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Uncovering Cryptic Asexuality in *Daphnia magna* by RAD Sequencing

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Abstract :

The breeding systems of many organisms are cryptic and difficult to investigate with observational data, yet they have profound effects on a species' ecology, evolution, and genome organization. Genomic approaches offer a novel, indirect way to investigate breeding systems, specifically by studying the transmission of genetic information from parents to offspring. Here we exemplify this method through an assessment of self-fertilization vs. automictic parthenogenesis in Daphnia magna. Self-fertilization reduces heterozygosity by 50% compared to the parents, but under automixis, whereby two haploid products from a single meiosis fuse, the expected heterozygosity reduction depends on whether the two meiotic products are separated during meiosis I or II (i.e., central vs. terminal fusion). Reviewing the existing literature and incorporating recombination interference, we derive an interchromosomal and an intrachromosomal prediction of how to distinguish various forms of automixis from self-fertilization using offspring heterozygosity data. We then test these predictions using RAD-sequencing data on presumed automictic diapause offspring of so-called nonmale producing strains and compare them with "self-fertilized" offspring produced by within-clone mating. The results unequivocally show that these offspring were produced by automixis, mostly, but not exclusively, through terminal fusion. However, the results also show that this conclusion was only possible owing to genome-wide heterozygosity data, with phenotypic data as well as data from microsatellite markers yielding inconclusive or even misleading results. Our study thus demonstrates how to use the power of genomic approaches for elucidating breeding systems, and it provides the first demonstration of automictic parthenogenesis in Daphnia.

Keywords : genome-wide heterozygosity, breeding system, inbreeding, automixis, tychoparthenogenesis, Daphnia magna, nonmale producers

INTRODUCTION

While humans and most other mammals reproduce exclusively by sexual reproduction with sexes being determined by the well-known XY sex-chromosome system, the breeding systems of many other organisms, including many pests and parasites, remain unknown (Bell 1982; Normark 2003). The breeding system *sensu lato*, (including details of meiosis, e.g., recombination patterns, and syngamy, e.g., levels of inbreeding, as well as their variants, e.g., modified meiosis in parthenogens) represents a key for understanding the biology of a species and has profound effects on its ecology, evolution, and genomics. Yet investigating breeding systems is often far from straightforward: Many species cannot easily be cultured and bred in the laboratory and observations of breeding behavior in nature are difficult. Even in species than can be bred in the laboratory, parts of the breeding system may be cryptic and not directly observable.

The advent of high-throughput genotyping methods opens an alternative possibility that can be used on a much larger array of species: indirect inference of the breeding system using genetic methods, which are based on differences among breeding systems in the transmission of genetic information from one generation to the next. In some cases, genome-wide information may not be needed. For instance, a few genetic markers such as microsatellites are sufficient to distinguish self-fertilization from outcrossing in hermaphrodites (e.g., David *et al.* 2007) or clonal from sexual reproduction in aphids (Delmotte *et al.* 2002). However, for a conclusive distinction between other breeding systems, a genome-wide approach may be essential. This is illustrated in the present paper for the distinction of self-fertilization vs. automictic parthenogenesis, comparing genomic data with microsatellite data and direct observations.

Self-fertilization and automictic parthenogenesis both reduce genome-wide heterozygosity among offspring compared to their parents, thereby increasing homozygosity

due to identity by descent (Hartl and Clark 2007; Charlesworth and Willis 2009). Under self-fertilization, in which male and female gametes produced by the same, hermaphrodite individual fuse, the expected reduction in offspring heterozygosity for diploid, autosomal loci is 50% per generation. A similar heterozygosity reduction also occurs under some forms of automictic parthenogenesis (also called "automixis"). Automictic parthenogenesis is a common form of parthenogenetic (i.e., female-only) reproduction (Bell 1982; Mogie 1986; Suomalainen *et al.* 1987), in which offspring are produced by fusion of two products of a single meiosis. Examples are intra-tetrad mating in fungi or fusion of an egg cell with a polar body in animals (Suomalainen *et al.* 1987; Hood and Antonovics 2004; Stenberg and Saura 2009). A more detailed account of the different processes that are summarized under "automixis" is given below.

The distinction between automixis and self-fertilization is subtle both in terms of the expected heterozygosity reduction among offspring as well as with respect to the processes that lead to it. Both involve the fusion of two meiotic products produced by a single individual. Self-fertilization involves fusion of products of different, independent meioses and therefore parental alleles are sampled with replacement. In contrast, automixis involves fusion of the products of a single meiosis and therefore parental alleles are sampled without replacement. Sampling of parental alleles with replacement leads to the well-known Mendelian expectations of genotype frequencies (50% heterozygotes, 25% of each homozygote) among self-fertilized offspring. However, to understand the consequences of sampling of maternal alleles without replacement during automixis, we have to distinguish two cases: Under "central fusion" two products that have been separated during meiosis I (the first meiotic division) fuse, and under "terminal fusion" two products that have been separated during meiosis II fuse. Because homologous chromosomes (carrying different alleles at heterozygous loci) are separated during meiosis I, and sister chromatids (carrying identical

alleles) are separated during meiosis II, central fusion tends to retains parental heterozygosity and terminal fusion tends to lead to fully homozygous genotypes. However, because recombination reshuffles alleles between homologous chromosomes, these expectations hold only for the centromere (at which sister chromatids are attached to each other). Expected offspring heterozygosity at loci far from the centromere attains 67% of parental heterozygosity for both central and terminal fusion. This is because, far from the centromere, alleles are distributed at random across sister and non-sister chromatids due to recombination, and therefore they are sampled randomly without replacement (once one meiotic product is chosen, two of the three remaining meiotic products contain the alternate allele, Rizet and Engelmann 1949; Barratt *et al.* 1954; Suomalainen *et al.* 1987; Pearcy *et al.* 2006; Engelstädter *et al.* 2011; Pearcy *et al.* 2011).

Patterns of heterozygosity reduction between parents and offspring can thus be used to distinguish self-fertilization from automixis and / or central from terminal fusion. This approach has previously been used in a few organisms to address the question whether automixis occurs via central or terminal fusion (Pearcy *et al.* 2006; Lampert *et al.* 2007; Oldroyd *et al.* 2008). However, differences in the realized levels of heterozygosity reduction among breeding systems depend on recombination rates and may be modulated by the degree of recombination interference and, if offspring heterozygosity is assessed at any later stage than the zygote, differential survival of heterozygotes vs. homozygotes (i.e., viability selection, Wang and Hill 1999).

We therefore first derive two specific theoretical predictions of how to distinguish self-fertilized from automictic offspring and central from terminal fusion based on heterozygosity patterns. We then use the freshwater crustacean *Daphnia magna* to empirically assess and compare the consequences of self-fertilization and automixis for offspring heterozygosity. We use known, self-fertilized offspring as controls and compare them with

offspring whose breeding system was initially unknown but could, by the present study be identified as automictic. *Daphnia magna* reproduces by cyclical parthenogenesis, in which clonal reproduction is intermitted by sexual reproduction. The clonal offspring may develop into males or females (environmental sex determination) and sexual reproduction always leads to the production of diapause stages ("ephippia": structures formed by maternal tissue, usually encapsulating two diapausing embryos). Hence, "self-fertilized" offspring in diapause can easily be generated by growing clonal cultures to high population densities and letting males mate with their genetically identical sisters. We acknowledge that within-clone mating (mating of a female with a genetically identical male) may only genetically but not ecologically be equivalent to self-fertilization (fertilization between male and female organs of a single, hermaphrodite individual), but for simplicity, we do not distinguish between these terms in the present paper.

