Sexual Reproduction in Bdelloid Rotifers

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ABSTRACT Nearly all eukaryotes reproduce sexually, either constitutively or facultatively, and nearly all that are thought to be entirely asexual diverged recently from sexuals, implying that loss of sex leads to early extinction and therefore that sexual reproduction is essential for evolutionary success. Nevertheless, there are several groups that have been thought to have evolved asexually for millions of years. Of these, the most extensively studied are the rotifers of Class Bdelloidea. Yet the evidence for their asexuality is entirely negative -- the failure to establish the existence of males or hermaphrodites. Opposed to this is a growing body of evidence that bdelloids do reproduce sexually, albeit rarely, retaining meiosis-associated genes and, in a limited study of allele sharing among individuals of the bdelloid *Macrotrachela quadricornifera*, displaying a pattern of genetic exchange indicating recent sexual reproduction. Here we present a much larger study of allele sharing among these individuals clearly showing the occurrence of sexual reproduction, thereby removing what had appeared to be a serious challenge to hypotheses for the evolutionary advantage of sex and confirming that sexual reproduction is essential for long term evolutionary success in eukaryotes. We also discuss the relation between bdelloid life history and population structure and the possible benefit of outcrossing in restoring advantageous genome-wide epistatic interactions disrupted by conversion and deletion.

KEYWORDS allele sharing, conversion, desiccation, epistasis, horizontal gene transfer, life history, outcrossing, population structure, Red Queen

MANY hypotheses have been advanced to explain why, despite its substantial costs, sexual reproduction is nearly universal in eukaryotes and why the loss of sex generally leads to early extinction--a major problem in evolution theory (1–5). Posing a challenge to all such hypotheses are a few groups of ancient origin that have been considered to be entirely asexual (6). Of these, the most extensively studied are the rotifers of Class Bdelloidea (7) (Fig. 1). First described nearly 350 years ago (8, 9), bdelloids are minute freshwater invertebrates commonly found in lakes, ponds and streams, swimming free or attached to surfaces, and in ephemerally aquatic habitats such as temporary pools and the water films on lichens and mosses. Characterized by their ciliated head and bilateral ovaries, they 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, they 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 (10–13).

The only observed means of bdelloid reproduction is from eggs mitotically produced in well-differentiated ovaries (14, 15). 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 (16, 17). Depending on species and conditions, the mean time from egg deposition to death is about a month (17–19). Bdelloids are eutelic, with no cell division after eclosion except in the germ line.

Bdelloids as a class 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 as they are encountered in all four bdelloid families (20–22). Bdelloids have an extremely effective system of anti-oxidant protection, as manifested by their extreme resistance to ionizing radiation and

IR-induced protein carbonylation (23, 24), apparently an adaptation to avoid the oxidative damage caused by desiccation in other systems (25, 26).

Although bdelloids have been systematically studied ever since adequate microscopes became available (27, 28), there is no confirmed evidence of males or hermaphrodites. 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 (29). 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” under the ice of a frozen lake in Denmark during a population bloom in November 1923, there being almost no bdelloids there the following summer (30). Sampling conducted there in January 2019 found few bdelloids, no evidence of males and no 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 rarely and under conditions not yet 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, owing to the active repression of meiosis which, however, can be relieved in the laboratory by growth in specific media (31, 32).

In the following we review findings once interpreted as evidence for bdelloid asexuality, but now known to have other explanations, and summarize recent genomic evidence for sexual reproduction. We then present an extensive study of allele sharing in the bdelloid *Macrotrachella quadricornifera* confirming the occurrence of sexual reproduction. We also discuss a possible benefit of outcrossing in restoring beneficial genome-wide epistatic interactions, the relation between bdelloid life history and population structure and its implications for how bdelloid males and mating might be discovered.

Findings formerly interpreted as evidence for ancient asexuality

Heterozygosity. In sexuals, heterozygosity caused by mutation is limited by drift of meiotic segregants. Assuming that without segregation the loss of heterozygosity by conversion would be much less than its generation by mutation, Mark Welch and Meselson proposed that bdelloids, if anciently asexual, might exhibit an unusually high level of heterozygosity (33). Finding very high values of difference at four-fold degenerate sites between copies of several genes in each of four bdelloid species but not in any of six facultatively sexual monogonont rotifers or in the reported sequences of any other invertebrate and also finding in relative rate tests that the synonymous substitution rate in bdelloids is not significantly different from that in monogononts and considering ancient polyploidy, unknown at the time in any animal, to be unlikely, they interpreted their findings as evidence that bdelloids had evolved asexually (33, 34). Continued investigation, however, showed that bdelloids are degenerate tetraploids, and that the highly diverged gene copies are homeologs, not homologs (10, 11). Bdelloid heterozygosity, the difference between homologs, lies within the range known for sexuals, providing no evidence for asexuality.

Meanwhile, mutation accumulation experiments with asexual *Daphnia pulex* and *Saccharomyces cerevisiae* unexpectedly found that the frequency with which a nucleotide site is covered by a conversion tract is much greater than the frequency of nucleotide substitution (35–38). In sexuals, heterozygosity lost by conversion can be regained by outcrossing but if bdelloids are ancient asexuals and conversion is more frequent than substitution, a lack of outcrossing should be manifested as unusually *low* heterozygosity, the opposite of what had been thought (32, 39). The observation that bdelloid heterozygosity is within the range known for sexual taxa therefore suggests that bdelloids may be sexual, with heterozygosity lost by conversion regained by occasional outcrossing. The moderate levels of heterozygosity seen in other putative ancient asexuals (6, 40) would then suggest that they too engage in outcrossing.

Paucity of retrotransposons. In another test for asexuality, based on the expectation that an asexual population would eventually either become devoid of pre-existing deleterious vertically-transmitted transposable elements or be driven to extinction by their increase (41, 42), Arkhipova and Meselson (43–45) surveyed the

genomes of 5 bdelloid species, 2 species of monogonont rotifers and 40 other sexually-reproducing species representing 23 animal phyla for sequences coding for the reverse transcriptases of two superfamilies of LINE-like retrotransposons. Such sequences were found to be abundant in all the sexually-reproducing taxa but were not detected in any of the bdelloids, while DNA transposons, transmitted horizontally, were found to be abundant. Nevertheless, while bdelloids are nearly devoid of LINE-like retrotransposons, later work showed that they are not entirely absent (46, 47) and that bdelloids have particularly effective retrotransposon silencing systems (48). Although unusual, the paucity of LINE-like retrotransposons is therefore non-evidentiary as regards bdelloid sexuality. Also consistent with asexuality but not decisive, is the presence of odd chromosome numbers in some bdelloid species (14, 49).

