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Published in:
Current Genetics

DOI:
10.1007/BF00357164

Link to publication

Citation for published version (APA):

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Download date: 16 Jan 2019
Mating-type differentiation and mate selection in the homothallic
Chlamydomonas monoica

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Received: 7 July 1993 / Accepted: 23 August 1993

Abstract. By using combinations of phenotypically-distinct – but sexually-compatible – mutant strains of C. monoica (zym-1, zym-27, and nit-2) and assaying for zygote genotypes in genetically-mixed mating populations (where gametes of the two parental homothallic strains were present at similar frequencies), we have found that matings occur preferentially between cells of the same genotype. Additional support for an hypothesis of non-random mate selection was provided by using an easily-selectable genetic marker (sup-l) to verify the frequent occurrence of matings between cells of identical genotype in populations where the selectable genotype was present at very low relative frequency (10^-2 or 10^-3) in a mixed mating population. Direct evidence for non-random mate selection was obtained by presenting nitro-gene-starved cells with limiting nitrate to synchronize gametic differentiation in wild-type strains. Under these conditions, the four, eight, or 16 mitotic daughters released from the same mother sporangium often immediately established mating pairs within the group. Thus successive mitotic divisions of a single mother cell yielded progeny of opposite expressed mating-type.

Key words: Chlamydomonas monoica – Homothallism – Mating-type differentiation – Mate selection

Introduction

The unicellular alga C. monoica is homothallic: sexually-compatible mating-types appear within a population derived from a single haploid cell (Strehlow 1929; VanWinkle-Swift and Bauer 1982; Van den Ende et al. 1992). Mating interactions resemble those of the heterothallic species C. eugametos (Musgrave et al. 1985; Van den Ende et al. 1988). Compatible gametes form vis-a-vis pairs with flagellar paralysis of one partner allowing the other to direct the movement of the pair. Although gametes remain walled, cell fusion is accomplished by partial anterior wall lysis and extension of the activated mating structures which establish a cytoplasmic connection (Shi and VanWinkle-Swift, in preparation). Uniparental inheritance of chloroplast genes also suggests that the gametes of C. monoica are equivalent to the opposite mating-types (mt+ and mt-) of heterothallic species (Van Winkle-Swift and Aubert 1983). However, nothing is known about the cellular and genetic mechanisms that give rise to opposite mating-types within a clonal population, i.e. from a single progenitor cell.

There are two necessary conditions for zygote formation in C. monoica: nitrogen starvation and the ability to divide (Van den Ende et al. 1992). Nitrogen-starved cells that are unable to divide do not mate. Thus, simply transferring vegetative cells to nitrogen-free medium (a technique routinely used in other Chlamydomonas species; Sager and Granick 1954; Trainor 1958) has not proven useful for gamete induction in C. monoica. Instead, media best described as nitrogen-limiting (VanWinkle-Swift and Bauer 1982; Van den Ende 1992) promote the differentiation of vegetative cells into mating-competent gametes. Upon transfer to nitrogen-limiting media, cells continue to undergo a few rounds of cell division before mating ensues. It is likely that upon attainment of mating competence, the cells are nitrogen-starved and incapable of further division.

Previous work on zygote formation in this species (Van den Ende et al. 1992) suggests that successful mating is not dependent upon random collisions but that some form of mate selection may be occurring. In particular, increasing the population density during gametogenesis did not result in an increase in zygote yields. The correlation between the potential for continued cell division and increased mating efficiency led to the hypothesis that mitotic daughters selectively establish mating pairs, perhaps initiating effective contact while still within the confines of the mother cell wall – a process we will henceforth refer to as sib-mating.