While diapause stages can be produced clonally in some species of *Daphnia* (Hebert and Crease 1980), they were hitherto thought to be always produced by sexual reproduction in *D. magna*. However, we have previously found that some strains of *D. magna* do not produce males ("non-male producing strains", NMP), even when stimulated with a "male-inducing" hormone (Innes and Dunbrack 1993; Galimov *et al.* 2011). In natural populations, these strains still participate in sexual reproduction, but only via the female function, that is, by producing diapause eggs that have to be fertilized by males from other, male-producing (MP) strains (i.e., strains that produce both males and females with sex determined by the environment). When grown in isolation (i.e., in NMP-only cultures), females still produce the diapause capsules, but these are usually empty (i.e., do not contain viable embryos). Yet, very rarely, a few offspring hatch from these ephippia, indicating that a very low percentage of them do contain viable embryos (Galimov *et al.* 2011). The offspring are diploid and show segregation of maternal alleles, indicating that they are not produced clonally (Galimov *et al.*

2011). They may thus be produced either by within-clone mating through rare and undetected male production in the maternal NMP strain or by automictic parthenogenesis (Galimov *et al.* 2011). To evaluate these possibilities, we used (i) direct testing for the presence of males by phenotypic screening of large samples, (ii) crossing attempts between different NMP strains (if rare males are present they are expected to fertilize females of other NMP strains as well as their own), and (iii) an assessment of the heterozygosity patterns among offspring by microsatellite genotyping and RAD-sequencing. Our results showed that only the genomic approach (RAD-sequencing) could provide conclusive evidence for the mode of reproduction by which these offspring had been produced. More generally, our study thus serves to illustrate the observed and expected genome-wide patterns of heterozygosity reduction under automixis and self-fertilization and to provide evidence for the great potential of genomic approaches for elucidating cryptic breeding systems.

Expected heterozygosity reduction under automixis

The expected heterozygosity reduction under automixis has been described before (Rizet and Engelmann 1949; Barratt *et al.* 1954; Suomalainen *et al.* 1987; Pearcy *et al.* 2006; Engelstädter *et al.* 2011; Pearcy *et al.* 2011). However, different aspects are discussed in different papers, and the literature on breeding systems is rather disparate from the literature on genetic mapping in fungi or on mapping of centromeres either by natural or artificial automixis. Furthermore, in addition to central and terminal fusion, a further term "random fusion" is sometimes discussed, but its definition and effects on heterozygosity reduction require clarification. Finally, the effects of recombination interference on heterozygosity reduction have only rarely been considered in the breeding systems literature (e.g., Asher 1970; Nace *et al.* 1970). For these reasons, we briefly review here the literature on expected heterozygosity reduction under automixis with the focus on the comparison with self-

fertilization. We identify two main predictions regarding expected heterozygosity patterns, an inter-chromosomal and an intra-chromosomal one, which allow distinguishing automictic from self-fertilized offspring using genomic data. We also mathematically derive predictions on the intra-chromosomal patterns of heterozygosity in offspring produced by terminal and central fusion, accounting for different degrees of recombination interference.

The terms “central fusion” and “terminal fusion” are derived from ordered tetrads (Tucker 1958; Suomalainen *et al.* 1987). In many fungi and algae, the four products of meiosis remain together in an envelope called ”ascus“, with some of them retaining a specific order (Bos 1996): the four meiotic products of a diploid parent heterozygous A1A2 at a centromeric locus are ordered along a sequence A1_A1_A2_A2, with meiosis I explaining the central division and meiosis II the two terminal divisions (each division is indicated by an underscore). Hence, fusion of neighboring meiotic products during within-tetrad mating can either be terminal (leading to homozygous centromeric regions A1A1 or A2A2) or central (leading to heterozygous centromeric regions A1A2). However, because the effects on offspring heterozygosity are identical, the term “central fusion” is often used to describe the fusion of any two meiotic products that have been separated during meiosis I (or where meiosis I is suppressed, Asher 1970). Equivalently, the term “terminal fusion” is used to describe the fusion of any products that have been separated during meiosis II (or where meiosis II is suppressed, Asher 1970), not only in ordered tetrads.

“Random fusion” can be defined as fusion of two randomly chosen products of a meiotic tetrad (Suomalainen *et al.* 1987; Pearcy *et al.* 2006; Lampert *et al.* 2007). Hence, with random fusion, 2/3 of the offspring are produced by central fusion and 1/3 by terminal fusion (once one meiotic product is chosen, only one of the three remaining products carries the same allele at the centromeric locus shown above, thus central fusion occurs with a probability of 2/3). Yet, in animals, meiosis typically leads to one oocyte and polar bodies,

and automictic fusion usually (but not always, e.g., Seiler and Schäffer 1960) occurs between the oocyte and one of the polar bodies. However, the first polar body often decays rapidly or does not undergo meiosis II (e.g., in *Daphnia*, Zaffagnini and Sabelli 1972), and these details of the reproductive mode may change the proportion of offspring produced by central vs. terminal fusion even under random expectations (i.e., without specific mechanism favoring one over the other). It may therefore be more useful to distinguish cases in which both central fusion and terminal fusion occur, possibly in different proportions (we term this "mixed fusion") from cases in which one of them is the exclusive mode of reproduction. With mixed fusion, any given offspring is produced by either central or terminal fusion (two specific meiotic products fuse or meiosis I or meiosis II is suppressed). This leads to a first general prediction, which should enable differentiating automixis from self-fertilization: Independently of whether automixis occurs by central or terminal fusion, the homozygosity of centromeric regions across different chromosomes should be 100% correlated within a given offspring (Fig. 1). That is, either all centromeric regions should become homozygous (offspring produced by terminal fusion) or they should all retain parental heterozygosity (offspring produced by central fusion). In contrast, under self-fertilization, each centromeric region is expected to become homozygous or retain parental heterozygosity with an independent probability of 0.5 (i.e., independently of the heterozygosity of other centromeric regions in the same individual, Fig. 1). A method to determine how the inter-chromosomal pattern can be assessed if the centromeric positions are unknown, is outlined in Supporting File S1.

Second, within each chromosome, heterozygosity is expected to gradually increase from zero (terminal fusion) or decrease from 100% (central fusion) to 67% of parental heterozygosity with increasing genetic distance from the centromere (Fig. 1, Rizet and Engelmann 1949; Engelstädter *et al.* 2011; Pearcy *et al.* 2011). The levelling off at 67% under

both terminal and central fusion occurs because, at large genetic distances from the centromere, recombination effectively distributes alleles at random across the sister and non-sister chromatids. Therefore, both terminal and central fusion result in random sampling without replacement of two alleles from four chromatids and thus to the expected heterozygosity of 67% (once one chromatid is chosen, two of the three remaining chromatids carry a different allele).

