Cytogenetic evidence for asexual evolution of bdelloid rotifers

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DNA sequencing has shown individual bdelloid rotifer genomes to contain two or more diverged copies of every gene examined and has revealed no closely similar copies. These and other findings are consistent with long-term asexual evolution of bdelloids. It is not entirely ruled out, however, that bdelloid genomes consist of previously undetected pairs of sequences so similar as to be identical over the regions sequenced, as might result if bdelloids were highly inbred sexual diploids or polyploids. Here, we employ fluorescent *in situ* hybridization with cosmid probes to determine the copy number and chromosomal distribution of the heat shock gene *hsp82* and adjacent sequences in the bdelloid *Philodina roseola*. We conclude that the four copies identified by sequencing are the only ones present and that each is on a separate chromosome. Bdelloids therefore are not highly homozygous sexually reproducing diploids or polyploids.

Sexual reproduction is nearly universal among multicellular organisms, and although asexual populations continually arise, they almost invariably suffer early extinction. The relatively brief evolutionary persistence of most asexual groups is seen in their failure to achieve high taxonomic rank and in the low degree of DNA sequence divergence between them and their nearest sexual relatives (1). Among higher-ranking animal taxa that have been considered candidates for ancient asexuality, many have proven on closer study to be sexual or of recent origin (1, 2). Thus, the abandonment of sexual reproduction is generally thought to be an evolutionary dead end. Against this generalization, however, rotifers of the class Bdelloidea stand out as an apparent exception. With some 370 described species (3, 4), they constitute the highest-ranking metazoan taxon in which males, hermaphrodites, and meiosis are unknown, eggs being formed from primary oocytes by mitosis (5, 6). The inference that bdelloids evolved asexually is further supported by the presence of chromosomes without morphological homologues and by the apparent lack of deleterious retrotransposons (5, 7, 8).

Bdelloid genomes also differ from those of typical meiotic diploids or polyploids in that, as might be expected for ancient asexual lineages, individual genomes contain two or more copies of every gene examined, each of which is moderately to highly divergent at synonymous sites from all of the others (9). In the bdelloid *Philodina roseola*, for example, sequencing of cloned amplicons and of clones from genomic libraries has identified four copies of the heat shock gene *hsp82*. Within ≈870 bp of coding sequence, copies 1 and 2 differ at fourfold-degenerate sites by 3.5%; copies 3 and 4 differ by 6.0%; and copies 1 and 2 differ from 3 and 4 by ≈47% (9).

Here, we ask whether, despite the diverse evidence for their ancient asexuality, bdelloids could be sexual diploids or polyploids that engage in some undiscovered form of genetic exchange with extreme inbreeding or in which extreme homozygosity is maintained by gene conversion, mitotic recombination, or occasional automixis. To test this possibility, we have used fluorescent *in situ* hybridization (FISH) to determine the copy number and chromosomal distribution of the heat shock gene *hsp82* and adjacent sequences in the bdelloid *P. roseola*.

Methods

Cosmid Library and Probes. All rotifers and probes were derived from a line propagated in our laboratory from a single egg. Rotifers were grown and cleaned as described (10) and ground in liquid nitrogen. High-molecular-weight DNA was prepared and partially digested with *Sau*3AI in agarose substantially following Sambrook *et al.* (11). DNA larger than 12 kb was selected by agarose gel electrophoresis, recovered by treatment with agarase and ethanol precipitation, dephosphorylated with shrimp alkaline phosphatase (USB), ligated into the SuperCos vector (Stratagene), and packaged in GigaPack III XL extracts (Stratagene). Phages were plated on the XL-1 Blue MR strain of *E. coli* to give a total of 240,000 colonies. Given the average cosmid insert size of ≈42 kb selected by the packaging reaction, the 240,000 colonies screened include 10^{10} bp, representing ≈5-fold average coverage of the 2×10^9-bp *P. roseola* genome (10, 12).

Colonies were screened by using the method of Hanahan and Meselson (13) with ^{32}P-labeled probes containing ≈870 bp of coding sequence from the 5′ end of *hsp82* copy 1 or 3 (9). *Eco*RI Southern analysis of each of the 25 hybridizing clones showed only a single band hybridizing to *hsp82* in each cosmid. The sequence of *hsp82* in each cosmid was determined by direct sequencing of PCR products.