Genome structure. Flot *et al*. (12) reported a draft genome sequence of the bdelloid *Adineta vaga* having numerous breaks in the collinearity of homologous regions and scaffolds containing numerous genes in direct or palindromic repeats but no copy elsewhere in the genome, a situation incompatible with the existence of homologous chromosome pairs and in which segregation with independent assortment would generally be lethal. These features of the *A. vaga* draft assembly led the authors to conclude that bdelloids had evolved ameiotically. But subsequent genomic sequencing of three other bdelloid species, including *Adineta ricciae*, a close relative of *A. vaga*, found no evidence for such repeats and frequent homology breaks, suggesting that their apparent presence in *A. vaga* resulted from mis-assembly (13).

Previous Evidence for Sexual Reproduction

Meiosis-related genes. Nowell *et al*. (13) sequenced the genomes of four bdelloid species belonging to two bdelloid families and searched for the proteomes of 12 genes considered to be meiosis-specific; *dmc1*, *hop1*, *hop2*, *mer3*, *mnd1*, *msh4*, *msh*5, *rec8*, *red1*, *sad1*, *spo11* and *zip1*. Proteomes of all but *red1* were found in all four species.But neither was *red1* found in *Drosophila melanogaster*, a sexual species known to lack it. The presence of proteomes of these 11 meiosis-related genes in each of the four bdelloid species suggests that they are under selection for the same functions as they

serve in sexuals and therefore that bdelloids engage in meiosis. Although Flot *et al*. (12) did not find *dmc1*, *mer3*, *hop2*, *mnd1*, or *zip*1 in their assembly of the *A. vaga* genome, their detection in all four bdelloid species examined by Nowell *et al*. suggests that they are present but were not detected in *A. vaga*.

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, as by sexual reproduction, homologous horizontal transfer, or parasexuality. Examining the sequences of both homologs in each of four genomic regions 2.4 to 9.7 kb in length, Signorovitch *et al*. (50) found at each region a striking example of allele sharing among three isolates of the bdelloid *M. quadricornifera*, designated MA, MM, and CR, collected at widely separated sites but all belonging to the same mitochondrial clade (*S1 Appendix*, Text 1 Methods). At each of the four regions, a homolog of MA was found to be identical to a homolog of MM while the other homolog of MA was identical to a homolog of CR in two regions and nearly so in the other two. All other inter-homolog differences, within and between isolates, almost entirely single nucleotide substitutions, were 1-3 percent, typical of large outbreeding populations. These findings argued strongly for recent sexual exchange but suggested that the four regions may not have recombined, as in certain plants in which entire haplotypes remain intact through meiosis (51). At the time, the suggestion of no recombination appeared to gain support from the earlier although subsequently challenged report that *A. vaga* lacks homologous chromosome pairs and has additional features incompatible with independent assortment and crossing-over (52).

The conclusion of Signorovitch *et al*. (50, 53) that the allele sharing they observed was the result of sexual reproduction was disputed by Debortoli *et al.* (54) who suggested that it resulted instead from horizontal transfer, a view supported by what they considered to be extensive homologous horizontal genetic transfer in the bdelloid *Adineta vaga*. Their evidence for horizontal transfer was challenged, however,

by Wilson *et al*. (55) who showed that their observations could be explained as the result of cross-contamination among the isolates they examined.

The allele sharing seen in *M. quadricornifera* and the conservation in diverse bdelloid species of numerous genes considered specific to meiosis are inconsistent with ancient asexuality and strongly suggest that bdelloids are facultatively sexual. The much larger study of allele-sharing in *M. quadricornifera* reported here clearly confirms the occurrence of sexual reproduction.

Results and Discussion

Alignments. As described more fully in Methods (*SI Appendix*, S1), we obtained phased sequences at a large number of genomic regions of *M. quadricornifera* isolates MA, MM and CR by aligning individual Nanopore reads of MA against megabubbles of the three isolates obtained by 10x sequencing and assembly with Supernova 2.1.1. From the resulting alignments, we selected those that were co-linear with the Nanopore read and which included both homologs of at least two of the three isolates. The megabubble sequences were then realigned among themselves and trimmed with Gblocks to remove overhangs and alignment errors caused by indels and repeats, but neither adding to nor rearranging sequences. We also rejected the shorter alignments when two or more overlapped and the five alignments in which both homologs of MM or CR differed by more than 10% from both homologs of MA. 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 gave a total of 1,177 non-overlapping alignments representing as many separate genomic regions, 331 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), altogether covering 14.7 Mb, approximately 4% of the *ca.* 360 Mb *M. quadricornifera* genome (*SI Appendix*, Table S2). Matrices giving pair-wise differences between homologous sequences, phylograms and “tic” plots of the distribution of differences between homologs are given in Table 1 for six representative regions and for all 1,177 regions in Supplemental Information (*SI Appendix*, Tables S1-3).

As expected, the sequences determined by Signorovitch *et al.* (51) were found to agree closely with the corresponding sequences determined by 10x, the mean difference being $2.9X10^{-4}$ /site (s.d.=$2.85 X 10^{-4}$) (*SI Appendix*, Table 4). The earlier sequencing was done by the Sanger method, for which an error rate of *ca*.10^{-4} is typical, while our 10x sequencing was done by the Illumina method at high coverage, suggesting that the error rate of our sequencing is considerably less than 10^{-4} /site.

Frequency of identical sharing. Inspection of the MA-MM and MA-MM-CR alignments shows that close to half of them, 315 of 622, comprise a discrete class in which a homolog of MA is identical to a homolog of MM (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) (Table 2).

In addition to the regions with identical sharing there is a relatively small number of alignments in which MA and MM differ by only one or a few substitutions, as may have arisen by mutation after the sharing event. In the alignments without identical or nearly 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 alignment in which we find them, as may be seen in a plot of the frequency of identical MA-MM sharing against alignment length in consecutive intervals each comprising 76-79 alignments (Fig. 4). The frequency of identical sharing is not significantly different from 50 percent in even the longest alignments.

MA and MM also share identical homologs with CR, but in a much smaller proportion of the alignments (Table 2; 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. While the number of nearly identical MA-MM sharers is small compared to the number of identical sharers, the proportion of nearly identical MA-CR and MM-CR sharers relative to the number of identical sharers is much larger.

Genealogy. MA and MM 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, a pattern of relationship expected for diploids related as grandchild and grandparent, half siblings, aunt and uncle or nephew and niece in a large genetically diverse panmictic population. As expected for a small sample of such a population there we found no reciprocal double sharers, a/b a/b. 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.

Counting only identical sharing, the degree of relationship between CR and MA and between CR and MM would suggest that CR identically shares 1/32 of its genome with MA and 1/32 with MM. But if the nearly identical MA-CR and MM-CR sharers are taken into account as the result of sharing followed by a low level of mutation, the relationship of CR to MA and to MM would be closer than indicated by identical sharing alone.