The transition from zero or 100% heterozygosity at the centromere and 67% heterozygosity in centromere-distant regions depends on the genetic map distance (i.e., the expected number of crossovers) and thus on the level of crossover interference (Fig. 1, Supporting File S2, Barratt *et al.* 1954; Nace *et al.* 1970; Zhao and Speed 1998). In Supporting File S2, we present an original derivation of this relationship, taking advantage of the flexibility of generalized Poisson distributions (Conway-Maxwell Poisson distribution, Sellers *et al.* 2012). With high degree of cross-over interference, this relationship may be non-monotonous (Fig. 1). However, the initial slope of the change in heterozygosity close to the centromere is $2d$ (where d is the genetic distance in Morgan) under terminal fusion and $-d$ under central fusion (Fig. 1, Supporting File S2), irrespectively of the degree of interference. In contrast, under self-fertilization, expected heterozygosity is 50% of the parental heterozygosity and does not depend on the distance from the centromere nor on the level of cross-over interference.

Several other forms of automixis are defined and discussed elsewhere (Bell 1982; Mogie 1986; Suomalainen *et al.* 1987; Stenberg and Saura 2009; Archetti 2010; Lutes *et al.* 2010; Neiman *et al.* 2014; Nougué *et al.* 2015). Their effects on genome-wide heterozygosity reduction are often very different from self-fertilization (e.g., complete loss or complete retention of parental heterozygosity).

METHODS

Origin of clones and outdoor experiments

We use the term "clone" to refer to a strain initiated by a single female and maintained by clonal reproduction. Clones used in this study originated from Russian populations known to contain NMP clones (Ast, BN, MZ, Vol, Galimov *et al.* 2011). They were classified as MP or NMP according to whether or not females of these clones produced male offspring during clonal reproduction when exposed to 400 nM methyl farnesoate, a juvenile hormone analog that has been shown to consistently induce male production in MP clones of *D. magna* (Olmstead and Leblanc 2002; see Galimov et al. 2011 for detailed methodology).

Outdoor mass cultures were carried out using two NMP treatments and two MP control treatments: (1) NMP single clone cultures ("NMP_single") each contained a single NMP clone. Because only one maternal clone was present, the ephippial offspring produced in these cultures were the result of within-clone mating (if rare males were present) or some form of parthenogenetic reproduction. This treatment was used (as in our earlier study, Galimov *et al.* 2011) to eliminate the possibility of clonal parthenogenesis by examining offspring for segregation of maternal alleles at microsatellite loci. Furthermore, the offspring of one culture (the culture that produced the largest number of offspring) were used to test for genome-wide patterns of heterozygosity using RAD-sequencing. (2) NMP multi-clone cultures ("NMP_mix") contained two to four different NMP clones (distinguishable at microsatellite loci). They were used to test for the presence of rare males by testing for the occurrence of outcrossed offspring (i.e., crosses between different NMP clones). Outcrossing should occur in the presence of rare males, but not under automixis. Control treatments (3) "MP_single" and (4) "MP_mix", containing single or two to four MP clones, respectively, were used to verify male production and outcrossing under the experimental conditions as well as to assess genome-wide heterozygosity in offspring produced by self-fertilization.

The outdoor cultures were set up under ambient conditions in the Botanical garden of Fribourg, Switzerland (46°48'6.00"N, 7°8'44.04"E) by transferring ~100 adult females of each clone into buckets containing 40 L of artificial *Daphnia* medium (Klüttgen *et al.* 1994) as well as a 50 ml initial inoculum of natural microalgae and bacteria (50-µm-filtered water from a local garden pond) as well as ~100 g of fresh horse manure to provide nutrients. Some fresh unicellular green algae, *Scenedesmus* sp., were added intermittently throughout the experiment to keep densities high, and natural rain water gradually filled the buckets to ~60 L.

The experiment took place in two parts: A first batch of cultures was grown outside from March to November 2011, and a second batch from March/April 2013 to October 2013 (Table 1). In both batches, the clones reproduced mostly asexually during summer and fall, with intermittent production of males observed in the MP cultures and ephippia, both in MP and NMP cultures. Even though there was no systematic quantification of ephippia production in this experiment, we did not notice any obvious differences in numbers of ephippia produced between NMP and MP cultures. However, all opened ephippia from NMP cultures were empty (i.e., did not contain embryos), whereas almost all ephippia from MP cultures contained embryos (several dozens of ephippia from each of the two culture types were opened). The results of the first batch suggested the possibility of clonal selection leading to substantially unequal clone frequencies in multi-clone cultures and thus reduced probabilities of outcrossing (assuming presence of males and random mating). We therefore intermittently (June, 15 July, 25 August, and 17 September 2013) re-stocked all multi-clone cultures of the second batch by adding up to 100 non-ephippial females of the less frequent clones, after estimating clone frequencies based on microsatellite genotypes of 25 individuals of each culture. The aim of this procedure was to equilibrate clone frequencies and thus to increase the likelihood of outcrossing if rare males were present. Finally, six NMP cultures of the first batch were used to phenotypically search for rare males using large samples (~4000

274 individuals) taken at the end of the growing season (November 2011), with sex identified
275 under a stereo microscope. The same was also done for two MP control cultures.
276 At the end of each growing season (mid November 2011, end of October 2013 for the
277 first and second batch, respectively), all ephippia that had accumulated at the bottom of the
278 buckets were collected and overwintered (which is necessary for later hatching).
279 Overwintering was done either outdoors in a small volume of water placed in the dark (first
280 batch) or in a dark cold-room at 0°C (second batch). In the subsequent spring, hatching tests
281 were carried out by transferring the ephippia to fresh *Daphnia* medium and keeping them
282 under warm and high-light conditions (ambient Fribourg spring conditions in the first batch,
283 ~20°C greenhouse conditions in the second batch). The containers were carefully checked for
284 hatchlings at least every third day, and hatchlings were removed and stored in ethanol at -
285 20°C for later genotyping or grown in isolation in order to establish cultures of offspring
286 clones. Overall, the 2011 batch yielded more hatchlings than the 2013 batch, likely due to
287 environmental effects during growth or hatching.
288
289 **DNA extraction and microsatellite analysis**
290 Genomic DNA was extracted using the HotSHOT protocol (Montero-Pau *et al.* 2008)
291 and nine diagnostic microsatellite loci (Supporting Table S1) were used to distinguish
292 outcrossed from non-outcrossed offspring (the latter resulting from within-clone mating or
293 parthenogenetic reproduction), as well as to check for segregation of markers that were
294 heterozygous in the parent clones. We set up PCR reactions of 10 µL, using the Qiagen
295 Multiplex PCR master mix (Qiagen, Venlo, The Netherlands). Cycling was performed
296 following the recommendations of the manufacturer. Fragment lengths were analysed using
297 GENEMAPPER Software version 4.0 (Applied Biosystems, Foster City, CA, USA) with
298 Gene Scan-500 LIZ as an internal size standard.

RAD-sequencing

To obtain markers throughout the genome, at which heterozygosity could be assessed, we used Restriction site-Associated DNA sequencing (RAD-sequencing, Baird *et al.* 2008), using eight hatchlings from a single clone NMP culture (clone AST-01-04, bucket V04), as well as 27 hatchlings from a single clone MP culture (clone RM1-18 MP, bucket B19). Only eight offspring of an NMP clone were used because this was the highest number of offspring from a single clone NMP culture that could successfully be grown in clonal culture in the laboratory before DNA-extraction (several other hatchlings died before reproduction or were sterile). We used a RAD-sequencing protocol based on Etter et al. (2011) with a few modifications as specified below. Two libraries were prepared: one containing the offspring of the NMP single clone culture, the other containing the offspring of the MP single clone culture, with each offspring individually labelled. Each library also contained two independent replicates of the parental clone. The details of the RAD-sequencing protocol and the analysis pipeline including quality checks, alignment, SNP-calling, and genotype calling, is explained in detail in Supporting File S3.