This hypothesis predicts that within a mixed population of genetically-distinct but sexually-compatible (and
homothallic) strains, mating within strains should be more common than mating between strains. In contrast, assuming an equal frequency of gametes of the two strains, a model invoking random mate selection would predict a 1:1 ratio of homozygous zygotes (from selfing) and heterozygous zygotes (from crossing). Figure 1 illustrates the two alternative hypotheses for mate selection in *C. monoica*. We describe below the application of a variety of phenotypically-distinctive mutant strains to test the hypothesis of non-random mate selection in genetically-mixed but homothallic populations. As predicted, the frequency of heterozygous zygotes produced by matings between the parental genotypes is always less than expected based on a model of random mate selection. Furthermore, we show that a given parental genotype can efficiently produce homozygous zygotes even when present at extremely low relative abundance in a genetically-mixed population. By shifting cells from nitrogen-free medium to nitrogen-limiting medium we have now been able to synchronize the gametogenic divisions and verify – by direct microscopic observation – extensive sib-mate selection.

Materials and methods

**Strains.** The *C. monoica* strains used in this study include the wild-type strains *WT15c* (VanWinkle-Swift and Burruscano 1983) and UTEX 220 (from the University of Texas Algal Culture Collection), a nitrate non-utilizing auxotroph (*nit-2*, unpublished), two recessive lethal zygote-maturation mutants, *zym-1* (VanWinkle-Swift and Bauer 1982) and *zym-27* (Parmelee and VanWinkle-Swift 1983), a germination-defective mating-type-limited strain *mtl-l-1* (carrying a more stringent mutant allele at the *mtl-l* locus described by VanWinkle-Swift and Hahn 1986), and a double mutant carrying the *mtl-l-2* lethal allele and a recessive suppressor, *sup-l*, of this lethality (VanWinkle-Swift et al., submitted).

**Media and culture conditions.** Mutant and wild-type strains are routinely maintained on agar-solidified nitrate-containing Bold's Basal medium (BM; Bischoff and Bold 1963) or on ammonium-containing High Salt medium (HS; Suocka 1960). Asynchronous mixed mating populations containing two genetically- and phenotypically-distinct strains were established in liquid nitrate-limited LPN medium (VanWinkle-Swift and Bauer 1982). Strains are maintained at 20–23°C under continuous cool white fluorescent illumination (30–60 μE m⁻² sec⁻¹).

**Mixed mating populations.** Mixed mating populations for the *zym-1*/*zym-27* or *nit-2*/WT15c combinations were established as follows: each parental strain was suspended in LPN medium to a final cell density of 1 x 10⁶ cells ml⁻¹; 1-ml mixed mating populations were created by mixing 200, 300, 400, 500, 600, 700 or 800 μl of one parental strain with 800, 700, 600, 500, 400, 300, or 200 μl of the other strain. Because the efficiency of gametogenesis varies from strain to strain, or from one experiment to another, a starting ratio of 1:1 vegetative cells of each strain will not necessarily produce a 1:1 ratio of parental gametes. Gamete genotype frequencies can be deduced from the resultant zygote genotype frequencies (see legend to Table 2). Only data from mating populations that were later deduced to have involved approximately-equal gamete genotype frequencies are reported here. Mixed mating populations for the *mtl-l-1*/*mtl-l-2* *sup-l* combination of parental strains were established by suspending vegetative cells of each parental strain in LPN to a final density of 1 x 10⁶ cells ml⁻¹. The *mtl-l-1*/*sup-l* suspension was then diluted 100 fold. To create the 100:1 biased mating culture, 500 μl of the undiluted *mtl-l-2* culture was mixed with 500 μl of the diluted *mtl-l-1* *sup-l* culture. To create the 1000:1 biased mating culture 900 μl of the undiluted *mtl-l-2* culture was mixed with 100 μl of the diluted *mtl-l-2* *sup-l* culture. In analyzing the data from these experiments (see Fig. 3 and Table 2), we assumed that the efficiencies of gametogenesis for the two strains were similar. (Zygote yields obtained in control self-matings of the individual parental cultures indicated mating efficiencies of 13% for *mtl-l-2* and 11% for the *mtl-l-1* *sup-l*). Analysis of these mixed mating populations also rests on assumptions concerning the viability (germination efficiency) of the *mtl-l-2* and *mtl-l-1* *sup-l* homozygotes. Zygote densities were determined by a hemacytometer count for self-matings of the *mtl-l-2* and *mtl-l-2* *sup-l* strains and known numbers of zygotes.
were plated, matured, and induced to germinate following standard procedures (VanWinkle-Swift and Bauer 1982). From 60,000 mtl-1-2 homozygotes plated, six colonies were recovered (germination efficiency = 10^{-4}). From 263 mtl-1-2 sup-1 homozygotes plated, 245 colonies were recovered (germination efficiency = 0.93). For simplicity, in making predictions (see Fig. 3 and Table 2) we have considered the germination efficiency of mtl-1-2 sup-1 to be equivalent to 1.0.