Inspection of tic plots for the triple alignments reveals a minority with long interior regions of MA-MM identity (*SI Appendix*, Table S 5). In most of these regions there is substantial divergence of MA and MM from CR, showing that such identity is unlikely to be a manifestation 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, the existence of alignments in which CR is identical to MA or MM over much but not all of the alignment may reflect similarly remote relationships. 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, for example, 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 the identically shared regions we see does not fall off with alignment length (Fig. 4).

Homozygosity and Conversion. In each of the three isolates there are a few regions that are entirely homozygous (Table 2). Finding such regions to be no more frequent in the shorter half of the alignments than in the longer half, we conclude that they are generally longer than the 20-30 kb alignments in which we find them. A suggestion that such regions result from conversion subsequent to the last outcross rather than from identity by descent is seen in the distribution of interhomolog differences. There a few with no difference, a smaller number with only one or a few differences, no regions with an intermediate number of differences and then many regions with numerous differences. As conversion tracts are erased by outcrossing, such a truncated distribution of differences is expected for tracts of recent conversion followed by infrequent mutation but not for the distribution of times back to the corresponding coalescents.

Among the 622 alignments without homozygosity of either MA or MM, 315 (50.6%) share an identical allele, whereas among the 23 regions in which either MA or MM is homozygous only 7 (30.4%) share an identical allele, consistent with the expectation that conversion would erase evidence of sharing in half of these 23 alignments. These few cases in which sharing may have been obscured by conversion are not included in the totals given above or in Table 2.

Recombination. That recombination occurs is seen in the observation that MA and MM do not share entire haplotypes but only a quarter of their genomes and share even less with CR. The pattern of sharing found by Signorovitch *et al*. (50) in which a homolog of MA was identical to a homolog of MM in each of the four 3-10 kb regions they examined and in which the other homolog of MA was identical to a homolog of CR in two regions and nearly so in the other two suggested to them that entire haplotypes may have passed through meiosis without recombination. In contrast, we find such a pattern of sharing in only a few of our much longer MM-MA-CR alignments. It may be that the pattern of sharing encountered in the earlier study resulted from identity and near identity by descent from more remote ancestors, in which case such regions would be too short to appear as regions of identical or near-identical sharing in our much longer alignments.

Horizontal transfer and parasexuality. Although horizontal transfer of alien genes into bdelloid genomes is well documented (51, 56), where located they are only in regions selected for being near chromosome ends (51, 57). Whether horizontal transfer occurs elsewhere in bdelloid genomes and, if so, is ever homologous is unknown and in any case cannot account for our results. The finding of identical MA-MM allele sharing at half (315/622 = 0.506) of the alignments in which MA and MM are represented is expected for diploids that share a quarter of their genomes by virtue of recent sexual reproduction. For this result to occur by HGT would require massive transfer of long DNA segments coincidentally at just half the regions examined and somehow prevented from ever transferring both homologs of MA to MM or *vice versa*, a configuration absent from the 622 alignments that include MA and MM.

Neither can the observed allele sharing 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 divisions undergo occasional misdivision, only rarely yielding viable diploids. In the present case, 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. Further, neither parasexuality nor horizontal transfer can account for our finding of identical sharing in half of the 1,177 regions examined, as expected for diploids that are half-sibs or double first cousins.

Number of 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 generations that would cause mutational reduction of the frequency of identical sharers to fall significantly below the observed value of 0.5; from the frequency of apparent conversion; and from the mitochondrial difference between MA and MM.

MA and MM differ by a single substitution in 2 of the 291 MA-MM alignments and in 4 of the 622 MA-MM-CR alignments, for an overall frequency of $6.6X10^{-3}$. For a mean alignment length of 15 kb this is a frequency of $0.44X10^{-6}$ per bp. Substitution rates

measured in accumulation experiments with *Caenorhabditis elegans*, asexual *D. pulex*, and *D. melanogaster range* from 2.3 to $5.5X10^{-9}$ per generation (37). Taking this as applicable to the substitution rate along the MA and MM lineages since their sharing event and assuming a Poisson distribution of the small differences between shared homologs, as expected for mutation, this suggests that the number of generations since the sharing event may be 100-200, summed over the MA and MM lineages.

If there were as many as 2,000 generations since the sharing event and assuming a substitution rate of $4X10^{-9}$ per generation, the expected number of substitutions in regions 18-33 kb in length, the longest interval in Figure 4, would be 0.14 – 0.26, reducing the proportion of identical sharers to 0.43 – 0.39, substantially lower than what we observe, suggesting that the summed number of generations between MA and MM is no more than 1,000.

As conversion tracts in a large essentially panmictic population are generally erased by outcrossing, the frequency of substitutions in such tracts will increase with the number of generations since the last outcross. Assuming a conversion rate of 3.3-4.8 X 10^{-5} per bp per generation (37, 58) and considering that the total length of MA regions is 13.4 Mbp of which perfectly homozygous regions comprise some 151 kbp, or about 1.1%, it would appear that there have been some 340-500 generations along the MA lineage since the sharing event. The corresponding estimate for the number of generations along the MM lineage is 550-800.

A fourth estimate of the number of generations since homologs of MA and MM separated may be obtained by assuming that they had the same mitochondria at the time of sharing. Taking the difference of 5 substitutions or 2.5 X 10^{-5} between their 14 kb mitochondria (59), the mitochondrial mutation rate of 1.5 X 10^{-7} found in mutation accumulation studies of asexual *Daphnia pulex* (58), would suggest that the sharing event occurred some 170 generations ago. Altogether, these various estimates suggest that the MA-MM sharing event may have occurred no more than about a thousand clonal generations ago and as few as about 100.

Abundance of close relatives in the sampled population. Isolates MA, MM and CR were obtained from ground moss at widely separated sites and are part of a collection

of 29 individuals having the morphology of Family Philodinidae (50)(*SI Appendix*,S1 Methods). What aspects of bdelloid life history could make finding such close relatives in so small and widely dispersed a sample of what must be an enormous population? It must be that the sampled population is largely made up of relatively few very large, widely dispersed clones descended from recent crossing.

Such an unusual population structure might result if sexual periods occur only rarely, during a population bloom at high density, with multiple rounds of random mass mating among two or more founding types and their progeny, producing large numbers of closely related bdelloids. This, together with subsequent dispersion and extensive clonal reproduction would give rise to very large, widely dispersed clones of the products of recent crosses. Meanwhile, lines that fail to outcross would suffer clonal erosion, possibly for reasons described below, driving them to extinction unless revived by timely outcrossing, leaving only widely dispersed descendants from recent crossing in a limited number of blooms.

