

Experimental sexual selection in *Chlamydomonas*

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Abstract

Sexual and asexual lines of the unicellular chlorophyte *Chlamydomonas reinhardtii* were propagated for about 100 sexual cycles and 1000 vegetative cycles in contrasted environments, liquid and solid growth media, in order to generate divergent natural and sexual selection. Sexual lines were transferred by many zygotes or by a single zygote in each sexual generation. By the end of the experiment zygote production was in the order sexual mass-transfer > sexual single-zygote > asexual > ancestor. The direct response to sexual selection was large, with zygote production increasing by about two orders of magnitude, mainly because mating had become spontaneous instead of being invoked by nitrogen starvation. Asexual lines became sexually sterilized by the fixation of a single mating type. Sexual selection caused a radical shift in the gender system, with homothallism spreading to high frequency in all sexual lines of this normally heterothallic species. This may have been caused by the transposition of a mating-type gene to an autosome. No substantial degree of environment-specific mating evolved, however, and thus no sexual isolation indicative of incipient speciation. It is possible that selection experiments of this kind are unlikely to induce sexual isolation because mating-type genes evolve in a saltatory fashion.

Introduction

Some characters seem to be exceptions to the general rule that organisms are well adapted to survive and grow in their customary environment. These include a wide range of structures and behaviours expressed in only one sex and involving visual, auditory and chemical signals that seem likely to make it more difficult for individuals to move and feed and more likely for them to be detected by predators. The evolution of such characters is explained by the theory of sexual selection through their manifold effects on obtaining or manipulating sexual partners (Andersson & Iwasa, 1996; Cunningham & Birkhead, 1998). The field has been and continues to be controversial. The mechanisms that are invoked as sources of sexual selection are often more subtle and indirect than those that are usually implicated in natural selection, and whether they have been correctly identified and interpreted is currently a matter for debate

(Cordero & Eberhard, 2003; Kokko *et al.*, 2003). The characters that respond to sexual selection have also been disputed: for example, Willson (1994) and others have interpreted flower structure and pollination in terms of sexual selection, whereas Grant (1995) denied that sexual selection operates at all in plants. Moreover, the apparently exceptional nature of the characters involved has led to the view that sexual selection is a special case of natural selection, so that Arnold (1994), for example, treats sexual selection as a component of fecundity selection. Sexual selection has always been recognized as crucial to the evolution of sexual isolation and species formation (Martin & Hosken 2003), but in more general treatments of evolutionary processes it is often marginalized.

Far from being a special case of natural selection, sexual selection can instead be interpreted as a qualitatively distinct and equally important process, the two kinds of selection together being responsible for causing adaptation in eukaryotes. The eukaryote life cycle consists of two phases: a vegetative phase of growth and reproduction, and a sexual phase of fusion and restitution. Selection causes adaptation in both phases. Natural selection acts through characters such as assimilation that

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affect vegetative growth. Sexual selection acts through characters such as motility that affect sexual fusion. In multicellular eukaryotes, however, this distinction is not always easy to make. The fundamental reason is that sex is almost always associated with reproduction. This is presumably because in a sexual life cycle the only way that an individual can develop as a genetically uniform community of cells is by development from a single sexually produced cell; if sexual processes occurred during growth then every cell in the body would be genetically different. This conflation of sexual and vegetative processes makes it difficult to differentiate between sexual and natural selection. The difficulty is compounded because sexual and vegetative processes often interact. In some cases, gamete character states such as flagellum length in *Drosophila* sperm are directly exposed to selection (Miller & Pitnick, 2002), but in most cases gamete fusion depends on attributes of the gamete-bearing individual. Sexual selection then causes the modification of secondary sexual characters such as colouration, size, song or pheromone production, which provide the subject matter for most investigations of sexual selection. The mechanisms that are responsible are poorly understood, in large part because very little experimental work has been undertaken. There is a tradition of selection experiments in *Drosophila* that has in the past involved characters such as mating speed (e.g. Manning, 1961), with more recent investigations of mate recognition (Blows & Higgin, 2002), sexual conflict (Pitnick *et al.*, 2001) and the interaction of sexual and vegetative components of fitness (Promislow *et al.*, 1998; Holland, 2002). The great bulk of the literature in the field, however, is comparative and speculative.