Assessing zygote genotypes and phenotypes. To determine the phenotypes (and genotypes) of zygotes produced in mixed mating populations containing the zym-1 and zym-27 strains, aliquots from 7-day-old LPN cultures were examined by phase-contrast light microscopy to determine zygote morphology (see text and Fig. 2 for further details). From 500 to 1,000 zygotes were scored for each zym-1/ zym-27 mating population analyzed. For mating populations containing the nit-2 and WT15e strains, aliquots from 7-day-old LPN cultures were plated on BM agar, incubated in the dark for 3–5 days and then induced to germinate as described previously (Van-Winkle-Swift and Bauer 1982). The latter procedure results in a color distinction between nit-2 (yellow) and wild-type (green) segregants from meiotic germination of zygotes (see text for further details). For each nit-2/WT15e mating population, 100–200 zygote clones were scored for color after 5–7 days post-germination growth. Viable zygotes produced in mixed mating populations containing the mtl-1-2 and mtl-1-2 sup-1 strains may be derived from rare germination of mtl-1-2 homozygotes (frequency 10^{-4}), rare germination of mtl-1-2 sup-1 heterozygotes (frequency 10^{-4}), or from normal germination of mtl-1-2 sup-1 homozygotes. The zygotes derived from rare germination of mtl-1-2 homozygotes are easily identified by “self-mating” each viable zygote clone and re-testing the second generation of zygotes for germination. To distinguish between mtl-1-2 sup-1 homozygotes and the rare germination of zygotes heterozygous for sup-1, 50 viable zygote clones derived from these populations were subcloned. Twenty-five subclones from each zygote clone were then self-mated and tested for germination. All viable zygote clones previously shown not to be mtl-1-2 homozygotes, proved to be homozygous for both the mtl-1-2 and sup-1 alleles, i.e., no segregation of the unsuppressed mtl-1-2 germination defect was observed.

Synchronization of gametogenesis. Details of the synchronization procedure are provided in the text. Briefly, vegetative cells are first transferred to nitrogen-free medium for 15–24 h in darkness (or under an alternating light/dark regime) to deplete intracellular nitrogen reserves. The starved cells are then shifted to a nitrate-limiting medium for 18–24 h under continuous illumination. At the end of this period the culture is comprised primarily of sporangia (containing four, eight, or 16 mitotic daughters) which hatch synchronously to release mating-competent gametes.

Photographic procedures. Gametogenic sporangia and mating cells were observed at 400 × magnification, and zygotes at 1000 ×, using a Zeiss Axioplan phase-contrast microscope equipped with an Hitachi color video camera (Model KP-C501). Images were viewed on an Hitachi color monitor (Model CT1365VM) and recorded on TDK-Extra High Grade VHS tape using an Hitachi Video Tape Recorder (Model VT-F430A). Images were captured as arrested single frames from the video tape, and were converted to a monochrome image using a Sony Video Graphic printer (Model UP-811).

Results

In mixed mating populations where two genotypes are present at similar frequencies, matings occur more frequently within strains (genotypes) than between strains.

To test the hypothesis that mate selection is non-random in genetically-mixed homothallic populations, we first used two mutant strains carrying zygote-specific lethal markers that alter zygote morphology in distinctive ways. Figure 2a–c compares the morphologies of wild-type, zym-1, and zym-27 homozygous zygotes. The zym-1 and zym-27 alleles are complementary and matings between the two strains yield wild-type heterozygous zygotes (Van Winkle-Swift and Burrascano 1983; VanWinkle-Swift 1989). As shown in Table 1a, the frequency of heterozygotes produced by matings between the two strains was always less than that predicted on the basis of random mate selection. Instead, matings occurred preferentially between gametes of the same genotype.