It may be that the production of males and mating occur only when particular mating types are present together, each producing a signal that causes the other to produce haploid eggs and haploid males, possibly *via* mictic females as with monogonont rotifers. At some stage of the process, from fertilization to zygote development, selfing may be prevented and heterozygosity thereby maintained. 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 (30).

Bdelloid life history and the benefit of sex. 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. 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. 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 (60), thereby escaping biotic antagonists in their initial habitat and transferring to an environment where antagonists may be less abundant. The combination of anhydrobiosis and dispersibility by wind, birds or other vectors therefore affords substantial protection from what would otherwise be co-evolving biotic antagonists (61–64), reducing the need for frequent recombination by largely eluding the “Red Queen” (64–67). 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 (68, 69).

The ability of bdelloid populations to avoid or escape from competition with co-evolving biotic antagonists and, by becoming temporarily dormant to survive transiently inhospitable environmental conditions without having to produce recombinant progeny in order to maintain the species, raises the question of why bdelloids nevertheless do reproduce sexually and what might cause rapid clonal erosion in the absence of sex. In addition to the well-known benefits of outcrossing in covering deleterious recessive mutations made homozygous by conversion or hemizygous by deletion, in restoring overdominance at individual loci, and, together with recombination, in purging deleterious mutations, an important further benefit of outcrossing may be the restoration of information lost by conversion or deletion and the consequent disruption of favorable genome-wide epistatic interactions (2, 70, 71). Although difficult to study, such genome-wide epistasis is to be expected in order to maximize the opportunity for favorable structural interactions between diverse gene products, the so-called “interactome” (72, 73) possibly also including spatial interactions between chromosome domains, influencing the regulation of gene expression (74). The enhanced opportunity for such epistasis made possible by diploidy may also contribute to its prevalence over haploidy in eukaryotes generally.

Summary

Our principal finding is that bdelloids are not entirely asexual, removing what has long been a challenge to hypotheses for the evolutionary benefit of sex. Contrary to their reputation as ancient asexuals, bdelloids of the population represented by the isolates MA and MM of *M. quadricornifera* and presumably bdelloids as a Class definitely engage in occasional sexual reproduction and outcrossing. It is suggested that the infrequency of sexual reproduction is an adaptation to the distinctive bdelloid life history. Being able to survive desiccation and other conditions lethal to most other taxa bdelloids have less need for recombination to maintain fitness, thereby greatly reducing the cost of sex. Nevertheless, occasional outcrossing is required to replace genetic information lost by conversion and deletion--to cover accumulated deleterious recessive mutations and to restore beneficial epistatic relations throughout the genome. The present evidence for sexual reproduction and outcrossing in a species of bdelloid rotifer, the group once considered the best example of ancient asexuality, makes it likely that there are no ancient asexuals and that sexual reproduction with outcrossing is universally essential for long-term evolutionary success in eukaryotes.

Figure Legends

Fig. 1. The bdelloid *M. quadricornifera*, an unhatched egg shown at the same scale and a metaphase nucleus showing the 10 chromosomes characteristic of the species.

Fig. 2. Histograms showing the distribution of divergence between the most similar homologs in each of the three pairs of isolates. (A) MA-CR, (B) MA-MM, (C) MM-CR. Alignments with identical or very nearly identical MA-MM sharing form a discrete class constituting almost exactly half of the regions. Bin size = 0.05 percent difference for the first bar in panel B. Otherwise 0.1 percent.

Fig. 3. Histograms showing the distribution of heterozygosity in each of the three isolates. (A) CR, (B) MA. (C) MM. Bin size = 0.1 percent difference.

Fig. 4. The mean frequency of identical MM-MA sharing in each of 8 bins of increasing length, each containing 76-79 intervals. Error bars represent standard errors.

Table 1. Representative matrices, phylograms and tic plots for MA-MM-CR alignments.