In unicellular eukaryotes the issues are much simpler because there is always a clear distinction between sex and reproduction. The vegetative individual is the *spore*, which grows to a certain size and then reproduces by division or budding to produce genetically identical offspring. The sexual individual is the *gamete*, a sexual cell that fuses, forming a zygote that sooner or later undergoes genetic reduction and recombination. Natural selection is then competition among spores for resources, whereas sexual selection is competition among gametes for fusion partners. Unicellular organisms lack most of the bizarre and fascinating attributes associated with sexual selection in animals and plants, unfortunately, but the clarity of the distinction between natural and sexual selection provides a good opportunity for experimental studies. This opportunity has not been taken. Natural selection in microbial populations has been the subject of a very large amount of experimental work during the last 15 years (Bennett & Lenski, 1999; Travisano & Rainey, 2000; Kassen, 2002). Most of this work has been carried out with bacteria such as *Escherichia* and *Pseudomonas*, asexual organisms in which the mechanics of adaptation and diversification can be studied without the complications introduced by sexuality. It is perhaps because of the

lack of sexuality of these favourite model systems that sexual selection in microbes has been almost completely neglected by experimentalists, although Da Silva & Bell (1992) used *Chlamydomonas* to show that natural selection and sexual selection act antagonistically. It is not even clear that sexual selection would be very effective. Natural selection is often capable of driving rapid adaptation, such that populations evolve within a few hundred generations the ability to flourish on substrates that were initially incapable of supporting growth. Whether there exists the same opportunity for enhancing sexual fusion has not been demonstrated.

The most fundamental feature of the sexual system, however, are the rules that govern sexual behaviour, and especially sexual fusion. These are expressed by the complementary concepts of gender and species: sexual fusion may occur only between gametes of like species and unlike gender. The central topic for experimental investigations of sexual selection is, therefore, whether, how and to what extent gender and species membership can be altered. The contribution of sexual selection to speciation in animals has been very extensively discussed (Price, 1998; Civetta & Singh, 1999; Wu, 2001) and has generated a large volume of comparative studies (e.g. Sauer, 1996; Carson, 1997). Again, there are very few experimental studies beyond an old *Drosophila* literature reporting the evolution of mating specificity through artificial selection (e.g. Crossley, 1974) or as the indirect response to divergent natural selection (e.g. Kiliars *et al.*, 1980). In particular, no study has yet reported any fundamental change in fusion rules, involving the appearance of a new gender system or a new species, as the result of selection in the laboratory. This should not be hopelessly impracticable, because genes related to sex and gender often evolve very rapidly (Civetta & Singh, 1998; Wyckoff *et al.*, 2000; Parsch *et al.*, 2001). Rice & Hostert (1993) reviewed the experimental literature and suggested that sexual isolation was most likely to evolve when divergent natural selection and sexual selection were applied simultaneously. I have not been able to trace any subsequent studies in which this approach was followed, however.

The genetic basis of sexual compatibility may be very complex in multicellular organisms, but is often quite simple in eukaryotic microbes and has been analysed in great detail for certain fungi (Kues & Casselton, 1992; Kronstad & Staben, 1997; Casselton, 2002). It has also been thoroughly elucidated in *Chlamydomonas reinhardtii*, a unicellular chlorophyte that has been extensively used to study photosynthesis, flagellar motility and gender. This is a heterothallic species with two morphologically similar gamete genders that are referred to as the *plus* and *minus* mating types, *mt+* and *mt-*. The regulation of sexual behaviour was described by Sager & Granick (1954). Cells differentiate as gametes when starved for nitrogen, and express mating-type-specific flagellar

