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Sister Chromosome Pairing Maintains Heterozygosity in Parthenogenetic Lizards

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Although bisexual reproduction has proven to be highly successful, parthenogenetic all-female populations occur frequently in certain taxa including the whiptail lizards of the genus *Aspidoscelis*. Allozyme analysis revealed a high degree of fixed heterozygosity in these parthenogenetic species1,2 supporting the view that they originated from hybridization events between related sexual species. It has remained unclear how the meiotic program is altered to produce diploid eggs while maintaining heterozygosity. Here we show that meiosis commences with twice the number of chromosomes in parthenogenetic versus sexual species, a mechanism that provides the basis for generating gametes with unreduced chromosome content without fundamental deviation from the classic meiotic program. Our observation of synaptonemal complexes and chiasmata demonstrate that a typical meiotic program occurs and that heterozygosity is not maintained by bypassing recombination. Instead, fluorescent *in situ* hybridization probes that distinguish between homologs reveal that bivalents form between sister chromosomes, the genetically identical products of the first of two premeiotic replication cycles. Sister chromosome pairing provides a mechanism for the maintenance of heterozygosity, which is critical for offsetting the reduced fitness associated with the lack of genetic diversity in parthenogenetic species.

True parthenogenesis, characterized by the complete absence of male contributions, has been described for various species of reptiles including whiptail lizards, geckos, blind snakes and rock lizards3. Whiptail lizards of the genus *Aspidoscelis*, formerly part of the genus *Cnemidophorus*4, are mostly native to the Southwestern United States and Mexico, and about one-third of the more than 50 species reproduce by obligate parthenogenesis.

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Morphological, karyotypic and biochemical studies provided strong evidence for hybrid origins of all parthenogenetic *Aspidoscelis* species examined1,5,6. While hybridization between individuals from distinct species can explain the initially high degree of heterozygosity across the genome, allozyme analysis demonstrated surprising persistence of heterozygosity over many generations in several parthenogenetic lineages of *Aspidoscelis*, including *A. tesselata and A. neomexicana*6. The acceptance of skin grafts between individuals of a parthenogenetic species7-10 and biochemical studies on several lab-reared lineages6 further support genetic uniformity.

The mechanism that underlies clonal reproduction and fixed heterozygosity has been the topic of much speculation. Most variations of the meiotic program that would produce diploid oocytes by skipping a division or by fusion of a haploid oocyte with a polar body cannot account for fixed heterozygosity unless recombination between homologs is suppressed. Based on the exclusion of alternative models and the observation of large numbers of chromosomes in two oocytes from *A. uniparens*11, premeiotic endoreplication of chromosomes was proposed as the most likely mechanism to produce mature oocytes that carry the complete complement of somatic chromosomes and maintain heterozygosity12. To test this hypothesis we set out to quantify the DNA content in oocytes of the diploid parthenogenetic species *A. tesselata* and the sexually reproducing control *A. gularis*. Extant *A. gularis* and *A. tigris* are closely related to the individuals that hybridized to generate the founding specimen of *A. tesselata* 1,2,13,14.

We isolated germinal vesicles (GVs), the oocyte nuclei, and visualized 4′,6-diamidino-2-phenylindole (DAPI) stained chromosomes by two-photon microscopy. Bivalents were readily observed in GVs from *A. gularis* and *A. tesselata*, and their morphology was consistent with the diplotene stage of prophase I (Fig. 1a, b). Visual inspection of three dimensional reconstructions of seven *gularis* and five *tesselata* GVs revealed a larger number of bivalents in *tesselata* GVs compared to *gularis*. Ambiguities in identifying the boundaries of individual chromosomes prevented accurate counting of bivalents at this stage. Instead, we quantified the volume occupied by chromosomes in each GV as an indirect measure of DNA content (Fig. 1c; Suppl. Table 1). Unlike measurements of fluorescence intensity, this approach is robust against changes in staining efficiency or laser intensity fluctuations. *A. tesselata* chromosomes occupied 2.24 +/-0.18 fold the volume of the averaged *A. gularis* samples. While indicative of a two-fold increase in the DNA content of the prophase oocyte in the parthenogenetic species, differences in genome size could also account for the increased chromosome volume.