Similarly, mixed mating populations were prepared using the nitrate non-utilizing nit-2 and the wildtype WT15e strains. After allowing for mating and zygote for-
Table 1. Zygote genotypes derived from mixed mating populations comprised of two homothallic parental genotypes in nearly equal abundance

(1) zym-1, zym-27 mixed mating populations

<table>
<thead>
<tr>
<th>Gamete genotype frequencies</th>
<th>Zygote genotype frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous</td>
</tr>
<tr>
<td></td>
<td>zym-1/zym-1</td>
</tr>
<tr>
<td>0.52</td>
<td>0.50</td>
</tr>
<tr>
<td>0.56</td>
<td>0.49</td>
</tr>
<tr>
<td>0.43</td>
<td>0.36</td>
</tr>
<tr>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>0.46</td>
<td>0.34</td>
</tr>
</tbody>
</table>

(2) nit-2, WT15c mixed mating populations

<table>
<thead>
<tr>
<th>Gamete genotype frequencies</th>
<th>Zygote genotype frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous</td>
</tr>
<tr>
<td></td>
<td>WT15c</td>
</tr>
<tr>
<td>0.53</td>
<td>0.41</td>
</tr>
<tr>
<td>0.56</td>
<td>0.49</td>
</tr>
<tr>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>0.55</td>
<td>0.50</td>
</tr>
<tr>
<td>0.63</td>
<td>0.58</td>
</tr>
</tbody>
</table>

a Relative gamete frequencies are calculated as, for example, \( \frac{[2(\text{no. of zym-1 homozygotes})+(\text{no. of heterozygotes})]}{\text{total no. of zygotes}} \) = original freq. of zym-1 gametes

b Predictions for homozygote and heterozygote frequencies are based on the assumption of random mate selection and are calculated, for example, as follows:

\[
\text{pred. freq. of zym-1 homozygotes} = (\text{freq. zym-1 gametes})^2,
\]

\[
\text{pred. freq. of zym-27 homozygotes} = (\text{freq. zym-27 gametes})^2,
\]

\[
\text{pred. freq. of heterozygotes} = 2 (\text{freq. zym-1 gamete}) (\text{freq. zym-27 gamete}),
\]

or \( 1-(\text{freq. of zym-1 homozygotes} + \text{freq. of zym-27 homozygotes}) \).

The observed zygote genotype frequencies are simply the number of zygotes showing the zym-1, zym-27 or wild-type (heterozygous) zygote phenotype divided by the total number of zygotes observed.

In a genetically mixed homothallic mating population, even an extremely rare genotype mates preferentially with cells of the same genotype.

To increase the stringency of our tests for non-random mate selection, a selectable genetic marker was needed to detect very rare mating events between cells of an uncommon genotype within a background of matings between cells of a more abundant genotype (or crosses between the rare and abundant genotypes). Mixed mating populations were established using cells of a germination defective strain, mtl-1-2, present in a 100- or 1 000-fold excess over those of a strain carrying – in addition to the lethal mtl-1-2 allele – a suppressor of the germination defect, sup-1. All matings in this population thus produce zygotes homozygous for mtl-1-2. Because the suppressor is recessive, matings between the rare mtl-1-2 sup-1 cells and the abundant mtl-1-2 cells do not produce a viable zygote. Only matings between the rare mtl-1-2 sup-1 cells themselves produce zygotes that germinate efficiently. However, the mtl-1-2 lethal allele shows a low level of leakiness such that one in 10 000 zygotes homozygous for mtl-1-2 is viable. Thus, the zygote genotype must be verified by subcloning (see Materials and methods).

Figure 3 shows the ways in which viable zygotes can be produced in these mixed populations according to either a random mating or sib-mating hypothesis for mate selection. The hypothesis of random mate selection predicts that many of the viable zygote clones recovered will be derived from rare germination of the more common.
Random Mate Selection: Expected freq. mating partners with apparent ease - an observation that Table 2, only 1% of the viable zygotes are expected to be homozygous for the population would be expected to be homozygous for genotype from a highly-biased mixed homothallic mating population. At ratio of 100:1, viability from Fig. 3. Predictions for recovery of zygotes homozygous for a rare genotype in highly-biased mixed mating populations. 