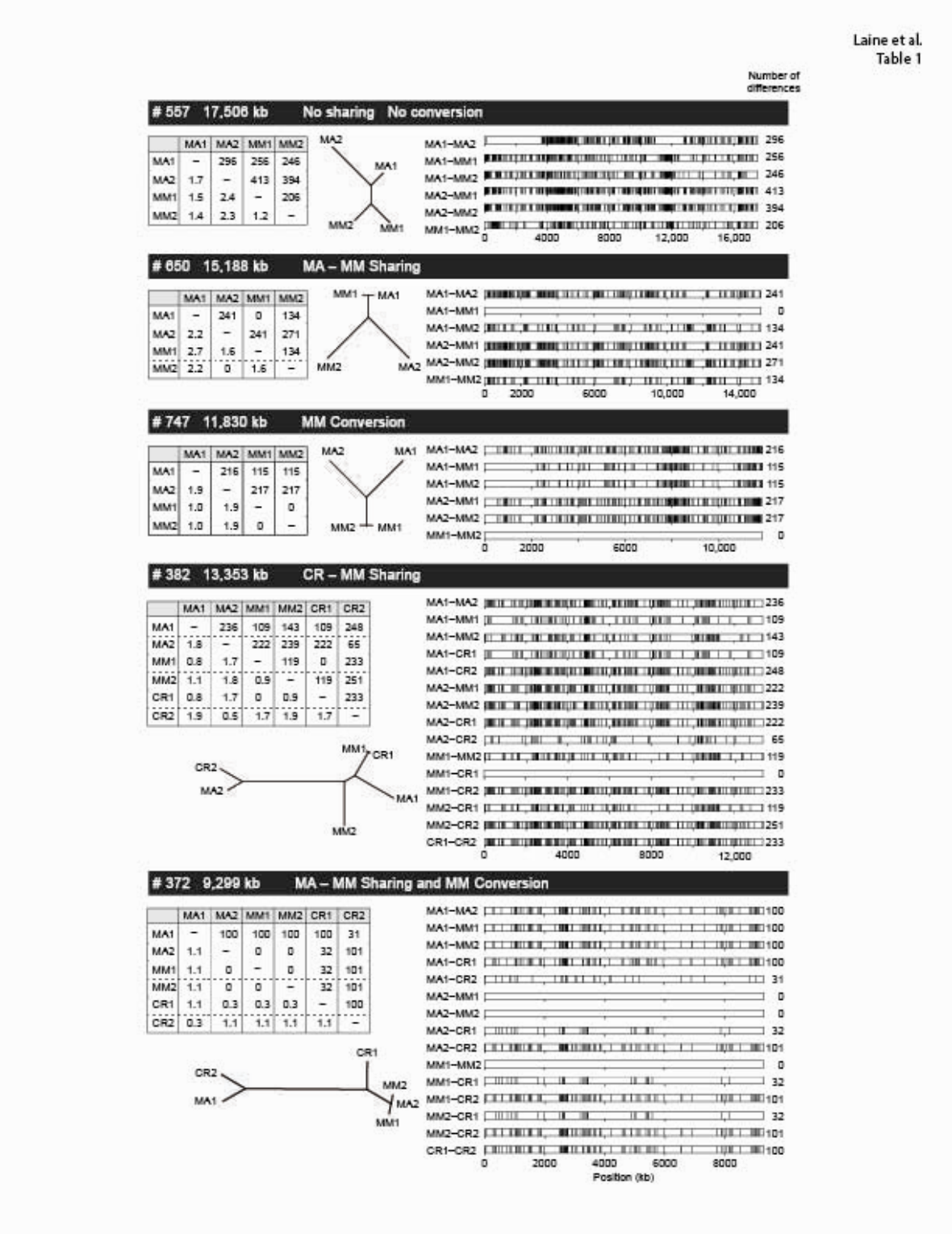


Table 2

Summary Statistics										
		Identical Sharing			Homozygous			Average Heterozygosity		
		Number / Proportion			Number / Proportion					
Alignment	Size Range (kb)	MA–MM	MA–CR	MM–CR	MA	MM	CR	MA	MM	CR
	# of Alignments									
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			

Fig. 1

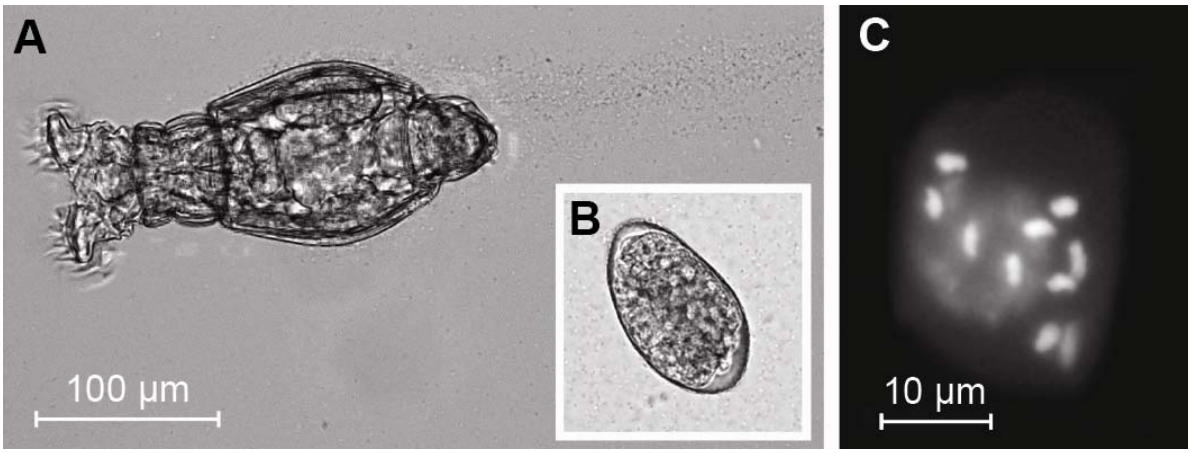


Fig. 2

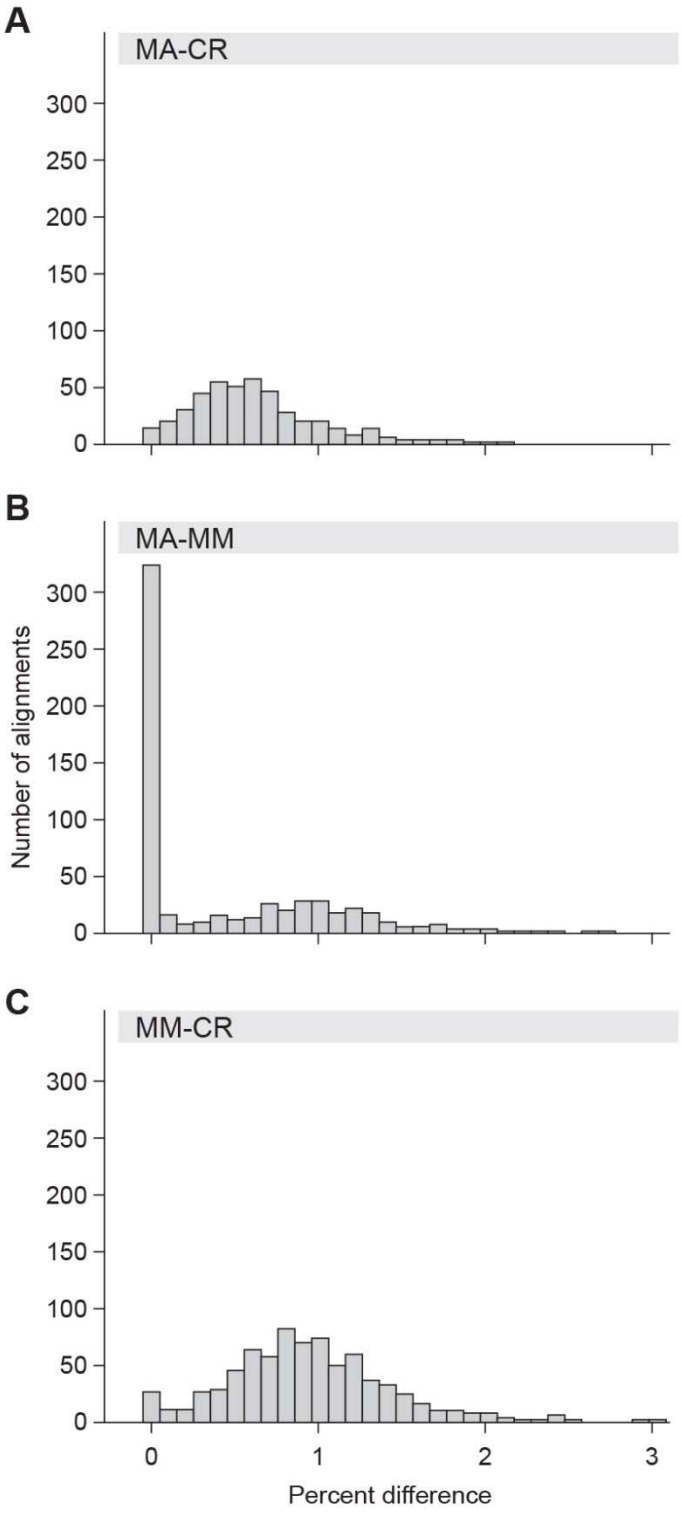

A
MA-CR
300
250
200
150
100
50
0
B
MA-MM
Number of alignments
300
250
200
150
100
50
0
C
MM-CR
300
250
200
150
100
50
0
0
1
2
3
Percent difference