Inter- and intra-chromosomal patterns of heterozygosity reduction

Putative centromere locations were inferred from the genetic map as corresponding to large, non-recombining regions, of which each linkage group contains exactly one, except linkage group 3 which has two such regions (*D. magna* genetic map v4.0.1, deposited on Dryad, Dukić et al., unpubl.). Centromeric regions were defined as consisting of all scaffolds (or parts of scaffolds) with the cM position of these non-recombining regions. Average heterozygosity as a function of the distance from the putative centromere was calculated for each chromosome arm separately by using a moving average, including markers within 5 cM

on either side of the focal marker (but in all cases excluding markers at a distance of zero cM from the centromeric regions). Subsequently, the averages and standard errors (SE) of these estimates were calculated across chromosome arms, and confidence limits calculated as 1.96 SE.

To estimate the distance from the centromere of microsatellite loci, we first mapped each primer pair to the current *D. magna* assembly v2.4. Subsequently, we retrieved the position on the genetic map v4.0.1 of the closest marker on the same scaffold. In this way, we were able to obtain estimated map locations for six of the microsatellite loci (Supporting Table S2).

Probability of within-clone mating in the presence of rare males

The absence of outcrossed offspring in NMP mixed clone cultures does not necessarily indicate the absence of rare males because a low number of offspring could, by chance be produced exclusively by within-clone mating. Hence, we calculated the probability of observing zero outcrossed offspring in the presence of rare males under the assumption of random mating among the clones present at the time of resting egg production in each NMP mixed clone culture. Under random mating, the probability of within-clone mating of a given clone i in a given culture j is equal to its squared frequency, f_i^2, and the overall expected frequency of within-clone mated offspring is $\Sigma(f_i^2)$, summed across all clones present in the culture. The probability of observing only offspring produced by within-clone mating among N offspring (i.e. the probability that despite the presence of males not a single outcrossed offspring was observed) then equals $pr_j = [\Sigma(f_i^2)]^N$, and the combined probability across all cultures is the product $\Pi(pr_j)$.

Because the frequencies of clones at the time of resting egg production were unknown, we assumed two contrasting scenarios: First, we assumed that all original parent clones were

still present at equal frequency at the time of resting egg production. This scenario maximizes the probability of outcrossing. Therefore, we also used a second, more conservative scenario: We assumed that the frequency of each parent clone at the moment of resting egg production was equal to its proportional contribution to the offspring generation. For this second scenario we only used buckets in which offspring from more than one parent clone were present (for the other buckets, the expected frequency of within-clone mated offspring under this scenario is 100%).

RESULTS

Sex ratios

We identified the sex of 25,925 NMP individuals of *D. magna*, sampled in late season from six outdoor cultures of NMP clones, but did not find a single male (Table 1). At the same time, cultures of MP clones contained between 10.8% and 38.8% males (mean across populations = 27.2%, SE = 6.1%, total N = 1502). Combined with data from our earlier study (Galimov et al. 2011), we have now identified the sex of 33,764 NMP individuals but did not find a single male. This yields an overall upper 95% confidence limit for the true proportion of males of ~10^{-4} (Clopper-Pearson confidence interval $1-(\alpha/2)^{1/N}$). However, the experiment involved many more individuals than the one that were checked (over 10^5 individuals across the whole duration of the experiment and all NMP cultures combined). Thus the presence of rare males cannot entirely be excluded through these phenotypic observations alone.

Number of hatchlings

A total of 110 hatchlings were found in NMP cultures (between zero and 28 per culture, Table 1). Of these, 61 were found in cultures containing just a single NMP clone and 49 in cultures containing multiple NMP clones. All MP control cultures except one yielded

more than 30 offspring (Table 1), some of them even many more. Even though numbers of hatchlings above 30 were not estimated systematically, this fits with our experience from similar experiments, where MP cultures usually yielded hundreds to thousands of hatchlings, though in rare cases only low numbers or even none (e.g., Haag and Ebert 2007).

Microsatellite genotypes of offspring from cultures containing single NMP clones

In total, we investigated microsatellite genotypes of 27 offspring from cultures containing single NMP clones. In all cases, these offspring showed segregation of maternal alleles (Supporting Table S1), thus excluding clonal parthenogenesis. Average heterozygosity across all cultures and loci was 0.61 (SE = 0.04), which is significantly different from 0.5 (N = 152, χ^2 = 6.7, P = 0.0094), but not from 0.67 (χ^2 = 2.9, P = 0.090). Nonetheless, two loci had heterozygosities that were significantly lower than 0.67 (locus B008: N = 5, heterozygosity = 0, binomial P = 0.005, locus B096: N = 21, heterozygosity = 0.36, SE = 0.10, χ^2 = 9.2, P = 0.0022) and, in one case, even significantly lower than 0.5 (locus B008: binomial P = 0.031, locus B096: χ^2 = 1.6, P = 0.20). Indeed heterogeneity among loci was significant (generalized linear model with binomial error distribution using Firth bias correction, likelihood ratio test, χ^2 = 23.6, *d.f.* = 6, P = 0.0006). In contrast, offspring from different cultures or different individuals within cultures did not significantly vary in heterozygosity (tested in the same model as the loci effects, cultures: χ^2 = 4.4, *d.f.* = 4, P = 0.35, individuals nested within cultures: χ^2 = 20.9, *d.f.* = 22, P = 0.53). The heterogeneity among loci was at least partly explained by the distance from the centromere: The two loci with heterozygosities significantly lower than 0.67 (loci B008 and B096) were the two loci estimated to be most closely linked to a centromere (at 25.8 and 3.6 cM, respectively). All other loci were had estimated distances from the centromere of >32 cM (Supporting Table S2).

Microsatellite genotypes of offspring from cultures containing multiple clones

We obtained microsatellite genotypes of 25 offspring from cultures containing multiple NMP clones (Table 1). Among these, not a single offspring resulting from outcrossing between two of the parent clones was observed. Rather, all 25 offspring were produced by self-fertilization or automictic parthenogenesis: They showed segregation of maternal alleles, just as offspring of the single clone cultures, but no sign of outcrossing between clones at diagnostic loci (Table 1, Supporting Table S1). In all but two of these cultures, all offspring found within the culture were produced by just one parent clone. Two cultures (V12 and B23) contained offspring from two different parent clones (Table 1), but nonetheless no outcrossed clone was observed (i.e., also these offspring were the result or self-fertilization or automictic parthenogenesis). In stark contrast, 23 of 24 genotyped offspring from cultures containing multiple MP clones were the result of outcrossing between two parent clones (Table 1, Supporting Table S1). The frequency of outcrossed offspring was even significantly higher ($\chi^2 = 5.6$, $P = 0.018$) than the 75% expected under random mating and equal frequencies of each of the four parent clones, likely due to inbreeding depression affecting hatching rates of inbred vs. outcrossed offspring.