Although somatic cells from *A. gularis* and *A. tesselata* both harbor 46 chromosomes15, a direct comparison of genome sizes was needed to inform our analysis. Taking advantage of the fact that reptilian erythrocytes are nucleated, we subjected blood samples to flow cytometry analysis and found that the nuclear DNA content in somatic cells differed by less than 1% between the two species (Fig. 1d). For comparison, samples from sexual diploid *A. tigris* and parthenogenetic triploid *A. exsanguis*[*] were also analyzed, with the latter showing

[*] *A. exsanguis* is the product of two consecutive hybridization events involving the sexual species *A. inornata*, *A. burti* and *A. gularis*13.

an approximately 50% increase in DNA content as expected (Fig. 1d). Independent confirmation for a doubling in chromosome number was obtained by examining GVs in late prophase. At this stage chromosomes are highly condensed and consistent with a doubling in chromosome number, and we were able to distinguish 46 bivalents in *A. tesselata* GVs (Suppl. Fig. 1).

Entering meiosis with an 8n chromosome complement would allow parthenogenetic animals to utilize the two normal meiotic divisions to generate diploid gametes. However, the long-term maintenance of heterozygosity across the genome is only ensured if cross-overs between homologs are suppressed. Two-photon imaging of diplotene chromosomes from *A. tesselata* and another parthenogenetic species, *A. neomexicana*, revealed no differences compared to sexual controls besides the increased DNA content. Notably, bivalents appeared to be connected by chiasmata in all samples, indicating that crossing-over is not abandoned (Fig. 2a, b).

To further examine chromosome pairing, thin sections of ovaries from *A. tesselata*, *A. tigris*, and *A. neomexicana* were examined by electron microscopy. Synaptonemal complexes (SCs), characterized by well-defined lateral and central elements, were observed in all species examined providing further support that a typical meiotic program is underway (Fig. 2c to f, Suppl. Fig. 2). Based on the presence of SCs in pachytene and chiasmata in diplotene, we surmise that meiotic chromosome pairing and recombination are not bypassed in parthenogenetic *Aspidoscelis* species.

The premeiotic doubling of chromosomes allows for bivalent formation to occur either between homologs as in normal meiosis or between sister chromosomes (Fig. 3). To distinguish between these possibilities we sought to identify probes that selectively recognize one particular chromosome in a pair of homologs. We discovered that 26 of the 46 *A. tigris* chromosomes, including all 22 macrochromosomes and 4 microchromosomes, harbor large tracks of internal telomeric repeats in addition to the signal at chromosome ends (Fig. 4a). In contrast, staining metaphases of *A. inornata* chromosomes with a telomeric protein-nucleic acid probe only revealed signal at the chromosome termini (Fig. 4b). Consistent with its hybrid origin from these two sexual species1,2,5, *A. neomexicana* chromosomes contained large internal repeats on 13 chromosomes, allowing us to unambiguously identify 13 chromosomes inherited from *A. tigris* in the original F1 hybrid (Fig. 4c). In the context of a bivalent, hybridization signals on both sides indicates sister chromosome pairing, whereas hybridization on only one side supports homolog pairing.

To preserve the three-dimensional arrangements of chromosomes in GVs and to provide better spatial resolution than commonly obtained in chromosome spreads, we adapted the FISH procedure to perform hybridization on intact GVs. At each site where chromosome internal hybridization was detected, a signal was observed on both sides of the bivalent (Fig. 4d, e). It is important to note that sister chromatids resulting from the most recent round of replication appear as one cytologically, as they are closely associated with each other along their length during this stage of meiosis. The exclusive presence of paired hybridization signals therefore strongly suggests that bivalents are composed of sister chromosomes, not homologs. Based on this experiment, we concluded that for the 13 chromosomes for which

the telomeric hybridization probe distinguishes sisters and homologs in *A. neomexicana*, sister chromosome pairing is the rule.