Fig. 3

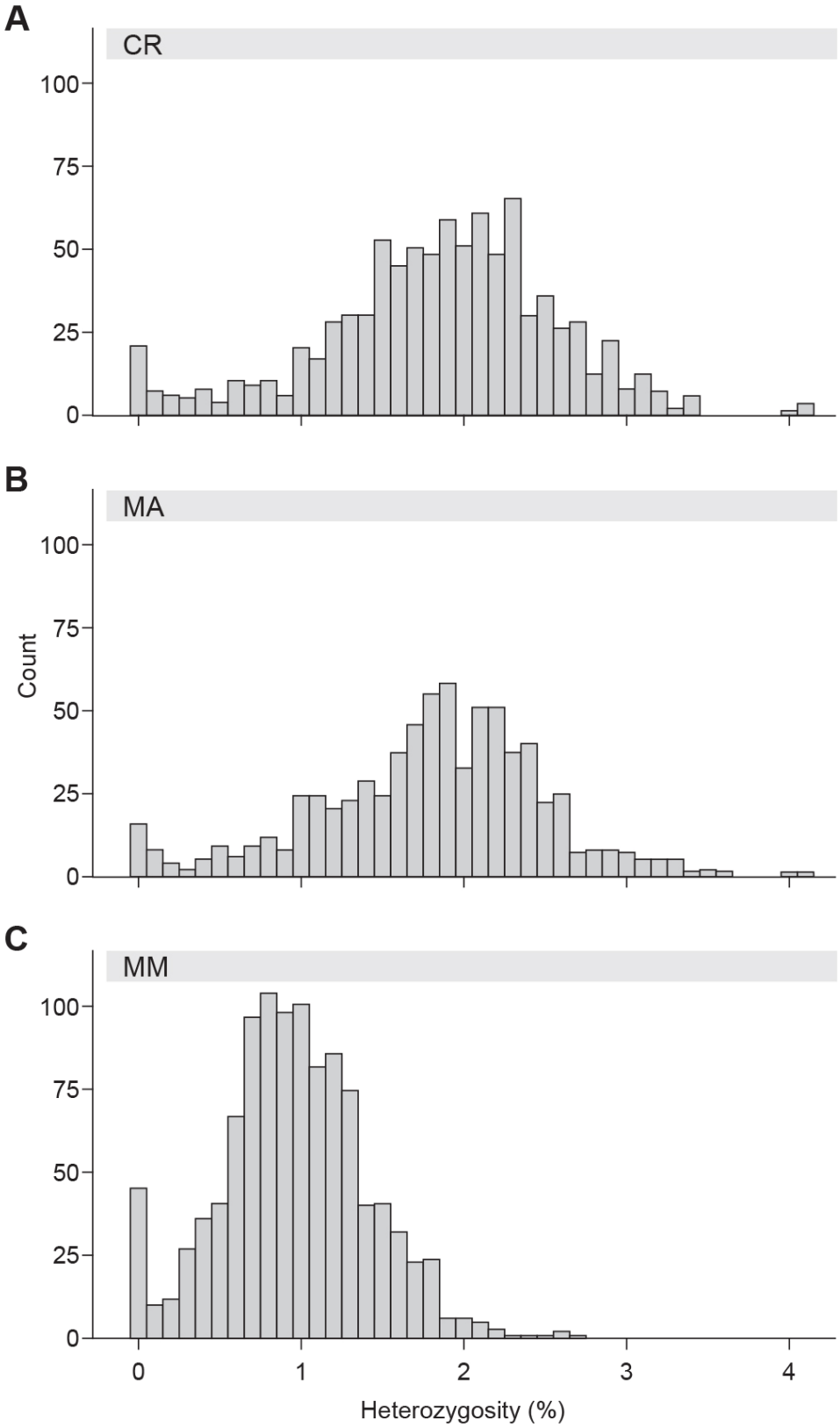
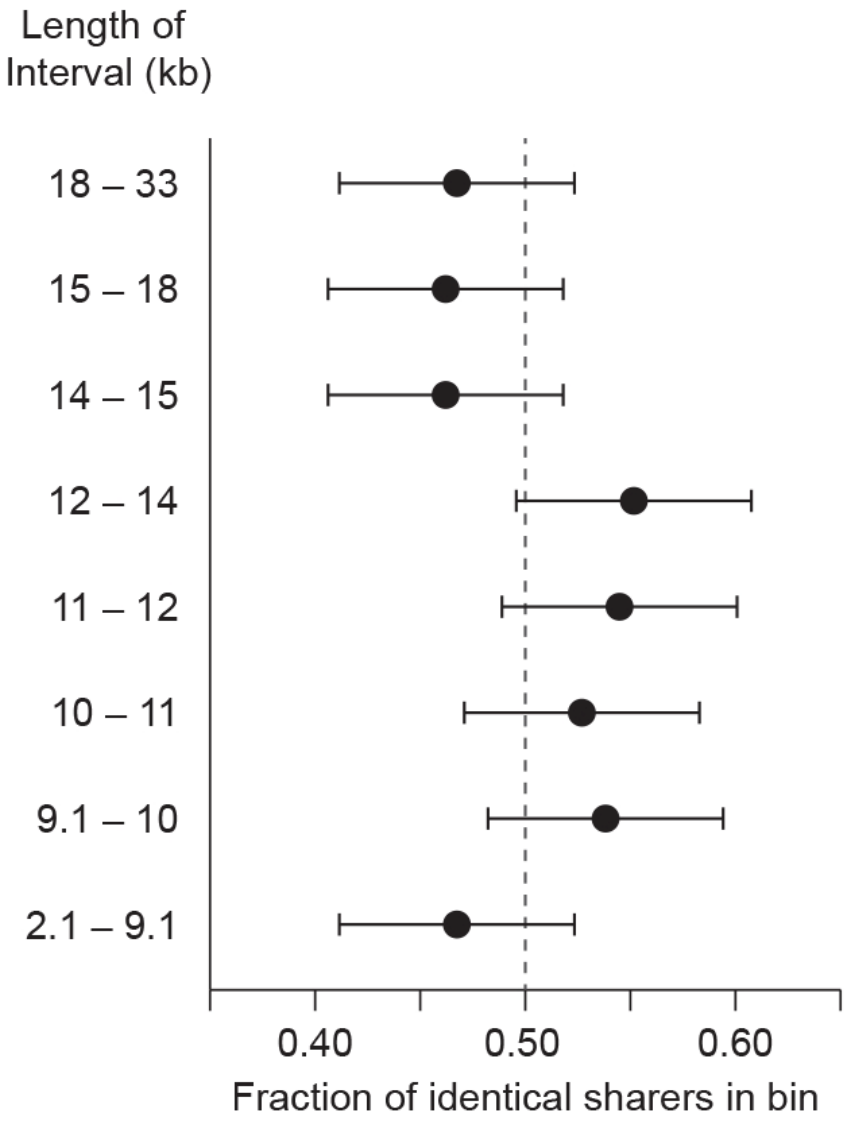