Even though no outcrossed offspring was observed in the cultures containing multiple NMP clones, there is still a possibility that they were produced by the mating of a rare male with a female of the same clone. Assuming random mating of rare males with all females, the overall probability of observing zero outcrossed offspring among the 25 genotyped individuals from the cultures containing multiple NMP clones was calculated under two extreme scenarios (see methods). Under the first scenario (assuming equal frequency of all introduced parent clones), this probability is very low ($\sim 10^{-9}$). The second scenario (frequency of parent clones equal to their contribution to the offspring generation) could be assessed only

for the five offspring from cultures V12 and B23, in which self-fertilized/automictic offspring from more than one parent were present. Under this scenario, the probability of observing zero outcrossed offspring among the five offspring in these cultures is 0.043.

Genome-wide patterns of heterozygosity assessed by RAD-sequencing

RAD sequencing was carried out on eight offspring of the AST-01-04 NMP clone (the remaining offspring of this clone died or did not reproduce and therefore it was not possible to obtain sufficient amounts of DNA). Average genome-wide heterozygosity of these eight offspring was 0.54 (N = 2523 loci). It ranged from 0.24 to 0.67 among linkage groups and from 0.40 to 0.73 among individuals (Supporting Table S3). The relatively high variation among individuals and linkage groups was expected because only few recombination events occur per meiosis and chromosome (Routtu *et al.* 2010; Routtu *et al.* 2014), so that many linked markers show identical inheritance patterns.

As a control, RAD-sequencing was also carried out on 27 offspring of the RM1-18 MP clone. Average genome-wide heterozygosity among these offspring was 0.60 (N = 1610 loci), varying among linkage groups between 0.46 and 0.71 (Supporting Table S3). This was significantly higher than 0.5 (linkage groups as independent replicates, t = 3.7, $d.f.$ = 9, P = 0.005), but also significantly lower than 2/3 (t = -2.7, $d.f.$ = 9, P = 0.023, though not quite significantly so when linkage groups were weighed according to the number of loci: P = 0.067).

Inter-chromosomal patterns of heterozygosity at putative centromere regions

The analysis carried out here requires knowledge of centromere regions. We use putative centromere regions (large, non-recombining regions as identified on each linkage group by the genetic map v4.0.1, which maps most scaffolds of the current *D. magna*

assembly, M. Dukić et al., unpubl.). An equivalent analysis that does not require assumptions on putative centromere regions is presented in Supporting File S1.

The putative centromere regions were either consistently homozygous (seven offspring) or heterozygous (one offspring: V04_04) across all ten linkage groups (Fig. 2). One of the linkage groups (LG3) contained two such regions, but only the region at 90.8 cM showed the same heterozygosity as the putative centromere regions of the remaining linkage groups. Hence, this rather than the region at 62.5 cM is the likely centromere region of LG3 (and only this region was considered for all other analyses). The inter-chromosomal pattern thus strongly suggests that V04_04 was produced by central fusion and the other seven offspring by terminal fusion.

In contrast, the putative centromere regions were not consistently homozygous or heterozygous across all linkage groups within individual offspring of the RM-1-18 MP clone, except for one individual, in which all 10 centromere regions were heterozygous (Fig. 2). Using the observed average heterozygosity of 0.60, the probability of observing this at least one time by chance among 27 offspring is ~0.15 ($p = 0.6^{10}$ is the probability that an individual is heterozygous for the 10 centromeric regions, $(1- p)^{27}$ that none of the 27 offspring is heterozygous for the 10 centromeric regions, and thus $1-(1-p)^{27}$ is the probability that at least one is heterozygous for the 10 centromeric regions).

Intra-chromosomal patterns of heterozygosity

Among the seven offspring of the NMP clone that were presumably produced by terminal fusion, heterozygosity gradually increased with distance from the centromere (Fig. 3), and reached an average heterozygosity of clearly more than 0.5 already at a distance of 50 cM. Heterozygosity in distal regions was even somewhat higher than the expected 0.67, though this was only marginally significant (the lower 95% confidence interval calculated by

using chromosome arms as independent replicates mostly included 0.67). Heterozygosity of the individual produced by central fusion (offspring V04_04) averaged across all markers and all chromosome arms was 0.73, and 0.67 if only markers located over 50cM from the centromere were considered. In contrast, heterozygosity among the 27 offspring of the RM1-18 MP clone did not vary in any systematic way along the chromosomes nor according to the distance from the centromere (Fig. 3).

DISCUSSION

Reliable distinction of automixis from self-fertilization requires genomic data

Our results demonstrate that the ephippial offspring of the *D. magna* NMP clone investigated by RAD-sequencing were produced by automixis, mostly but not exclusively by terminal fusion. The microsatellite results on the offspring of the other clones strongly suggest that this was also the case for the offspring of the other clones (lower heterozygosity at centromere-proximal markers). However, an unequivocal distinction from self-fertilization was only possible owing to the use of genomic data, which also allowed confirmation of the centromere locations. While the phenotypic results were suggestive of automixis (no males detected in NMP cultures, no cross-breeding observed in cultures containing multiple NMP clones), they were nonetheless not entirely conclusive. Very low male frequencies could not be excluded, despite large sample sizes, and the expectation that the presence of males would lead to outcrossed offspring was based on specific assumptions that could not be verified. Moreover, the average microsatellite heterozygosity was similar to the average genome-wide heterozygosity of the self-fertilized controls and therefore also inconclusive. Also at RAD-loci, automictic offspring retained 54% of parental heterozygosity, due to a predominance of terminal rather central fusion and due to inclusion of both centromere-distal and centromere-proximal markers. If only average heterozygosity had been assessed, this could easily have

been mistaken as consistent with self-fertilization rather than automixis (average observed heterozygosity in self-fertilized offspring was 60%). This shows that average offspring heterozygosity is not necessarily a reliable indicator of the breeding system.

Unequivocal evidence for automixis was obtained only when the inter-chromosomal and intra-chromosomal patterns of genome-wide heterozygosity were analyzed. These patterns are clearly inconsistent with self-fertilization, as shown by our parallel analysis of self-fertilized controls. In addition, the inter-chromosomal patterns provide a direct estimate of the proportion of offspring produced by terminal vs. central fusion: Offspring produced by terminal fusion are homozygous at the centromeric regions of all chromosomes, offspring produced by central fusion retain full parental heterozygosity at all these regions (see also, Oldroyd *et al.* 2008). Even if the positions of the centromeres are unknown, the inter-chromosomal patterns can be analyzed by investigating if specific segregation patterns (among individuals) occur consistently on all chromosomes (Supporting File S1).

The intra-chromosomal patterns of heterozygosity in automictic offspring can be used to map the centromeres, an approach that has been used both in natural automicts (Barratt *et al.* 1954; Baudry *et al.* 2004) and in organisms, in which automixis can be induced artificially (or meiotic tetrads or half-tetrads can be recovered by other means, Lindsley *et al.* 1956; Eppig and Eicher 1983; Johnson *et al.* 1996; Zhu *et al.* 2013). Also in our study, the intra-chromosome heterozygosity patterns among offspring produced by terminal fusion confirm the presumed locations of centromeres in *D. magna* and indicate that all *D. magna* chromosomes are metacentric. Furthermore, our results also indicate that the centromere on LG3 is located at 90.8cM rather than 62.5cM (two large, non-recombining regions are found on this linkage group, Dukić et al., unpubl).