Screening of several tri-, tetra-, and hexanucleotide repeat probes identified $(CCAAGG)_n$ as an additional marker for at least nine chromosomes in *A. neomexicana* that are of *A. tigris* origin (Suppl. Fig. 3a to c). When hybridized to diplotene chromosomes in acrylamide-embedded GVs, only paired signals were observed (Suppl. Fig. 3d and e). In summary, two independent probes enabled us to distinguish sister chromosomes from homologs, and for over 20 bivalents examined, pairing occurred exclusively between sister chromosomes.

Entering meiosis with twice the usual number of chromosomes allows parthenogenetic species to produce oocytes carrying the complete somatic chromosome complement while preserving the established meiotic program. There are two principal pathways by which a diploid species' premeiotic oocytes may acquire eight rather than four sets of chromosomes. One is the process in which chromosome duplication occurs without cytokinesis; this has been termed endomitosis or endoreplication16. Alternatively, 8n germ cells may arise by fusion of two cells either before or after the final premeiotic doubling of chromosomes. There is ample precedent for either mode of genome amplification in plants and animals, but the regulatory mechanisms are largely unclear.

In sexual species, homologous chromosomes form bivalents, and meiotic recombination promotes genetic diversity while ensuring orderly segregation of chromosomes during the first meiotic division. The same mechanism would result in loss of heterozygosity in parthenogenetic species, whereas formation of bivalents from genetically identical sister chromosomes preserves heterozygosity. Interestingly, this same variation of the meiotic program appears to enable parthenogenetic reproduction in widely diverged species. Premeiotic doubling of chromosomes has been documented in triploid ambystomatid salamanders17 as well as a parthenogenetic grasshopper (*Warramaba virgo*)18. In both cases, sister chromosome pairing was suggested based on bivalent morphology. Although the lack of molecular markers in these studies precludes definitive conclusions, the striking parallels with whiptail lizards strongly indicate that a common mechanism enables parthenogenetic reproduction in diverse groups of animals. It seems likely that a relatively simple deviation from the established program of oogenesis is sufficient to permit parthenogenesis. However, loss of heterozygosity, paternal inheritance of centrosomes, and a requirement for fertilization in triggering completion of female meiosis are seemingly unconnected obstacles to parthenogenetic reproduction. A better understanding of the changes that permit a small but diverse group of animals to reproduce without males is clearly needed and may well be the Rosetta stone that sheds light on the overwhelming success of sexuality.

Methods

Laboratory colonies of *Aspidoscelis* species were from animals collected in Texas and New Mexico under a permit from the New Mexico Department of Game and Fish (permit # 3199 and 3395).

GV isolation and quantification of chromosome volume

Ovaries from adult and sub-adult lizards were isolated, stained with 4′,6-diamidino-2-phenylindole (DAPI) and imaged using a Zeiss LSM 510 system in two-photon excitation mode. An nonparametric and unsupervised, automatic threshold selection method developed by Otsu was employed to obtain an unbiased measurement of chromosome volumes.

Fluorescent *in situ* hybridization (FISH)

Colcemid-treated embryonic fibroblasts were harvested, fixed and used to prepare metaphase spreads. FISH was performed on dried coverslips using AlexaFluor-labeled peptide-nucleic acid (PNA) and locked-nucleic acid (LNA) probes. Samples were imaged on a fluorescence microscope and images were analyzed with AxioVision software. FISH on meiotic chromosomes was performed after embedding GVs in an acrylamide gel.