Fig. 4


Length of
Interval (kb)
18 – 33
15 – 18
14 – 15
12 – 14
11 – 12
10 – 11
9.1 – 10
2.1 – 9.1
0.40
0.50
0.60
Fraction of identical sharers in bin

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References

1. A. Weismann, On the significance of the polar globules. *Nature* **36**, 607–609 (1887).
2. J. Maynard Smith, *The Evolution of Sex* (Cambridge University Press, 1978).
3. G. Bell, *The Masterpiece of Nature: the evolution and genetics of sexuality* (University of California Press, 1982).
4. N. H. Barton, B. Charlesworth, Why sex and recombination? *Science.* **281**, 1986–1990 (1998).
5. S. P. Otto, The Evolutionary Enigma of Sex. *Am. Nat.* **174**, S1–S14 (2009).
6. I. Schon, K. Martens, P. van Dijk, Eds., *Lost Sex: The evolutionary biology of parthenogenesis.* (Springer, 2009).
7. D. B. Mark Welch, C. Ricci, M. Meselson, Lost Sex. *Media*, 259–279 (2009).
8. A. van Leewenhoeck, 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 (1677).
9. A. van Leewenhoeck, Letter to the Royal Society December 25, 1702. *Proc. R. Soc. London B* **23**, 1291–1311 (1702).
10. D. B. Mark Welch, J. L. Mark Welch, M. Meselson, Evidence for degenerate tetraploidy in bdelloid rotifers. *Proc. Natl. Acad. Sci.* **105**, 5145–5149 (2008).
11. J. H. Hur, K. Van Doninck, M. L. Mandigo, M. Meselson, Degenerate tetraploidy was established before bdelloid rotifer families diverged. *Mol. Biol. Evol.* **26**, 375–383 (2009).

12. J.-F. Flot, *et al.*, Genomic evidence for ameiotic evolution in the bdelloid rotifer Adineta vaga. *Nature* **500**, 453–457 (2013).
13. R. W. Nowell, *et al.*, Comparative genomics of bdelloid rotifers: Insights from desiccating and nondesiccating species. *PLOS Biol.* **16**, e2004830 (2018).
14. W. S. Hsu, Oogenesis in the Bdelloidea rotifer Philodina roseola Ehrenberg. *Cellule* **57**, 283–296 (1956).
15. W. S. Hsu, Oogenesis in Habrotrocha tridens (Milne). *Biol. Bull.* **111**, 364–374 (1956).
16. N. D. Meadow, C. H. Barrows Jr., Studies on aging in a bdelloid rotifer .I. The effect of various culture systems and longevity and fecundity. *J. Exp. Zool.* **176**, 303–314 (1971).
17. C. Ricci, Life histories of some species of Rotifera Bdelloidea. *Hydrobiolgia* **180**, 175–180 (1983).
18. C. Ricci, U. Fascio, Life-history consequences of resource allocation of two bdelloid rotifer species. *Hydrobiologia* **299**, 231–239 (1995).
19. M. Pagani, C. Ricci, A. M. Bolzern, Comparison of five strains of a parthenogenetic species, Macrotrachela quadricornifera (Rotifera, Bdelloidea) .II. Isoenzymatic patterns . *Hydrobiologia* **211**, 157–163 (1991).
20. C. Ricci, Anhydrobiotic capabilities of bdelloid rotifers. *Hydrobiologia* **387/388**, 321–326 (1998).
21. C. Ricci, M. Caprioli, Anhydrobiosis in bdelloid species, populations, and individuals. *Integr. Comp. Biol.* **45**, 759–763 (2005).
22. C. Ricci, F. Perletti, Starve and survive: stress tolerance and life-history traits of a bdelloid rotifer. *Funct. Ecol.* **20**, 340–346 (2006).
23. E. Gladyshev, M. Meselson, Extreme resistance of bdelloid rotifers to ionizing radiation. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 5139–5144 (2008).
24. A. Krisko, M. Leroy, M. Radman, M. Meselson, Extreme anti-oxidant protection against ionizing radiation in bdelloid rotifers. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2354–2357 (2012).
25. M. B. França, a D. Panek, E. C. a Eleutherio, Oxidative stress and its effects during dehydration. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **146**, 621–

631 (2007).

26. J. K. Fredrickson, *et al.*, Protein oxidation: key to bacterial desiccation resistance? *ISME J* **2**, 393–403 (2008).
27. C. G. Ehrenberg, *Die Infusionsthierchen als vollkommene Organismen. EinBlick in das tiefere organische Laben der Natur* (L. Voss, 1838).
28. C. T. Hudson, P. H. Gosse, *The Rotifera or Wheel-Animalcules* (Longmans, Green, 1886).
29. C. W. Birky, Positively negative evidence for asexuality. *J. Hered.* **101**, 42–45 (2010).
30. C. Wesenberger-Lund, 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 (1930).
31. D. M. Ruderfer, S. C. Pratt, H. S. Seidel, L. Kruglyak, Population genomic analysis of outcrossing and recombination in yeast. *Nat. Genet.* **38**, 1077–1081 (2006).
32. P. M. Magwene, *et al.*, Outcrossing, mitotic recombination, and life-history trade-offs shape genome evolution in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **108**, 1987–1992 (2011).
33. D. B. Mark Welch, M. Meselson, Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. *Science* **288**, 1211–1215 (2000).
34. D. B. Mark Welch, M. S. Meselson, Rates of nucleotide substitution in sexual and anciently asexual rotifers. *Proc. Natl. Acad. Sci.* **98**, 6720–6724 (2001).
35. A. R. Omilian, M. E. A. Cristescu, J. L. Dudycha, M. Lynch, Ameiotic recombination in asexual lineages of Daphnia. *PNAS* **103**, 18638–18643 (2006).
36. S. Xu, A. R. Omilian, M. E. Cristescu, High rate of large-scale hemizygous deletions in asexually propagating Daphnia: Implications for the evolution of sex. *Mol. Biol. Evol.* **28**, 335–342 (2011).
37. J. M. Flynn, F. J. J. Chain, D. J. Schoen, M. E. Cristescu, Spontaneous mutation accumulation in daphnia pulex in selection-free vs. competitive environments. *Mol. Biol. Evol.* **34**, 160–173 (2017).