Even though each centromeric region did contain markers that were homozygous in all individuals produced by terminal fusion, there were also, in each of these regions, some

markers that were not fully homozygous (average heterozygosity across centromeric regions was 12%, Fig. 3). These 12% of unexpected genotypes are likely due to a combination of genotyping error (false heterozygote calls), erroneous mapping of reads from paralogous loci to single loci (e.g., centromeric markers that were heterozygous in all individuals, as found on several linkage groups, Supporting Figure S1), errors in the genetic map and/or non-collinearity between chromosomes in our study population compared to the clones on which the *D. magna* map is based. Errors in the genetic map and non-collinearity would have the effect that loci that were mapped by us to the centromeric regions are in reality not in these regions, which could explain their non-zero heterozygosity.

Automixis and diversity of breeding systems in *Daphnia*

While most *Daphnia* species have so far been thought to produce diapause stages exclusively by sexual reproduction, a few species regularly produce parthenogenetic diapause stages (e.g., obligate parthenogenetic strains of *D. pulex*, Hebert and Crease 1980). Yet, in these cases, offspring do not show segregation of maternal alleles and are therefore believed to be clonal offspring, just as the offspring resulting from parthenogenetic production of subitaneous (directly-developing) eggs during the regular asexual part of the life cycle in *Daphnia* (Hebert and Ward 1972, but see comments on clonality below). Hence our results constitute the first demonstration of classical automixis in *Daphnia*.

The finding of rare automixis in NMP clones of *D. magna* is important for our understanding the NMP/MP polymorphism. Non-male producing clones of *D. magna* participate in sexual reproduction only through their female function (Galimov *et al.* 2011), and were therefore speculated to be unable to colonize new populations on their own. However, rare automixis may allow these populations to persist through the first period of

548 diapause and may therefore allow single NMP clones to colonize new populations. This may
549 be especially important in environments with frequent extinction-colonization dynamics.
550 However, there is no reason to believe that rare automixis is limited to NMP clones.
551 Production of empty ephippia (shells of resting stages not containing embryos) is frequently
552 observed also in MP clones, when females are grown in the laboratory in isolation from males
553 and even in nature (Ebert 2005). Yet, possible rare automictic reproduction in these cultures
554 would be much more difficult to detect than in NMP clones because MP clones regularly
555 produce males under the conditions needed to stimulate ephippia production. Indeed, rare
556 automictic parthenogenesis occurs in a large number of organisms in the form of rare,
557 spontaneous hatching of unfertilized eggs ("tychoparthenogenesis") with diploidy restored via
558 automixis (Bell 1982; Schwander *et al.* 2010; Neiman *et al.* 2014).
559 In other species, automixis is a regular form of reproduction, but often exclusively or
560 almost exclusively with central fusion. Examples are many fungal species, including yeast, in
561 which central fusion during within-tetrad mating (= automixis) is assured by a mating type
562 locus (unless there is a mating type switch or recombination between the centromere and the
563 mating type locus, Antonovics and Abrams 2004). Another example comes from several
564 social insects, which can reproduce parthenogenetically by central fusion, and, additionally,
565 show very low rates of crossover within the chromosomes (Baudry *et al.* 2004; Oldroyd *et al.*
566 2008; Rey *et al.* 2011). With very low recombination rates, central fusion effectively
567 approaches clonality because central fusion assures that parental heterozygosity is retained at
568 the centromeres, and low rates of crossover result only in a slow decay of heterozygosity in
569 the centromere-distal regions.
570 Automixis has been hypothesized to represent an intermediate step in the evolutionary
571 pathway from sexual to clonal reproduction (Schwander *et al.* 2010). According to this
572 hypothesis, rare automictic reproduction ("tychoparthenogenesis") with "mixed fusion" may

become more frequent, with subsequent selection for increased rates of central fusion and repression of recombination. Parthenogenetic reproduction in *Daphnia* has indeed been termed an intermediate between clonal and automictic reproduction because subitaneous, parthenogenetic eggs are produced by a modified meiosis rather than by mitosis (Hiruta *et al.* 2010; Hiruta and Tochinai 2012). Specifically, the homologs pair and start to separate, but meiosis I is not completed, and sister chromatids of a diploid set of chromosomes are separated during meiosis II (Hiruta *et al.* 2010). In other words, meiosis I is suppressed, which is identical to central fusion (Asher 1970), but it is also indistinguishable from purely clonal reproduction as long as no recombiantion occurs during the paring of homologs (a low degree of exchange occurs in *D. pulex*, Omilian *et al.* 2006; Tucker *et al.* 2013). More generally, this suggests that the mechanism of clonal parthenogenesis may, also in other diploid organisms, be an extreme form of automixis (central fusion with no or very low levels of recombination) and thus a meiosis-derived process rather than production of fertile eggs by mitosis. This distinction is important because it implies that the evolution of asexuality in these taxa may have involved strong heterozygosity-reducing processes (terminal fusion, central fusion with recombination). Hence the classical view that clonal diploids maintain their heterozygosity at least on short evolutionary timescales (thus for instance avoiding inbreeding, e.g., Haag and Ebert 2004) may not have been true during the initial evolution of asexuality. Hence it may be necessary to more explicitly account for the mechanism of this transition in order to fully understand the selection pressures acting during the evolution of parthenogenesis from sexuality. Furthermore, the same processes may also be important for understanding the maintenance of asexuality: If clonal parthenogenesis is indeed meiosis-derived, there may be residual rates of recombination during homologue pairing (Hiruta *et al.* 2010), such that transitions to homozygosity and loss of complementation may occur at higher

rates than under purely mitotic parthenogenesis (Archetti 2004; Archetti 2010; Nougué *et al.* 2015).

Inbreeding depression in self-fertilized and automictic offspring

In the self-fertilized offspring, observed heterozygosities were higher than the expected 50 % for the majority of the linkage groups. This suggests that the parent clone carried loci contributing to inbreeding depression, that is, loci with recessive or partly recessive deleterious alleles on these linkage groups (Fu and Ritland 1994b). Indeed, the realized heterozygosities can deviate from the expected ones in inbred individuals due to selection and such deviations are a form of inbreeding depression (Fu and Ritland 1994a; Wang and Hill 1999). Also the higher than expected number of outcrossed offspring in the cultures containing multiple MP clones is evidence for inbreeding depression in the control cultures.

The automictic offspring also showed signs of inbreeding depression: Only few hatchlings survived to adulthood and were sufficiently fecund so that they could successfully be taken into clonal culture (Table 1). Furthermore, observed offspring heterozygosities also tended to be higher than the expected ones, even after accounting for high levels of cross-over interference. A closer examination of the contribution of selection to the genome-wide patterns of observed heterozygosity is not possible due to the low number of automictic offspring investigated, and also due to complicating effects of possible genotyping errors and other possible errors (alignment, mapping, collinearity, see above) in our analysis. Due to these uncertainties, our prediction that the initial increase in heterozygosity at short distances from the centromere should be $2d$ (where d is the genetic distance in Morgan), if it is not influenced by selection, could not be evaluated with the present data. Nonetheless, the strong initial increase in heterozygosity at distances up to 100 cM from the centromere is

inconsistent with the absence of both recombination interference and selection, but rather indicates the action of one or both of these processes. If a larger number of offspring is analyzed and selection is estimated independently (e.g., by analyzing loci at >100 cM from the centromere) or can be excluded (e.g., by investigating zygotes), the analysis of heterozygosity patterns among automictic offspring may be used to investigate the degree of crossover interference.