Cosmids were purified by CsCl density-gradient equilibrium centrifugation and labeled by nick-translation to incorporate the green fluorophore Alexa 488-dUTP or the red fluorophore Alexa 568-dUTP (Molecular Probes), under conditions adjusted to give 100- to 300-nt fragments. Salmon testes DNA digested to 100–400 nt was added as a blocking agent at 80-fold mass excess.

Embryos and FISH. Bdelloids are eutelic; all nuclear divisions take place in embryos. Well resolved chromosomes were most often seen in the youngest embryos, those having only a few nuclei. Typically, one to two well resolved chromosome sets were found per 100 embryos. Sedimented material from an actively growing culture was treated 2–4 min with 0.5% sodium hypochlorite (10% Clorox) to lyse adults and then rinsed five to six times with distilled water by decantation. Embryos were pipetted onto a poly(L)-lysine-treated microscope slide and covered with a coverslip. Slides, each with ≈1,000 embryos, were inverted onto a pad of five to six tissues, and the embryos were squashed by gentle pressure, rupturing the egg cases in which they are enclosed. With minor variations, the following procedure, derived from Lichter *et al.* (14) and Manuelidis (15, 16), was used for further processing. Slides were placed in liquid nitrogen for 10 sec, after which cover slips were removed and slides were kept 15 min in 4% freshly depolymerized paraformaldehyde in PBS (100 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/7 mM Na_2HPO_4; filter sterilized). Slides were then passed through a series of

Abbreviation: FISH, fluorescent *in situ* hybridization.

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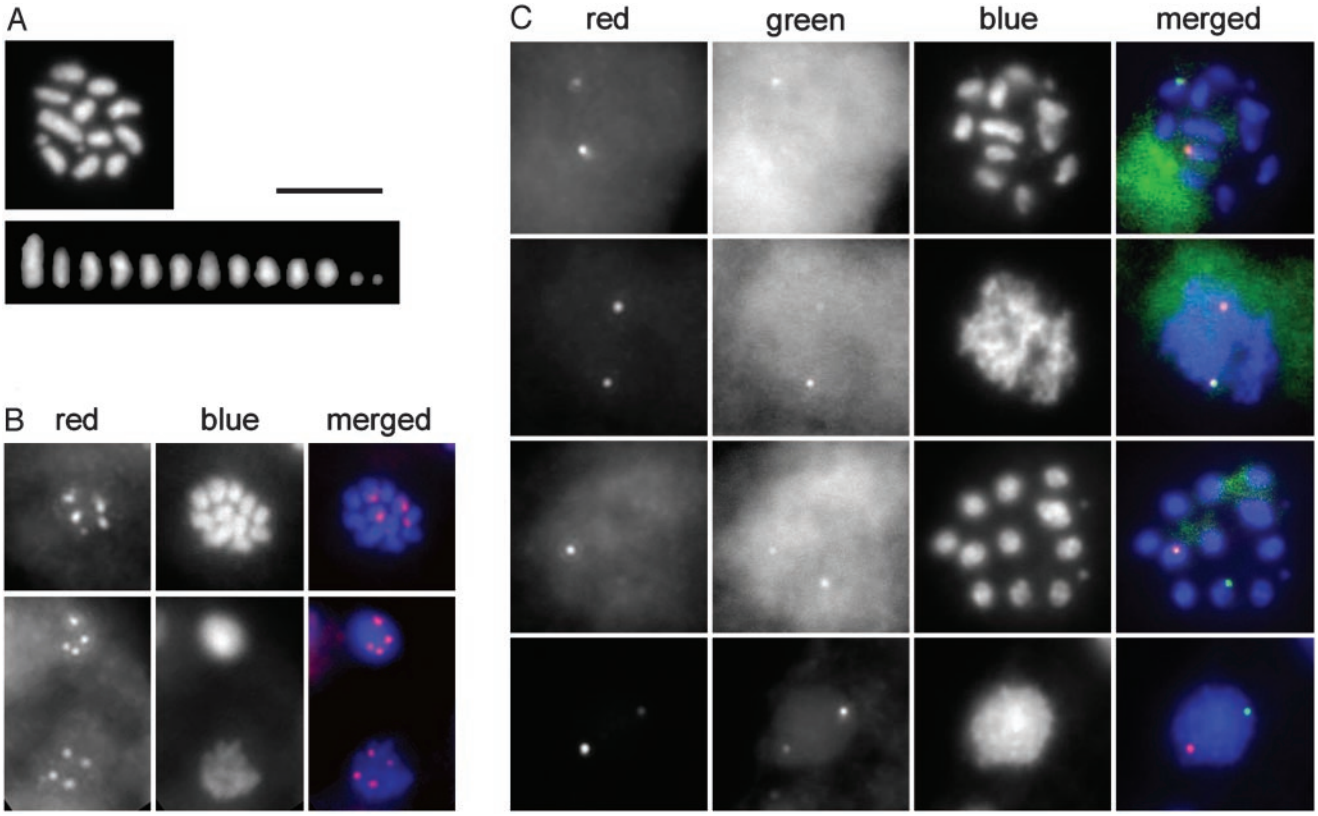