Transmission electron microscopy

Ultrathin sections of epoxy-embedded ovaries were collected on copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were photographed using a FEI transmission electron microscope at 80 kV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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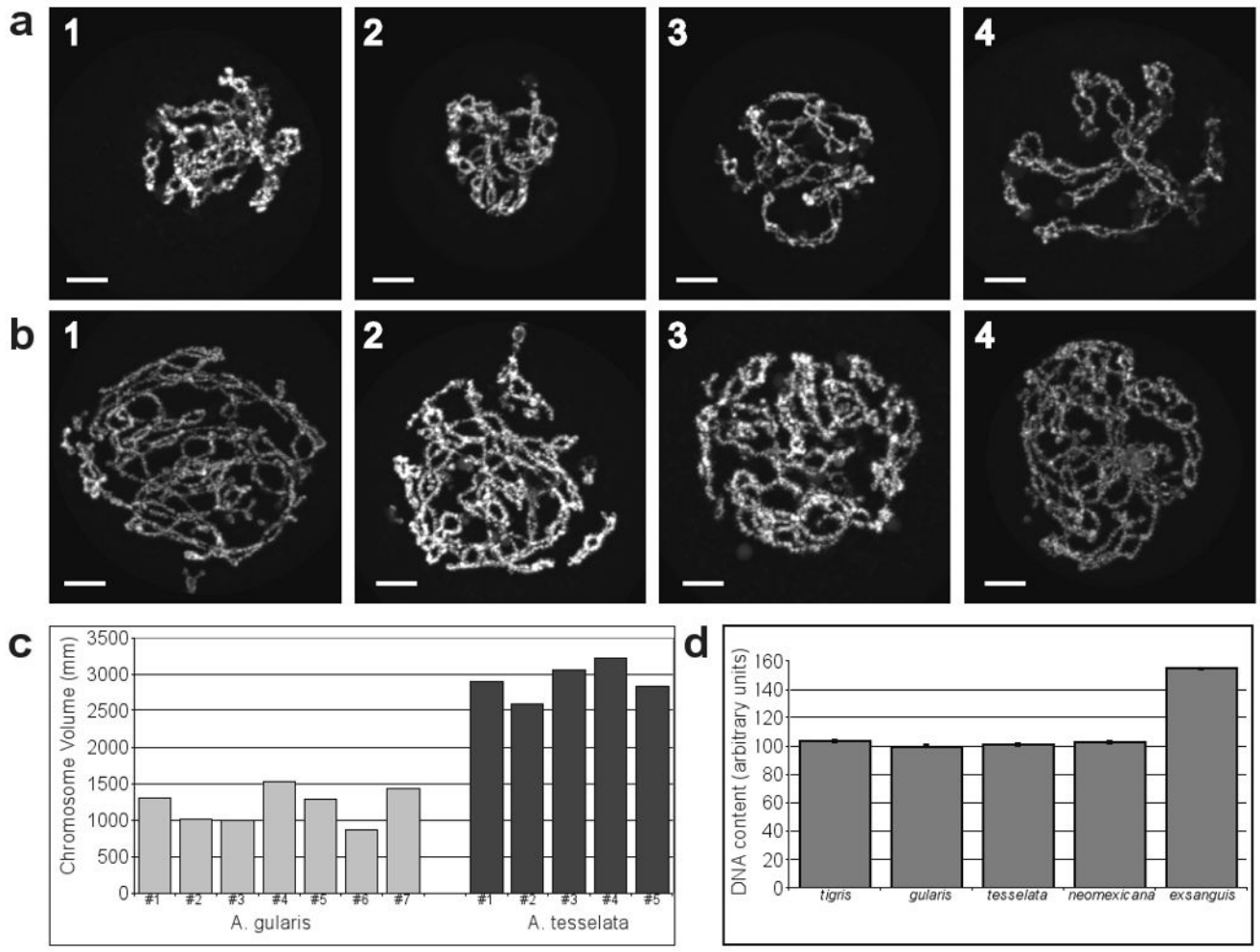

Figure 1.

Oocytes from parthenogenetic *A. tesselata* contain twice the amount of chromosomal DNA compared to sexual *A. gularis*. **(a)** DAPI-stained chromosomes in germinal vesicles (GVs) from *A. gularis*. 3D projections of four GVs are shown, details on size and quantifications are available as Suppl. Table 1. Scale bars correspond to 10 μm. **(b)** GVs from *A. tesselata*. **(c)** Quantification of chromosome volumes. **(d)** Quantification of DNA content in somatic cells by flow cytometry. Fluorescence intensities from biological triplicates of ∼50,000 cells were averaged and normalized against samples from *A. gularis* which was set at 100 to facilitate comparison.