Conclusions

Overall, our study shows that the mode of reproduction in automictic vs. self-fertilizing species can be inferred from the heterozygosity patterns among offspring. However, our study also illustrates that it was only due to the availability of genomic rather than sparse marker data that these inferences were robust to the complicating effects of recombination interference and selection. The same applies to the distinction between terminal and central fusion in species that use a mix of these two modes of reproduction (not necessarily in the ratios corresponding to "random fusion"). More generally, our findings support the idea that obtaining genome-wide heterozygosity data from mothers and a limited number of offspring may be a widely applicable and accessible approach to study breeding systems in species with cryptic or mixed modes of reproduction.

DATA ACCESS

All demultiplexed read data used for genotyping were submitted to NCBI SRA: BioProject ID PRJNA279333. The reference genome used for mapping and annotation is available on http://wfleabase.org/ (dmagna_v.2.4_20100422). The full raw and corrected SNP data sets, as well as the genetic map v4.0.1 are available as supporting information (Supporting Files S4, S5, and S6).

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DISCLOSURE DECLARATION

The authors declare no competing financial interests.

665 **TABLES**

666 Table 1: Origins of clones, sex rations, number of hatchlings, as well as numbers of within-
667 clone and outcrossed offspring in each of the cultures. Empty cells indicate values that were
668 not assessed in a given culture. (Table continued on next page).

Bucket ID	Batch	Treatment	Origin of clones	*N* males	*N* females	*N* hatchlings	*N* genotyped	*N* within-clone offspring	*N* outcrossed offspring
V02	2011	NMP_single	Vol			7	2	2	0
V03	2011	NMP_single	MZ			0			
V04	2011	NMP_single	Ast			28	14	14	0
V08	2011	NMP_single	Vol	0	4629	3	3	3	0
V10	2011	NMP_single	Ast	0	5370	3	3	3	0
V21	2011	NMP_single	MZ			8	5	5	0
B11	2013	NMP_single	Ast			11			
B12	2013	NMP_single	Vol			0			
B13	2013	NMP_single	MZ			1			
B14	2013	NMP_single	Ast			0			
B15	2013	NMP_single	Ast			0			
V01	2011	NMP_mix	MZ, Vol			13	3	3 (same parent)	0
V05	2011	NMP_mix	BN, Vol			11	7	7 (same parent)	0
V06	2011	NMP_mix	MZ, Vol			1	1	1	0
V07	2011	NMP_mix	BN, Vol	0	5105	1			
V09	2011	NMP_mix	MZ, Vol	0	4256	10	3	3 (same parent)	0
V11	2011	NMP_mix	BN (2x)	0	5550	0			
V12	2011	NMP_mix	Ast, BN, MZ, Vol			2	2	1+1 (two different parents)	0
V15	2011	NMP_mix	MZ (2x)	0	1015	1			
V17	2011	NMP_mix	MZ (2x)			4	4	4 (same parent)	0
V19	2011	NMP_mix	Ast, BN, MZ, Vol			1			
V20	2011	NMP_mix	BN (2x)			0			
B20	2013	NMP_mix	Ast, MZ, Vol			1	1	1	0
B21	2013	NMP_mix	Ast (3x)			0			
B23	2013	NMP_mix	Ast, MZ, Vol			3	3	1+2 (two different parents)	0
B24	2013	NMP_mix	Ast (3x)			0			
B26	2013	NMP_mix	Ast, MZ, Vol			1	1	1	0
B27	2013	NMP_mix	Ast (3x)			0			

Table 1 continued.

Bucket ID	Batch	Treatment	Origin of clones	N males	N females	N hatchlings	N genotyped	N within-clone offspring	N outcrossed offspring
B17	2013	MP_single	MZ			0			
B18	2013	MP_single	MZ			>30			
B19	2013	MP_single	MZ			>30			
D069	2011	MP_mix	BN, Vol	53	440	>30			
D096	2011	MP_mix	BN, Vol	82	232	>30			
D141	2011	MP_mix	BN, Vol	142	224	>30			
D202	2011	MP_mix	BN, Vol	109	220	>30			
B22	2013	MP_mix	MZ (4x)			>30	8	0	8
B25	2013	MP_mix	MZ (4x)			>30	8	1	7
B28	2013	MP_mix	MZ (4x)			>30	8	0	8