agglutinins. These cause adhesion of gametes of unlike mating type, which proceeds to pairwise fusion mediated by a second agglutinin system. The zygote is a resting cell resistant to drying and freezing that undergoes a conventional meiosis to liberate recombinant haploid spores when light and mineral nutrients become available. The proteins that regulate gamete behaviour are encoded by idiomorphic mating type regions about 2000 kb in extent that occupy corresponding regions of chromosome VI and each bear several genes, some specific to one mating type and others not. These regions have been sequenced and interpreted by Ferris & Goodenough (1997) and Ferris *et al.* (2002). The *mt*-region includes *mid*, encoding a regulatory protein that determines differentiation as a minus gamete, and *sad1*, which causes expression of a sexual adhesion agglutinin in the presence of the Mid gene product. The *mt*+ region includes *fus1*, encoding the plus agglutinin. These genes are chiefly responsible for gamete behaviour, although autosomal genes, regulated by mating-type genes, are also involved. The simplicity of the sexual system, the ease with which it can be manipulated, and the degree of understanding of the genes involved, combine to make *Chlamydomonas reinhardtii* a good model system for experimental investigations of sexual selection.

In *Chlamydomonas*, gametogenesis and fusion are normally induced by nitrogen starvation in liquid medium. I serendipitously observed zygote formation on solid medium among old plates of an unrelated study. This provided an opportunity to test the suggestion of Rice & Hostert (1993) by propagating sets of lines that grew and mated either in liquid or on solid medium. Although chemically identical except for the addition of agar, conditions of growth are quite different, the genetic correlation of growth in liquid and solid medium among arbitrary wild-type isolates being low (G. Bell, unpublished observations). The conditions for fusion might also be expected to differ, since the flagellar agglutinin systems essential for efficient mating in liquid medium might not be required, or might function differently, for immobilized cells on solid medium. An experiment was therefore devised that would estimate the quantitative response of mating to intense sexual selection, identify any modification of gender, and evaluate the extent of sexual isolation.

Materials and methods

Base population

The base population descends from the cross ($A \times [B \times (B \times C)]$) made by Clifford Zeyl in October 1992, where A is CC-253 *nit1-305 mt*-, a nitroguanisine-derived mutant of CC-1640 wild-type *mt*-, B is CC-1952 wild-type *mt*- and C is CC-2343 wild-type *mt*+. All three are standard laboratory strains of *C. reinhardtii* that are completely interfertile. This population of full sibs was

propagated for 16 sexual cycles in nitrate-minimal medium (Bold's; for standard techniques see Harris, 1989). It mated vigorously in liquid medium under conventional conditions of nitrogen deprivation, but was chosen as the base population because scattered zygotes were observed in cultures grown on solid medium. Two independent replicates of the base population were stored on agar slants in dim light throughout the experiment.

Selection lines

Two sets of selection lines were established, one cultured in liquid minimal medium (Bold's) and the other on solid minimal medium (Bold's and 1.5% agar). In each case, 12 lines were set up:

- Sexual mass-transfer*: four obligately sexual mass selection lines, propagated from many zygotes at each transfer. At least 100 zygotes were transferred on each occasion, usually many more. Unmated vegetative cells are killed by chloroform vapour; only zygotes are transferred.
- Sexual single-zygote*: four obligately sexual isolate selection lines, propagated from a single randomly-chosen zygote at each transfer.
- Unselected*: two optionally sexual mass selection lines, in which mating is permitted but vegetative cells are not killed at transfer.
- Asexual*: two asexual mass selection lines, in which gametogenesis is not induced and mating does not occur.

The experiment was initiated on 5–9 September 1997 and suspended for assay in November 2002. During this time the liquid lines went through 113 sexual cycles and the solid lines through 72 sexual cycles. The number of vegetative divisions in each line is roughly ten times as great. The general course of the experiment is illustrated in Fig. 1.