Fig. 1. (*A*) The 13 chromosomes of *P. roseola*. (*Upper*) Embryo nucleus stained with DAPI (4′,6-diamidino-2-phenylindole) and visualized by fluorescence. (*Lower*) Karyotype. The nuclear DNA content determined by photometry of G_1 oocyte nuclei is ≈2.2 pg (12). (*B*) *P. roseola* embryo nuclei hybridized at low stringency to a mixture of all four *hsp82*-containing cosmids labeled with Alexa 568. Red, fluorescent signals; blue, DAPI-labeled DNA; merged, false-color superposition. (*Upper*) A metaphase nucleus. (*Lower*) Two interphase nuclei. Each nucleus shows four signals. Near the four prominent signals, fainter signals sometimes appear, often not directly over the chromosome, possibly because of dispersal of the ≈40-kb target DNA. An example is seen in *Upper Left*. (*C*) *P. roseola* embryo nuclei hybridized at high stringency to pairs of differently labeled probes. Red, fluorescent signal from Alexa 568-labeled probe; green, fluorescent signal from Alexa 488-labeled probe; blue, DAPI-labeled DNA; merged, false-color superposition. Autofluorescence from cytoplasm is particularly visible in the green channel. First row, chromosomes hybridized to a mixture of probe 1 green and probe 2 red; second row, interphase nucleus hybridized to a mixture of probe 1 red and probe 2 green; third row, chromosomes hybridized to a mixture of probe 3 red and probe 4 green; fourth row, interphase nucleus hybridized to a mixture of probe 3 green and probe 4 red. Each probe hybridizes preferentially to a single site. (Scale bar, 5 μm.)

5-min rinses: two times in PBS; one time in 100 mM Tris (pH 7.4); one time in PBS; three times in 0.5% Triton X-100/0.5% saponin in PBS; and three times in PBS. After equilibration for 30 min in 20% glycerol in PBS, slides were subjected to three freeze–thaw cycles with liquid nitrogen, rinsed 5 min three times in PBS, treated 1 h at 37°C with RNase at 32 Kunitz units/ml in PBS, rinsed two times in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), desiccated through an ethanol series, air-dried, and stored over desiccant (Drierite, Xenia, OH).

For each slide, 250 ng of each nick-translated probe and 20 μg of digested salmon DNA were ethanol-precipitated together, resuspended in 12.5 μl of deionized formamide, and mixed with an equal volume of 4× SSC/20% dextran sulfate containing 0% (low stringency) or 40% (high stringency) formamide. The probe mixture was kept at 85°C for 10 min, placed on ice, and dispensed at 25 μl per slide. Each slide was quickly covered with a 22 × 40-mm plastic coverslip (HybriSlip, Molecular Probes), sealed with rubber cement, and kept 30 min at room temperature. Slides were then kept 6 min on the heat block of a thermal cycler at 86°C, quickly transferred to a 37°C incubator, and incubated for at least 16 h in a dark humid chamber.

After removal of cover slips, slides were washed 5 min three times in 50% formamide, 2× SSC at 45°C, and 5 min three times in 1× (low stringency) or 0.1× (high stringency) SSC at 60°C. They were then incubated 5 min in 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in 1× or 0.1× SSC, rinsed in 1× or 0.1× SSC and mounted with ProLong antifade solution (Molecular Probes).