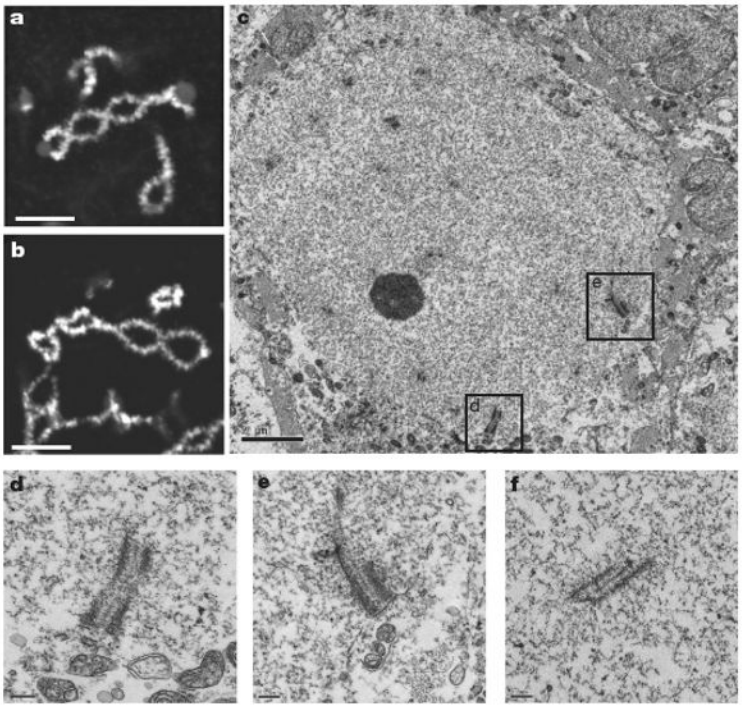

Figure 2.

Visualization of chiasmata and synaptonemal complexes in parthenogenetic *A. tesselata* and sexual *A. tigris*. Projection of bivalents from *A. tigris* **(a)** and *A. tesselata* **(b)** GVs in diplotene. Scale bars correspond to 10 μm. **(c)** EM image of *A. tesselata* GV in pachytene. Several sections of synaptonemal complexes are visible. Scale bar corresponds to 2 μm. Close-ups for two areas are shown in **(d)** and **(e)**. A close-up of a SC from *A. tigris* is shown in **(f)**. Scale bars correspond to 200 nm.