Liquid culture

The liquid culture lines were at first transferred using conventional protocols. The vegetative cultures were grown in Erlenmeyer flasks holding 300 mL of sterile medium and bubbled with sterile air under ordinary fluorescent light on open shelves at ambient temperature. After 7 days a 40-mL sample was extracted and centrifuged twice, with the pellet being resuspended in nitrogen-free medium. The population was then allowed to mate for 2 h, after which a 250- μ L sample was spread on each of two agar plates. These were placed in a dark drawer for 4–5 days to permit zygote maturation, then exposed to chloroform vapour for 45 s to kill unmated cells. The zygotes were then placed in the light, where they germinated and the spores were allowed to proliferate for 3–4 days. The plate was then respread by loop and used to inoculate a fresh flask of liquid medium. This

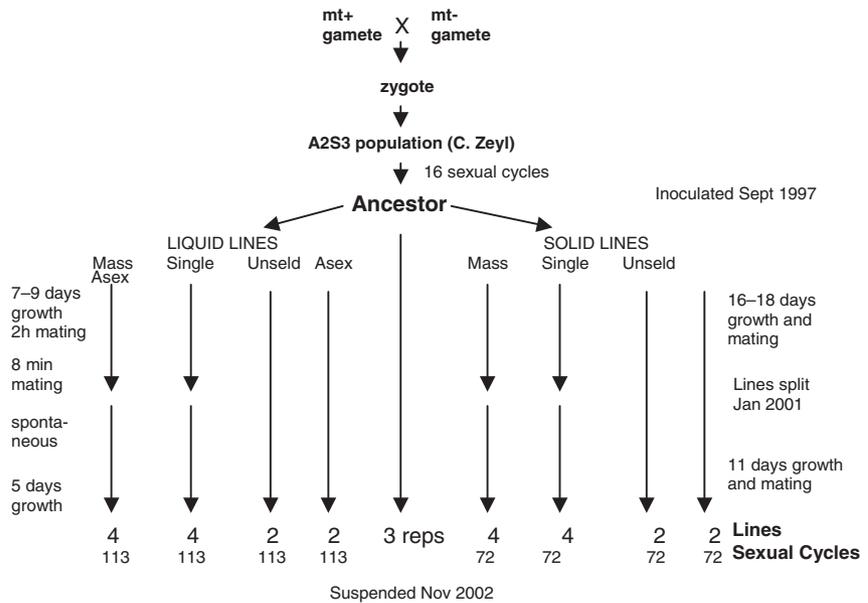


Fig. 1 The general conduct of the experiment.

basic procedure was modified for the isolate lines (no respreading, single zygote colony used to inoculate flask) and the optionally sexual (no chloroforming) and asexual (no nitrogen starvation, no chloroforming) lines. The time allowed for mating was reduced during the course of the first 3 years of the experiment from 2 h to 8 min in order to intensify sexual selection for rapid gamete fusion. Thereafter samples were spread directly from the flask, with no nitrogen starvation, so that only zygotes formed by spontaneous mating during culture growth would survive transfer.

Solid culture

The solid culture lines were grown on agar under continuous fluorescent illumination on the same shelves as the liquid lines for about 2 weeks, at which time zygotes were usually visible. New plates were then respread from areas of the old plate where zygotes could be seen, and placed in the dark for 4–5 days. They were then chloroformed and placed in the light for germination and proliferation. New plates were then spread from all colonies (sexual mass-selection lines) or a single random colony (sexual single-zygote lines), taking care to mix the cells thoroughly. Two lines were treated in the same way, but without chloroforming (unselected lines) and two were transferred directly, without dark incubation or chloroforming (asexual lines). These treatments are not completely equivalent to those imposed in liquid medium, because of the inability to impose a strict regime of nitrogen starvation, but in practice seem to achieve the same result, with spontaneous zygote production soon becoming imperceptible in the Unselected and asexual lines.

Yellow marker

The ancestor and all selection lines grow as yellow colonies when cultured in acetate-supplemented solid medium in the dark. This phenotype is commonly encountered in *Chlamydomonas* and is caused by an accumulation of protochlorophyllide following failure of the final step in chlorophyll biosynthesis in the dark; such strains grow as normal green cultures in the light. To investigate its genetic basis, one of the liquid and one of the solid mass selection lines were crossed with *mt+* isolates (CC-1010, CC-2343, CC-2932, CC-2936) and three *mt-* isolates (CC-1009, CC-2931, CC-2342). After zygote germination, individual zygote colonies were restreaked onto acetate-supplemented agar and cultured for 14 days in the dark. The results were as follows.