Table 2. Recovery of zygotes homozygous for a rare genotype in highly-biased mixed mating populations.

<table>
<thead>
<tr>
<th>Parental genotypes ratios</th>
<th>No. of viable zygotes homozygous for sup-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed (vs total viable)</td>
</tr>
<tr>
<td></td>
<td>RM</td>
</tr>
<tr>
<td>100:1</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>1000:1</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
</tbody>
</table>

*See Fig. 3 for methods for predicting the frequencies of viable zygotes that will be homozygous for mtl-1-2 versus mtl-1-2 sup-1. RM, prediction based on assumption of random mating. SM, predictions based on assumption of sib-mate selection. A and B are separate experiments using independently-isolated alleles at the sup-1 locus that similarly suppress the germination defect of mtl-1-2 strains.*

mtl-1-2 homozygous for mtl-1-2 sup-1 proved upon further analysis (see Materials and methods) to be homozygous for mtl-1-2 and arose as a consequence of the low level of leakiness associated with this lethal mutation. As expected, based on the recessive nature of the sup-1 allele (Van Winkle-Swift et al., submitted), matings between the rare mtl-1-2 sup-1 gametes and the abundant mtl-1-2 gametes (if such matings occurred at all) were not a source of viable zygotes.

**Direct microscopic observation of sib mate selection**

These genetic observations emphasized the need to synchronize gametogenic divisions in order to observe directly whether the hypothesized sib-mate selection was occurring. The routine procedure for genetic analysis in *C. monoica* utilizes a low nitrate medium that promotes asynchronous gamete formation 24–48 h after the transfer of parental vegetative cells to the nitrogen-limiting environment. We report here two alternative protocols that improve synchronization of gamete formation in *C. monoica*:

1. Cells grown in nitrate-limited continuous culture (Van den Ende 1992) are subjected to a 15 h dark period in nitrogen-free Bold’s Basal medium. Nitrate is then added to a final concentration of 0.1 μmoles per 10^5 cells. After 18 h of continuous illumination, the population undergoes synchronous mitotic divisions giving rise to four-, eight- and 16-celled sporangia. A detailed description of this protocol will be presented elsewhere (Van den Ende, submitted). As the daughter cells are released by partial autolysis of the sporangial wall they agglutinate immediately by their flagella. (Mating pairs fuse within 1 h to produce the quadriflagellate zygote which then matures into the thick-walled zygospore.)

2. Vegetative cells maintained on HS agar for a minimum of 14 days are suspended in nitrogen-free Bold’s Basal medium and are placed in an alternating 15 h light/
Fig. 4a–e. Sib-mating in *C. monoica*. Panels are numbered to indicate temporal progression of the gametic interactions. *a* and *b* the first cell released from the mother sporangium has escaped without interacting with its sibs (data not shown). The remaining three siblings show typical gametic flagellar agglutination which assists in the escape of the fourth gamete from the sporangial wall. These gametes can ultimately yield only one mating pair. *c* The first two cells released from the sporangial wall have escaped (data not shown). The third gamete exits and is shown here assisting the fourth gamete to escape the mother cell wall. These two siblings then establish a vis-a-vis mating pair. *d* The first gamete released assists in the release of the second gamete and proceeds toward pair formation via extensive flagellar agglutination with its sibling. This pair ultimately moved away from the sporangium. The third gamete escaped leaving the fourth entrapped within the sporangial wall (data not shown). *e* As hatching progresses, all four gametes (including one still within the sporangial wall) are seen to be interacting via their flagella. After the fourth gamete is pulled free of the wall by its siblings, flagellar agglutination reactions proceed to the establishment to two sib-mating pairs.