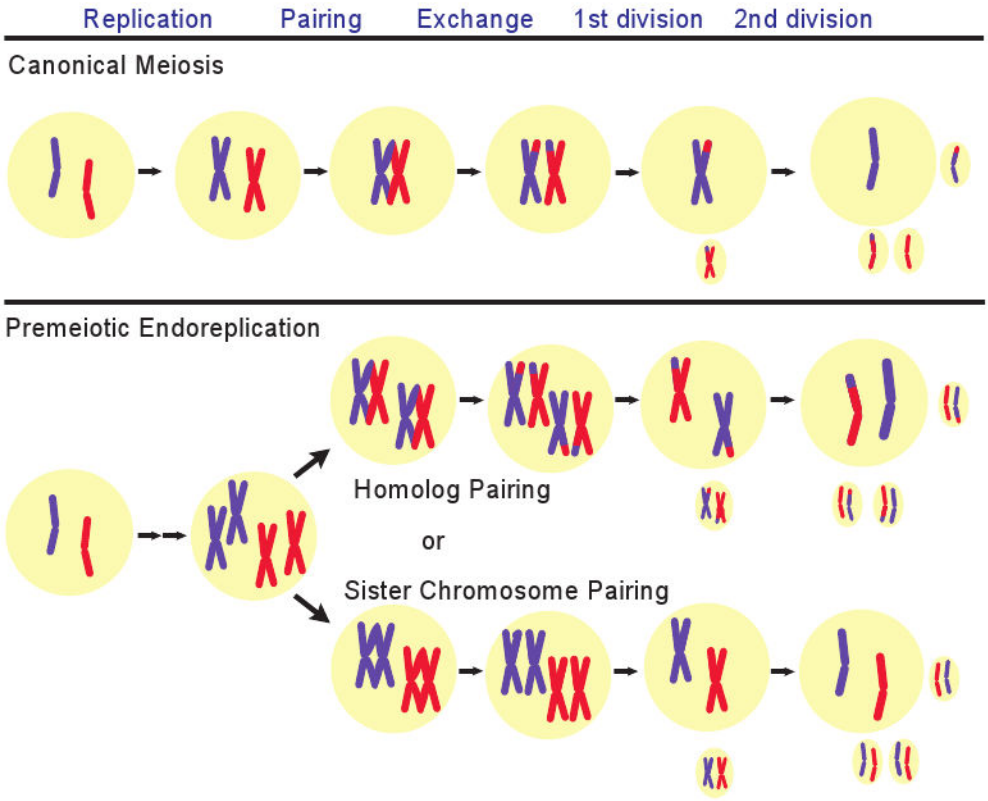

Figure 3.

Meiosis in sexual and parthenogenetic *Aspidoscelis* species. In normal meiosis a single round of DNA replication is followed by two consecutive divisions that result in a haploid gamete and three polar bodies. Homologs are shown in red and blue. Recombination generates chimaeric chromosomes. Premeiotic doubling of chromosomes allows for pairing of homologous or sister chromosomes. Homolog pairing and recombination result in some loss of heterozygosity in the mature oocyte. Recombination between pairs of sister chromosomes maintains heterozygosity at all loci.

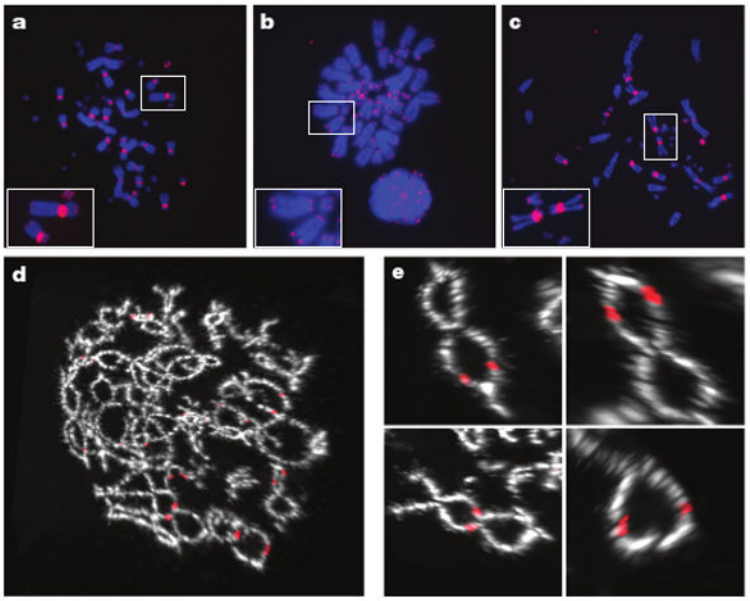

Figure 4.

Internal telomeric repeats distinguish homologs in *A. neomexicana* and demonstrate sister chromosome pairing. **(a)** A $CCCTAA_{(3)}$ peptide nucleic acid probe (red) identifies chromosome termini and large internal telomere repeat regions on metaphase spreads of *A. tigris* chromosomes prepared from fibroblast cultures. DAPI stained chromosomes are shown in blue. **(b)** Chromosome terminal signals, but no nternal telomeric repeats are detected in *A. inornata*. **(c)** Chromosomes inherited from *A. tigris* but not from *A. inornata* are identified by internal telomeric repeats in *A. neomexicana*. **(d)** Projection of a subset of images from an *A. neomexicana* GV visualized by confocal microscopy. DAPI-stained chromosomes in white and the telomeric probe in red. **(e)** Close-up of four representative areas showing paired fluorescence signals. The differences in resolution stem from differences in projection angles.