- Mating within the liquid selection line or within the solid selection line gave yellow colonies exclusively (in all the very many plates cultured from all the selection lines, no reversion to green in the dark has ever been observed).
- Matings between either selection line and the base population gave exclusively yellow colonies.
- Crosses of either selection line with any *mt-* tester gave exclusively yellow colonies.
- Crosses of either selection line with an *mt+* tester usually gave either completely green or completely yellow colonies. The exceptions were two crosses involving CC-2932, one with each selection line, which gave some zygotes that produced both green and yellow colonies.

These results are consistent with a nonreverting mutation in the chloroplast genome, which is usually transmitted by the *mt+* parent; in a few per cent of

matings the genome is transmitted biparentally or by the *mt*⁻ parent. Thus, mixing a selection line with a *mt*⁺ tester will give some matings between *mt*⁻ and *mt*⁺ from the selection line, yielding all yellow progeny, and others between *mt*⁻ from the selection line and the *mt*⁺ tester, yielding all green colonies.

Assay procedure

To evaluate the sexual behaviour of the lines at the end of the experiment, each was cultured together with a roughly equal quantity of a wild-type *C. reinhardtii* strain. The strains used as testers were CC-2932 *mt*⁺ and CC-2936 *mt*⁺, chosen because they grow vigorously as green colonies on acetate-supplemented solid medium in the dark. The principle of the assay is that when a *mt*⁺ tester is cultured with a selection line, the zygotes produced that yield green colonies in the dark are from matings between the tester and *mt*⁻ gametes in the selection line, whereas yellow colonies are from matings between *mt*⁺ and *mt*⁻ gametes within the line. Thus, when a sample from a mixed population is spread on a plate, chloroformed, and incubated in the dark, the number of the colonies that are formed is an estimate of the overall intensity of mating, and the colour of these colonies is an estimate of the fraction of matings that occur between *mt*⁺ and *mt*⁻ gametes from the selection line, rather than with the *mt*⁺ tester. These assays have three potential sources of experimental error. First, the number of zygotes formed will to some extent reflect the initial density of vegetative cells, and thus of gametes. To minimize this effect, each assay was conducted over a complete growth cycle, being inoculated with 25 μ L each of selection line and tester from preinoculation cultures grown for 2 days. Initial cell density is thus similar and very low, and increases through the assay period; what is measured is the overall yield of zygotes during this period. Secondly, zygotes tend to clump, so that a single colony may represent more than one zygote. We confirmed visually that zygotes were scattered singly on the plates after chloroforming, and recorded few colonies of mixed colour, so this did not represent a substantial source of error. Finally, the repeated exposure to chloroform might have provoked the evolution of chloroform resistance in the sexual lines, leading to an overestimate of their zygote production. This possibility has not been investigated directly, but the unselected testers produce abundant crops of zygotes after chloroforming.

To test the liquid lines, the mixtures were cultured in 3 mL of liquid medium on 24-well microtitre plates. A sample of 200 μ L was withdrawn every day for 5 days, spread on acetate-supplemented agar, chloroformed and incubated in the dark for about 10 days. To test the solid lines, mixtures were spread on minimal agar and sampled by loop five times during a growth period of 14 days, chloroforming after transferring the samples to acetate-supplemented plates for incubation in the dark. In both

cases, two replicate plates for each tester were prepared on each sampling date. Two independently maintained cultures of the base population were included in each assay. The testers were cultured alone to detect colony survival in the absence of mating, and with wild-type testers of opposite mating type (CC-2342 *mt*⁻ and CC-2931 *mt*⁻) to check for reliability of green colony colour. The entire assay procedure was attempted twice. In the first assay, the solid lines were not scored because many of the plates were contaminated by a fungus, so I report two complete assays for the liquid selection lines and one for the solid lines. A total of 41 632 zygotes were scored from 2200 matings. A single colony was recorded from pure cultures of the *mt*⁺ testers, showing a very low rate of false positives. In mixtures of *mt*⁺ and *mt*⁻ testers, yellow colonies represented two of 238 in the liquid assays and five of 2801 in the solid assay, showing misidentification of mating between selection lines and testers of about 1% or less.