9 h dark cycle for 24–72 h. At the dark-to-light transition, cells are recovered by centrifugation, resuspended in LPN medium (0.6 mM nitrate; VanWinkle-Swift and Bauer 1982) to a final density of approximately $5 \times 10^6$ cells ml$^{-1}$, and placed under continuous illumination. Synchronous mitotic divisions to yield four- or eight-celled sponrangia occur 18–21 h later. Upon release of the daughter cells from the mother cell sporangium, immediate flagellar agglutination among siblings often occurs.
Discussion

In mixed mating populations containing genetically-distinct strains, the frequency of mating within strains is often higher than expected if mate selection were random; conversely, matings between strains often occur less frequently than expected. Although the increased frequency of intra-strain mating observed in many populations may be largely a consequence of sib-mating, such inbreeding can also be enhanced by differences in the timing of attainment of mating competence by the two strains. If one strain acquires agglutinability prior to the other, gametes of the precocious strain are likely to have chosen their partners before the other strain attains competence. The problem is exacerbated by the rapid loss of agglutinability in *C. monoica*. Soon after gamete formation (Van den Ende 1992). Gametes must find partners quickly.

The most reasonable explanation for the apparent ease with which very rare genotypes within a population find mating partners of the same genotype is to assume that mating-competent gametes can be produced prior to release of mitotic daughters from the mother sporangial wall, and that these gametes establish effective flagellar agglutination prior to or at the time of sporangial wall lysis. Crowding of gametes within the sporangium makes it unlikely that effective flagellar agglutination could occur there unless cell division positions opposite mating-types such that their flagella can make contact. However, immediate agglutination upon sporangial wall lysis – including flagellar interactions between the first gametes released and their sibs still within the sporangium – has been observed (Fig. 4). In cases where mating competence is not fully established at the time of sporangial wall lysis (e.g., in the absence of light), the subsequent selection of mating partners will occur essentially at random, i.e., gametes will be more likely to select non-sib partners – which may or may not be derived from the same genetic background.

Sib-mating may be common among immotile homothallic organisms. For example, in *Saccharomyces cerevisiae* the proper orientation of two budding sister cells can result in the formation of two zygotes (Hicks and Herskowitz 1976; Nasmyth 1982). However, it is remarkable that mating between sibs is not avoided in a motile haploid organism. The nature of cellular division in *C. monoica* results in the transient retention of fully-differentiated gametes of opposite mating-type within the mother sporangium and promotes non-random, sib-mate selection (via flagellar agglutination) upon lysis of the sporangial wall. Although sib-mating may occur at variable frequencies depending upon environmental conditions that affect the synchronization of gametic divisions and gamete release, the fact that it occurs at all is of tremendous survival value to the species. Although homothallism per se reduces the potential difficulty in finding a mate of opposite sex, sib-mating reduces even further the distances that need to be travelled to find a partner and accentuates the inbreeding associated with homothallism in this species.

This mating strategy – which is essentially independent of cell density – differs from that used by heterothallic species such as *C. eugametos* where gamete encounters are strongly dependent upon the density of opposite mating-type cells in the population (Lewin 1956; Tomson et al. 1986). The potential for sib-mate selection may be particularly advantageous for a species colonizing an empty niche or one whose numbers have been greatly reduced. Nevertheless, the question remains as to why cells which propagate very efficiently vegetatively use sex at all. Many procaryotes and lower eucaryotes (including Chlamydomonas) survive under conditions where vegetative propagation is not possible by sporulation and entrance into dormancy. For Chlamydomonas, then, the more relevant question may be why it uses sex to produce the spore (see Goodenough 1985 for discussion). One might imagine that diploidy per se is of selective value for survival under environmental stress where chromosomal damage and mutation may occur at increased frequency. Diploidy not only allows masking of mutations arising in the spore but, in species where diploidy is achieved by sex and subsequently terminated by meiosis, the potential also exists for recombinational or meiotic repair regardless of how inbred the original mating strategy may have been.

Acknowledgements. This work was supported, in part, by NSF grant 9106008 to K.V.W.S. Technical assistance was provided by Ms. Amy Parks-Ritter and Julie Flynn. We thank Drs. A. Gibor, R. F. Hoekstra, and A. Musgrave for helpful conversations.

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Communicated by R. W. Lee