FIGURES AND FIGURE LEGENDS

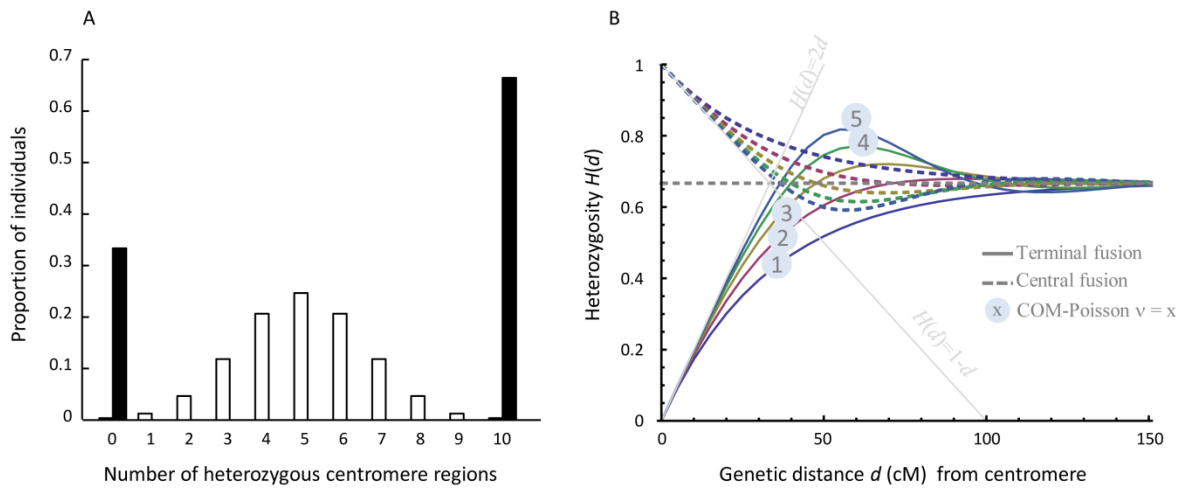
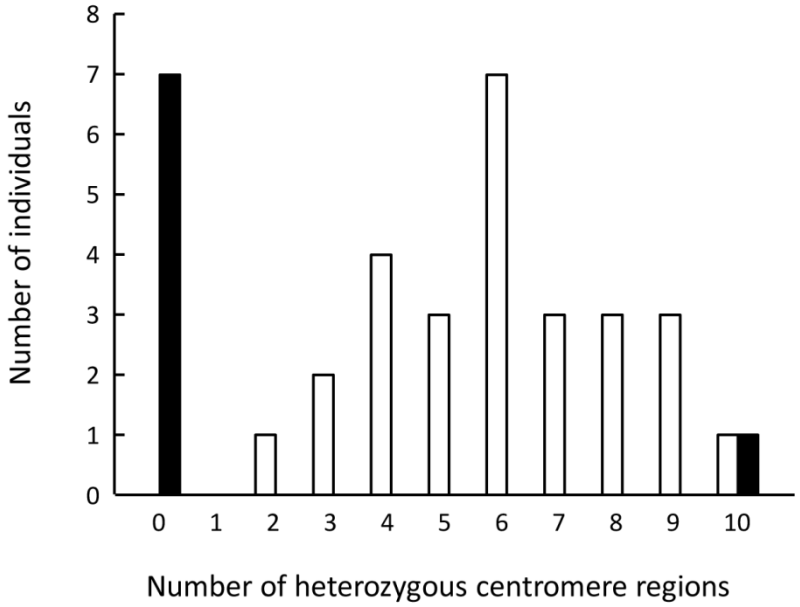
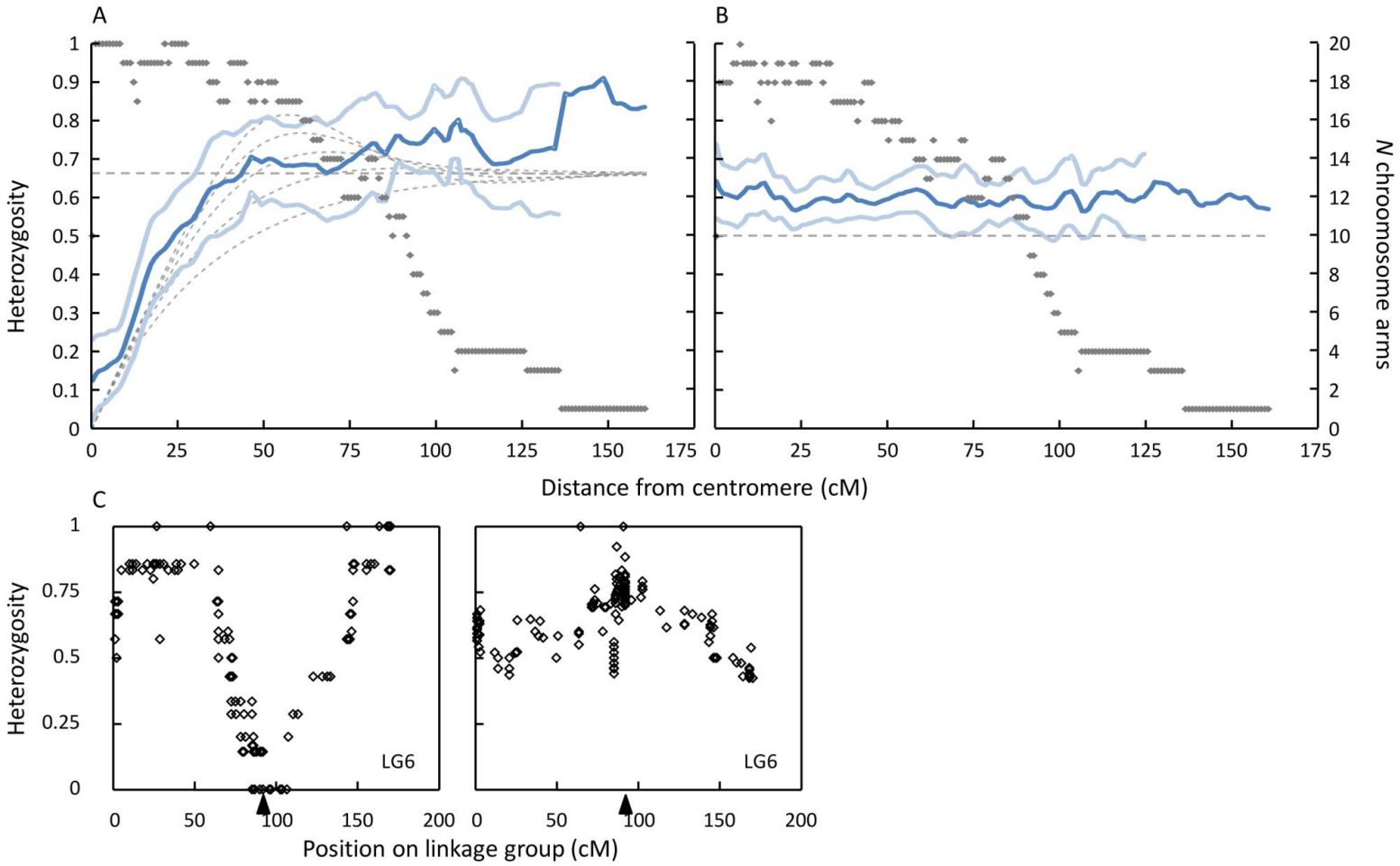


Fig. 1: Expected inter-chromosomal (A) and intra-chromosomal patterns (B) of heterozygosity reduction in automictic offspring. (A) shows the proportion of individuals that retain parental heterozygosity at a given number (out of ten) centromeric regions. Black bars represent automictic offspring, which should always have either zero or ten heterozygous centromeric regions (the relative proportion of individuals with heterozygous vs. homozygous regions depends on the proportion of offspring produced by central vs. terminal fusion, here 2/3 central fusion is assumed). The empty bars represent self-fertilized controls. (B) shows expected offspring heterozygosity as a function of the genetic distance from the centromere under central and terminal fusion and different degrees of crossover interference (Supplement 2). $\nu = 1$ corresponds to no interference, and the two gray lines correspond to complete interference. The dashed line gives the expected heterozygosity for centromere-distal markers (2/3).



673

674 Fig. 2: Observed number of individuals that retained parental heterozygosity at a given
675 number (out of ten) of centromeric regions. Black bars represent offspring of the AST-01-04
676 NMP clone, empty bars offspring of the RM-1-18 MP clone. For LG3 only the region at 90.8
677 cM was considered.



678

679 Fig. 3: Heterozygosity as a function of the distance from the centromere under (A) automixis (terminal fusion only, N = 7 offspring) and (B) self-

680 fertilization (N = 27 offspring). Dark blue lines represent averages across all chromosome arms with N chromosome arms (gray dots) according

to the secondary y-axis. Light blue lines represent the 95% confidence limits, and the dashed lines the expected heterozygosity and asymptotes under different degrees of recombination interference (see Fig. 1). (C) Realized heterozygosity along linkage group 6 (automictic offspring left, self-fertilized offspring right) for illustration. The black triangle shows the presumed centromere position. The patterns of all linkage groups are shown in the supplementary Figure S1. All heterozygosities are expressed in percentage of parental heterozygosity.

SUPPORTING INFORMATION CAPTIONS

Supporting File S1: Assessing the inter-chromosomal pattern when centromere locations are unknown

Supporting File S2: Expected offspring heterozygosity under central vs. terminal fusion

Supporting File S3: Detailed RAD-sequencing protocol and analysis of RAD-sequencing data

File S4: .xlsx file containing the raw and corrected SNP data set for all offspring of the AST-01-04 NMP clone.

File S5: .xlsx file containing the raw and corrected SNP data set for all offspring of the RM-1-18 MP clone.

File S6: .xlsx file containing the genetic map v4.0.1.

Supporting Figure S1: Heterozygosity depending on marker position along each linkage group

Supporting Figure S2: Mapping functions under different degrees of interference

Supporting Table S1: Microsatellite genotypes of parents and offspring

Supporting Table S2: Heterozygosity of microsatellite loci in relation to the distance from the centromere

Supporting Table S3: Number of RAD loci and average heterozygosity per linkage group

Supporting Table S4: The ten most frequently observed segregation patterns

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