Experimental matings

To investigate sexual isolation directly, we scored mating within and among the selection lines. The assays using standard testers had identified one Liquid selection line (LM3) and two Solid selection lines (SM1 and SM4) as being especially prolific maters in the environment of selection. Twelve spores were isolated from LM3 and crossed in all pairwise combinations in liquid medium, to yield two pairs of spores of different mating type which gave strong and consistent mating reactions. Six zygotes from each of SM1 and SM3 were restreaked to isolate random spores and two spores of opposite mating type selected from one zygote for each line on the basis of mating on solid medium. This yielded four Liquid and four Solid testers for investigating mating reactions within and among lines. Screens for mating reactions were carried out by isolating spores from a selection line, expanding them and then adding the appropriate testers in liquid or solid medium. The strength of mating in liquid medium was scored by two methods. First, a 200- μ L sample from the surface was spotted onto agar after 1 h, stored in the dark for 4 days, chloroformed and germinated in the light; the number of zygotes can then be counted. Secondly, cultures were scored visually for the formation of a zygote mat after 24 h. The strength of mating in solid medium was assessed by spotting out isolates opposite a series of all four testers and scoring the intensity of bands visually after 10 days, after the colonies had made contact.

Results

Novel phenotypes

After about 3 years of propagation, dark green clumps and strands appeared after 4–5 days of culture in the



Fig. 2 'Banded' phenotype appearing in Sexual Mass-selection lines on solid medium after about 50 sexual cycles.

liquid sexual lines. These were found to be zygotes, showing that gamete formation and fusion were taking place within the liquid culture. After nearly four years of culture, a striking new phenotype was observed in the solid sexual lines; short dark lines forming an incomplete meshwork over much of the surface of the plate (Fig. 2). On investigation, it was found that a band of zygotes was being formed at the junction of colonies of different mating type. The phenotype varies in expression among lines, and is usually intensified by storage in dim light for 2 days or so. These novel phenotypes herald the evolution of enhanced spontaneous mating in the lines.

Direct response to sexual selection

Zygote production by liquid selection lines mating in liquid medium, and by solid selection lines mating on solid medium, is shown in Fig. 3. Log-transformed values were analysed using the among-line variance to test effects, where sexual mass, sexual single-zygote, unselected, asexual and ancestral are the treatments. Unselected lines became fixed for mating type 1–2 years after the beginning of the experiment, and when assessed periodically never produced zygotes (data not shown). Consequently, they have been pooled with the asexual lines. In both liquid assays there is significant added variance associated with treatment ($F_{4,9} = 19.4$, $P < 0.001$ for the first assay; $F_{4,9} = 8.1$, $P < 0.005$ for the second assay). Pairwise comparisons suggest that the sexual mass-transfer lines exceeded the ancestral population ($F_{1,4} = 16.9$, $P < 0.025$ for the first assay; $F_{1,4} =$