Slides were examined with a Zeiss Axioplan 2 equipped with a mercury arc lamp, a Plan-Apochromat 100×/1.4 numerical aperture objective, a triple bandpass filter (61000 V2 BS&M 710, Chroma Technology, Rockingham, VT), and a black-and-white cooled charge-coupled device (CCD) camera (Micromax, Princeton Instruments, Trenton, NJ). Data were collected with METAMORPH software (Universal Imaging, Downingtown, PA). Exposure times were 1–5 sec. Photography of 0.5-μm TetraSpeck beads (Molecular Probes) verified the absence of significant chromatic aberration. For each combination of probes, 20–40 nuclei with condensed chromosomes were photographed. Brightness levels of the grayscale images were adjusted so that the range of pixel brightness occupies the entire range of output levels; grayscale images were not otherwise adjusted. False-color superpositions of the grayscale images were created with PHOTOSHOP (Adobe Systems, San Jose, CA); levels in each channel of the color images were further adjusted for better contrast.

Results and Discussion

As shown in Fig. 1*A* and as described earlier (5, 7), *P. roseola* has 13 chromosomes: 1 that is long, 10 of intermediate size, and 2

unequal dots. While morphologically unique chromosomes and odd chromosome numbers are suggestive of ameiotic reproduction, there are species in which a single chromosome pairs in meiosis with two partners (17).

To determine whether bdelloid chromosomes are present as nearly identical pairs of homologs, we conducted FISH on embryo nuclei of *P. roseola*. Cosmid probes were used to ensure that signal strength would be well above background. We constructed a cosmid library of *P. roseola* genomic DNA and screened it for clones containing *hsp82*, the best-characterized gene in diverse bdelloid species. Two of the most divergent copies of *hsp82* were used as probes to isolate all four copies from the library. No cosmid contained more than one copy. Four cosmids, designated 1–4 according to the particular *hsp82* gene present (9), were chosen as probes. The genomic inserts in the cosmids are 43, 40, 40, and 45 kb long, respectively. The inserts in cosmids 1 and 2 overlap by 29 kb and differ by 4.3%; the inserts in cosmids 3 and 4 overlap by 40 kb and differ by 5.0%. There are ≈100 indels in each of the two alignments, nearly all of which are <10 nt in length. As with the copies of *hsp82* they contain (9), the divergence of cosmids 1 and 2 from cosmids 3 and 4 is much greater than that between 1 and 2 or between 3 and 4. In addition to a single copy of *hsp82*, each cosmid insert includes a number of other coding regions, as judged by significant BLASTX scores to protein-coding genes in GenBank. These putative coding regions are present in the same order and orientation on each cosmid, but because of extensive sequence divergence cosmids 1 and 2 cannot be confidently aligned with cosmids 3 and 4 outside of these regions. The complete sequence of each cloned segment will be reported elsewhere.

When hybridization is performed with a mixture of all four cosmid probes, four signals are seen, each on a separate intermediate-size chromosome (Fig. 1*B*). The finding of four rather than eight hybridizing chromosomes rules out the possibility that there are four closely homologous pairs of chromosomes, each with a copy of *hsp82* and its adjacent regions. Moreover, if there is only a single copy of a probe sequence on each of the four hybridizing chromosomes, all four chromosomes must be divergent.

There remains, however, the complex possibility that there is more than one region homologous to a probe sequence on individual chromosomes. In this case, the four hybridizing chromosomes could comprise two nearly identical pairs. Because the cosmid inserts are nonrepeating, any such repeated regions would have to be sufficiently long or far apart that both are not present in any one cosmid, yet close enough together as not to have been resolved by FISH. An example of such an arrangement would place the sequences of probes 1 and 2 on one chromosome and nearly identical sequences on a homolog, with a corresponding arrangement for copies 3 and 4. The possibility of arrangements such as this was investigated by two-color FISH.

The top row of Fig. 1*C* shows the result of hybridization with a mixture of cosmid 1 labeled with the green fluorophore and cosmid 2 labeled with the red fluorophore. Two chromosomes are labeled, each at a single site, one preferentially red and the other preferentially green. The same pattern, one red and one green, is seen when the labeling is reversed, as seen in an interphase nucleus in the second row of Fig. 1*C*. Similar results are seen with cosmids 3 and 4 (Fig. 1*C*, bottom two rows). Thus, all four of the hybridizing chromosomes are sufficiently different to be told apart under the hybridization conditions employed.