38. J. St. Charles, T. D. Petes, High-Resolution Mapping of Spontaneous Mitotic Recombination Hotspots on the 1.1 Mb Arm of Yeast Chromosome IV. *PLoS Genet.* **9**, e1003434 (2013).
39. M. Hartfield, S. I. Wright, A. F. Agrawal, Coalescence and linkage disequilibrium in facultatively sexual diploids. *Genetics* **210**, 683–701 (2018).
40. I. Schaefer, *et al.*, No evidence for the “Meselson effect” in parthenogenetic oribatid mites (Oribatida, Acari). *J. Evol. Biol.* **19**, 184–193 (2006).
41. D. A. Hickey, Selfish DNA: a sexually-transmitted nuclear parasite. *Genetics* **101**, 519–531 (1982).
42. E. S. Dolgin, B. Charlesworth, The fate of transposable elements in asexual populations. *Genetics* **174**, 817–827 (2006).
43. I. R. Arkhipova, M. Meselson, Transposable elements in sexual and ancient asexual taxa. *Proc. Natl. Acad. Sci.* **97**, 14473–14477 (2000).
44. I. R. Arkhipova, M. Meselson, Diverse DNA transposons in rotifers of the class Bdelloidea. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11781–11786 (2005).
45. I. Arkhipova, M. Meselson, Deleterious transposable elements and the extinction of asexuals. *BioEssays* **27**, 76–85 (2005).
46. E. A. Gladyshev, M. Meselson, I. R. Arkhipova, A deep-branching clade of retrovirus-like retrotransposons in bdelloid rotifers. *Gene* **390**, 136–145 (2007).
47. E. Gladyshev, I. Arkhipova, 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 (2010).
48. F. Rodriguez, I. R. Arkhipova, Multitasking of the pirna silencing machinery: Targeting transposable elements and foreign genes in the Bdelloid Rotifer Adineta vaga. *Genetics* **203**, 255–268 (2016).
49. J. L. Mark Welch, M. Meselson, Karyotypes of bdelloid rotifers from three families. *Hydrobiologia* **387/388**, 403–407 (1998).
50. A. Signorovitch, J. Hur, E. Gladyshev, M. Meselson, Allele Sharing and Evidence for Sexuality in a Mitochondrial Clade of Bdelloid Rotifers. *Genetics* **200**, 581–590 (2015).
51. E. A. Gladyshev, M. Meselson, I. R. Arkhipova, Massive horizontal gene transfer

in bdelloid rotifers. *Science.* **320**, 1210–1213 (2008).

52. J. F. Flot, N. Debortoli, B. Hallet, K. Van Doninck, Response to Signorovitch et al. *Curr. Biol.* **26**, R755 (2016).
53. A. Signorovitch, J. Hur, E. Gladyshev, M. Meselson, Evidence for meiotic sex in bdelloid rotifers. *Curr. Biol.* **26**, R754–R755 (2016).
54. N. Debortoli, *et al.*, Genetic Exchange among Bdelloid Rotifers Is More Likely Due to Horizontal Gene Transfer Than to Meiotic Sex. *Curr. Biol.* **26**, 1–10 (2016).
55. C. G. Wilson, R. W. Nowell, T. G. Barraclough, Cross-Contamination Explains “Inter and Intraspecific Horizontal Genetic Transfers” between Asexual Bdelloid Rotifers. *Curr. Biol.* **28**, 2436-2443.e15 (2018).
56. I. Eyres, *et al.*, Horizontal gene transfer in bdelloid rotifers is ancient, ongoing and more frequent in species from desiccating habitats. *BMC Biol.* **13**, 90 (2015).
57. E. A. Gladyshev, I. R. Arkhipova, Genome structure of bdelloid rotifers: shaped by asexuality or desiccation? *J. Hered.* **101 Suppl**, S85-93 (2010).
58. S. Xu, *et al.*, High mutation rates in the mitochondrial genomes of Daphnia pulex. *Mol. Biol. Evol.* **29**, 763–769 (2012).
59. E. Lasek-Nesselquist, A Mitogenomic Re-Evaluation of the Bdelloid Phylogeny and Relationships among the Syndermata. *PLoS One* **7**, e43554 (2012).
60. D. Fontaneto, T. G. Barraclough, K. Chen, C. Ricci, E. A. Herniou, Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. *Mol. Ecol.* **17**, 3136–3146 (2008).
61. C. G. Wilson, Desiccation-tolerance in bdelloid rotifers facilitates spatiotemporal escape from multiple species of parasitic fungi. *Biol. J. Linn. Soc.* **104**, 564–574 (2011).
62. C. G. Wilson, P. W. Sherman, Spatial and temporal escape from fungal parasitism in natural communities of anciently asexual bdelloid rotifers. *Proc. R. Soc. B Biol. Sci.* **280**, 20131255 (2013).
63. D. Fontaneto, T. G. Barraclough, K. Chen, C. Ricci, E. A. Herniou, Molecular evidence for broad-scale distributions in bdelloid rotifers: Everything is not everywhere but most things are very widespread. *Mol. Ecol.* **17**, 3136–3146

(2008).

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

65. L. T. Morran, O. G. Schmidt, I. A. Gelarden, R. C. Parrish, C. M. Lively, Running with the Red Queen: Host-parasite coevolution selects for biparental sex. *Science.* **333**, 216–218 (2011).

66. M. A. Brockhurst, *et al.*, Running with the Red Queen: The role of biotic conflicts in evolution. *Proc. R. Soc. B Biol. Sci.* **281** (2014).

67. T. L. F. Leung, K. C. King, J. Wolinska, Escape from the Red Queen: An overlooked scenario in coevolutionary studies. *Oikos* **121**, 641–645 (2012).

68. A. Aguilera, *et al.*, Distribution of Eukaryotes in an extremely acidic, high-metal river. *Mol. Biol.*, 1–41 (2007).

69. R. Marotta, A. Uggetti, C. Ricci, F. Leasi, G. Melone, Surviving starvation: Changes accompanying starvation tolerance in a bdelloid rotifer. *J. Morphol.* **273**, 1–7 (2012).

70. P. C. Phillips, Epistasis - The essential role of gene interactions in the structure and evolution of genetic systems. *Nat. Rev. Genet.* **9**, 855–867 (2008).

71. M. S. Breen, C. Kemena, P. K. Vlasov, C. Notredame, F. A. Kondrashov, Epistasis as the primary factor in molecular evolution. *Nature* **490**, 535–538 (2012).

72. M. Y. Hein, *et al.*, A Human Interactome in Three Quantitative Dimensions Organized by Stoichiometries and Abundances. *Cell* **163**, 712–723 (2015).

73. J. S. Hawe, F. J. Theis, M. Heinig, Inferring interaction networks from multi-omics data. *Front. Genet.* **10**, 1–13 (2019).

74. O. Delaneau, *et al.*, Chromatin three-dimensional interactions mediate genetic effects on gene expression. *Science.* **364**, 452–462 (2019).