M. quadricornifera*. (A) Adult. The animal has attached itself to

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.

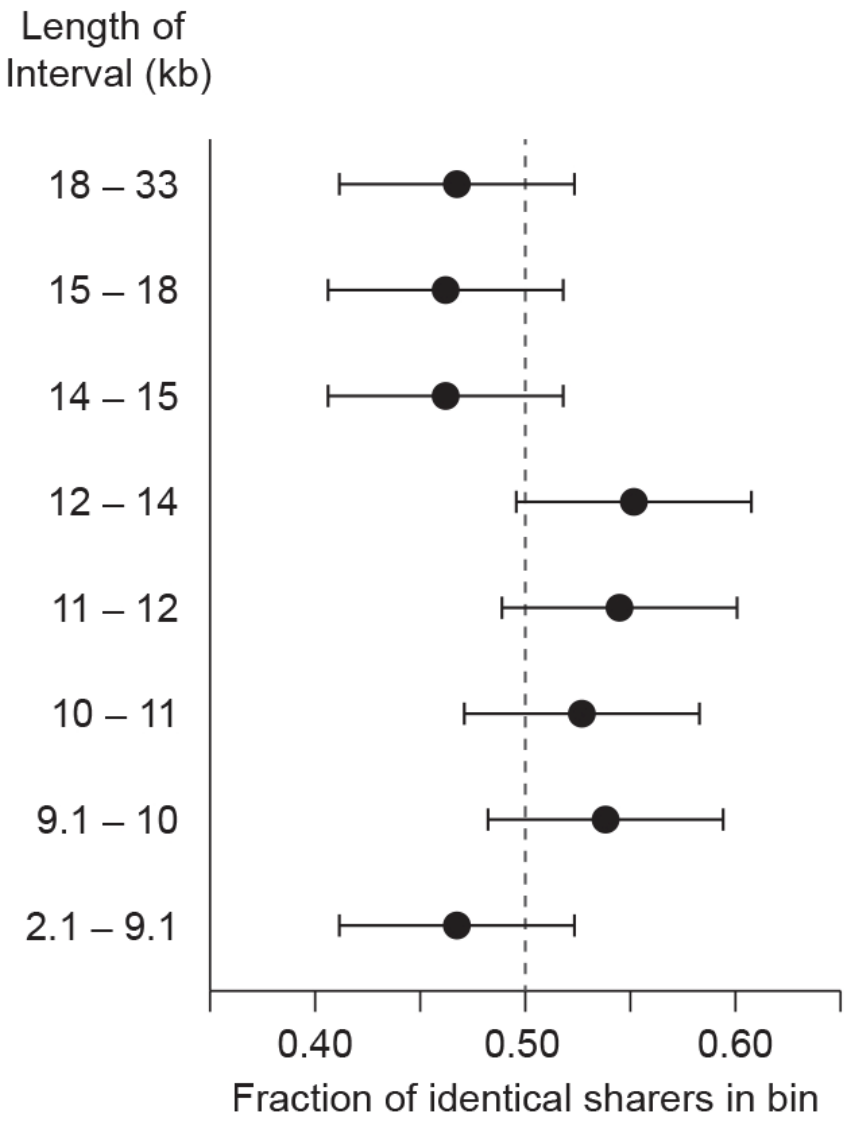
630



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



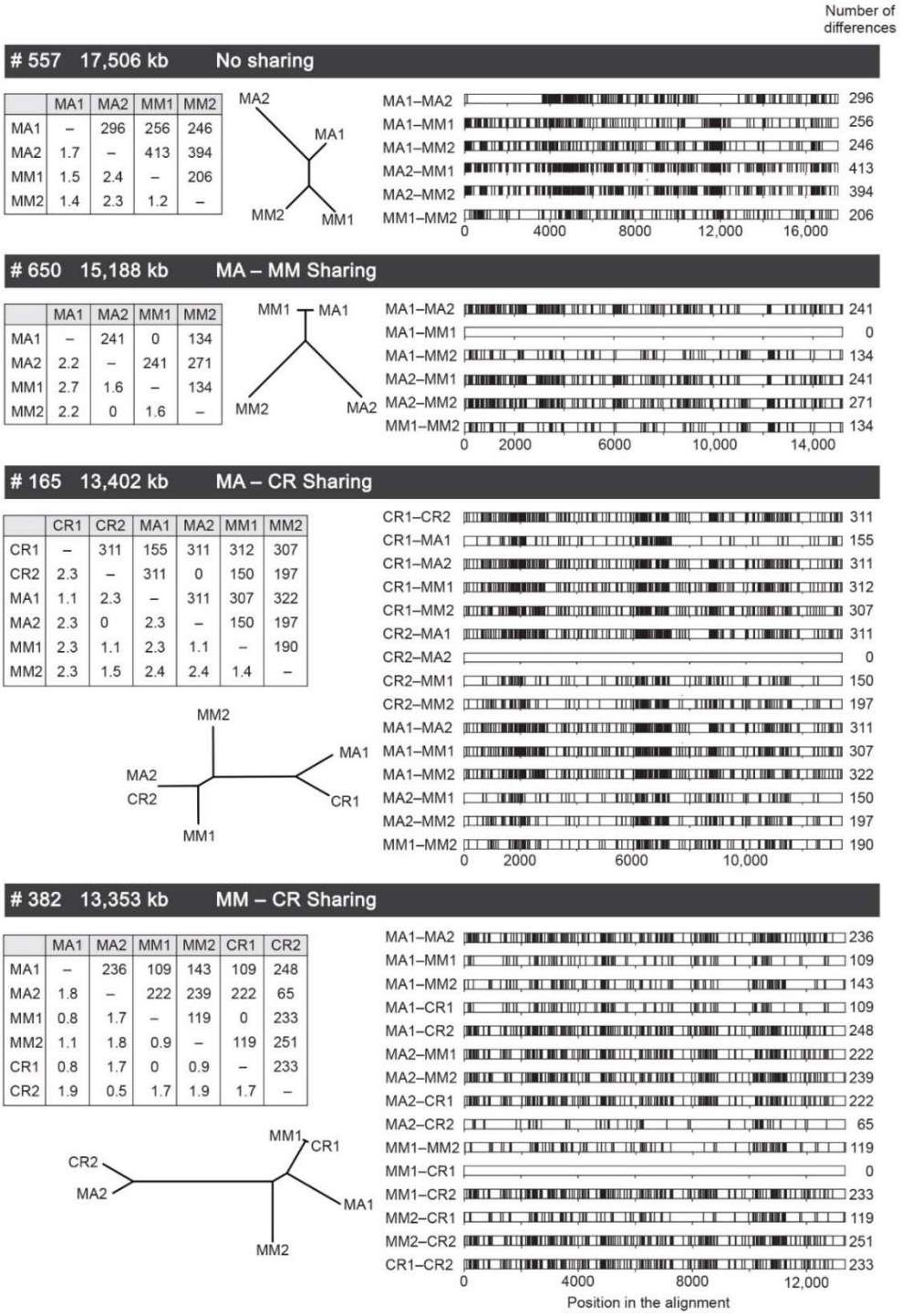
638

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

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657

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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