The simplest explanation of these results is that the *P. roseola* DNA cloned in each of the four cosmids is present only once per genome, with each copy on a separate chromosome. One might imagine other situations in which multiple nearly identical copies are arranged on each of four chromosomes in such a way as to produce the observed results. All such arrangements, however, would require the presence in the genome of at least eight copies of *hsp82*, a number far outside the range of gene copy numbers indicated by the agreement between the estimate of 1.9–2.4 pg of DNA per G_1 oocyte nucleus, obtained by photometry (12), and the estimate of 1.7–2.8 pg of genomic DNA per 4 copies of *hsp82,* obtained by quantitative membrane hybridization (10). The agreement between these two values also shows that the chromosomes we see are not synapsed pairs of homologs, because that too would require the existence of eight copies of *hsp82* in oocytes.

We conclude that there are only four copies of *hsp82* and adjacent sequences in the genome and that each copy resides on a different chromosome. Because the four probe sequences are all divergent, *P. roseola* is not a highly homozygous sexually reproducing diploid or polyploid.

Our results and earlier findings are consistent with the possibility that the class Bdelloidea evolved from an ancient diploid or polyploid ancestor, perhaps a species hybrid, that abandoned meiosis and syngamy. With the cessation of meiotic segregation and haplotype drift, former alleles would be inherited together and mutational divergence would accumulate between them, limited by certain events that give rise to identical copies which themselves then diverge over time. Such events might include nondisjunction, polyploidization, segmental duplication, conversion, or mitotic crossing-over. The degree of divergence between the most similar copies of a given gene within a bdelloid genome would reflect the time since the duplicate sequences arose or were last homogenized. Conversely, the most highly divergent sequences within individual genomes may have escaped such events throughout bdelloid evolution and may descend from pairs of homologs or homeologs present in the ancient sexual ancestor. As explained in the following paper (18), whether the most highly divergent copies of bdelloid genes separated before the bdelloid radiation, as we originally suggested (9), or only later, within bdelloid families, cannot be distinguished on the basis of available sequence data.

The present results have implications for the origin of the multiple copies of *hsp82* and other genes in bdelloids and for the processes that account for their genealogies. The finding that each of the four probe sequences is on a separate chromosome suggests that the multiple copies of *hsp82* arose by a mechanism or mechanisms involving whole chromosomes.

Although it could be imagined that the four copies of *hsp82* in *P. roseola* represent two pairs of meiotically segregating alleles within an exceptionally heterozygous sexual population, this interpretation must contend with substantial countervailing evidence. Of the eight additional cases in which individual genomes of bdelloid species have been screened for specific genes, PCR found only two copies of the gene in six cases, differing at fourfold degenerate sites (D4) by 12%, 14%, 16%, 44%, 54%, and 73%. In the two remaining cases, three copies were found, two of which differed by 6–7% and a third that differed from these by 25–30% (9). For allelic pairs to exist, one would have to postulate an extraordinary range of heterozygosity within every bdelloid genome examined or accept that duplications are unusually common in bdelloids and that PCR screens found numerous highly diverged copies, while missing more closely similar allelic copies. PCR screens can fail to detect copies that are not efficiently amplified; indeed, our initial PCR screens for *hsp82* in *P. roseola* identified only copies 1 and 3, while copies 2 and 4 were first found in genomic libraries. With the same degree of sequencing effort as for the bdelloids, PCR screens of seven sexually reproducing rotifer species for *hsp82* found two copies in each of five species, with D4 ranging from 0.7 to 2.4%, and a single sequence (possibly representing two copies homozygous over the region examined) in each of two other species, for an overall average D4 of 1% (9), a value typical of sexually reproducing diploids.

Whatever the processes by which the four probe sequences arose, they cannot have occurred recently. This is seen in the 4.3% difference of the most similar pair of cosmid inserts, 1 and 2, and in the 1.9% fourfold-degenerate difference between the complete coding regions of the *hsp82* genes they contain. Assuming that the rate of fourfold-degenerate divergence of the *hsp82* genes in *P. roseola* is comparable to that of *hsp82* in dipterans and vertebrates (19–21), even these two closest copies of the gene separated one to four million years ago. If conversion or mitotic crossing-over occur, they must be infrequent in this region.

In sum, our results eliminate the possibility that bdelloids are highly homozygous sexual diploids or polyploids and add to the considerable body of other evidence that bdelloids have evolved without sexual reproduction.

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