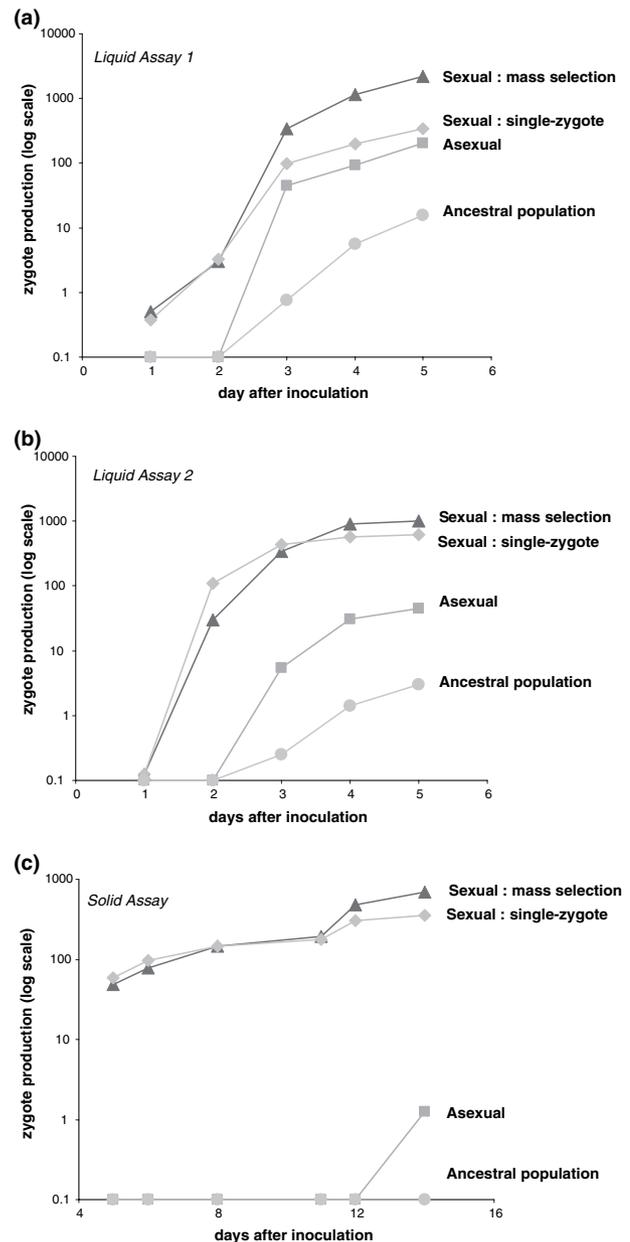


Fig. 3 The direct response to sexual selection. The lines show the overall (total) cumulative mean zygote production per line in sexual mass-transfer, sexual single-zygote, asexual and ancestral lines as a function of days since inoculation. (a) Liquid mating, first assay. (b) Liquid mating, second assay. (c) Solid mating.

21.8, $P \approx 0.01$ for the second assay), the asexual lines ($F_{1,4} = 12.3$, $P \approx 0.025$ for the first assay; $F_{1,4} = 10.3$, $P \approx 0.03$ for the second assay) and the sexual single-zygote lines ($F_{1,4} = 32.2$, $P < 0.001$ for the first assay; $F_{1,4} = 5.5$, $P \approx 0.06$ for the second assay). In the solid assay the analysis is less conclusive because the variance

among the sexual mass lines was greater: two lines produced very many zygotes whereas the other two were much less productive. Conventional significance is thereby difficult to demonstrate although hardly any zygotes were produced by the asexual or the ancestral lines. The overall effect was barely significant ($F_{4,9} = 3.9$, $P < 0.05$). The sexual mass-transfer lines exceeded the ancestor ($F_{1,4} = 12.0$, $P \approx 0.025$) and perhaps the asexual lines ($F_{1,4} = 7.7$, $P \approx 0.05$), but not the sexual single-zygote lines ($F_{1,4} = 1.9$, $P \approx 0.25$). In all three assays the order of zygote production is the same: sexual mass-transfer > sexual single-zygote > asexual > ancestor. The three independent assays can therefore be combined to yield a combined estimate for the overall treatment effect of $\chi^2 = 30.4$, d.f. = 6, $P < 0.001$. By the same procedure, the sexual mass-transfer lines exceed the ancestor ($\chi^2 = 24.0$, $P < 0.001$), the asexual lines ($\chi^2 = 20.4$, $P < 0.005$) and the sexual single-zygote lines ($\chi^2 = 22.2$, $P \approx 0.001$).

Fixation of mating type in asexual lines

Mating with the testers isolated from the selection lines revealed only a single mating type in all asexual and unselected lines, and all are presumed to be fixed for mating type (six for *mt-* and two for *mt+*). Previous assays had shown that seven of eight lines were fixed within a year of the beginning of the experiment, with the remaining line fixing in the following year.

Mating specificity

Combining both liquid assays, 53 of 236 (22.5%) zygotes produced by the ancestor represented mating between gametes from the ancestral population, rather than mating between an ancestral *mt-* gamete and the *mt+* tester, as evaluated by colony colour. In the liquid mass-transfer lines, 11 744 of 15 052 (78.0%) matings occurred between gametes from the selection lines. A similar excess was observed in the sexual single-zygote lines, with 3641 of 3895 (93.5%) within-line matings. In the asexual lines, by contrast, only 46 of 791 (5.8%) zygotes were the result of within-line mating. In the solid assay, no zygotes were produced by the ancestor. The frequency of within-line mating was 5368 of 6873 (78.1%) for the sexual mass-transfer lines and 858 of 987 (86.9%) for the sexual single-zygote lines. Only eight zygotes were produced by the asexual lines, all from mating with the tester. Consequently, when the frequency of within-line mating is plotted against the total production of zygotes, the ancestral and asexual lines fall to the lower left of the diagram, with low productivity and low specificity, whereas the sexual lines fall to the upper right, with high productivity and high specificity (Fig. 4). The frequency of within-line mating tended to fall with time in both liquid assays, whereas it remained constant in the solid assay (Fig. 5).

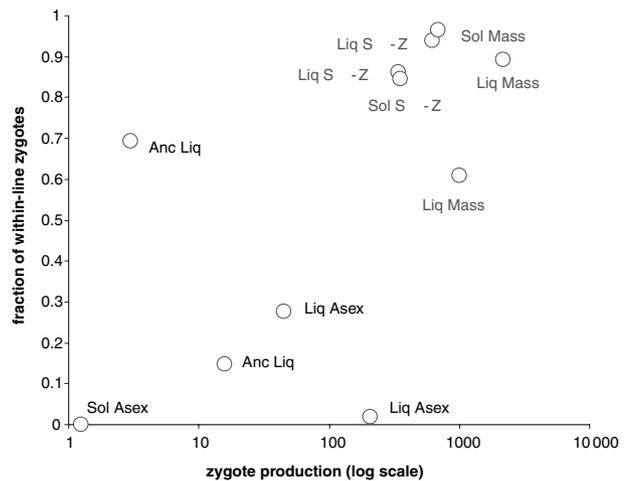


Fig. 4 Within-line mating. Line totals for overall (total) zygote production and the fraction of zygotes produced by mating within the line, rather than with the tester, in the environment of selection.

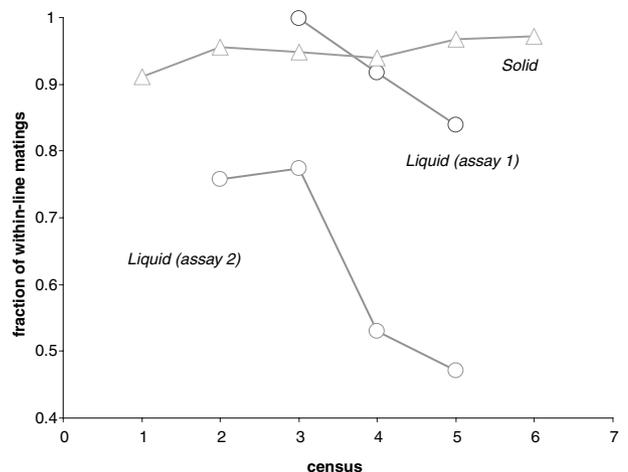


Fig. 5 Within-line mating as a function of time since inoculation. 'Census' indicates the consecutive assays, extending over 5 days for liquid and 14 days for solid cultures.

Spread of homothallism in sexual lines

Sexual lines comprised a mixture of mating types. Many spores isolated from these lines, however, showed a mating reaction when mixed with either tester. Culturing these spores clonally, without adding a tester, gave an equally strong mating reaction. Spores that mated only with testers of a single mating type never showed a mating reaction when cultured clonally. Consequently, the sexual selection lines comprise a mixture of homothallic and heterothallic genotypes ('homothallic' refers to the ability of gametes from the same clone to

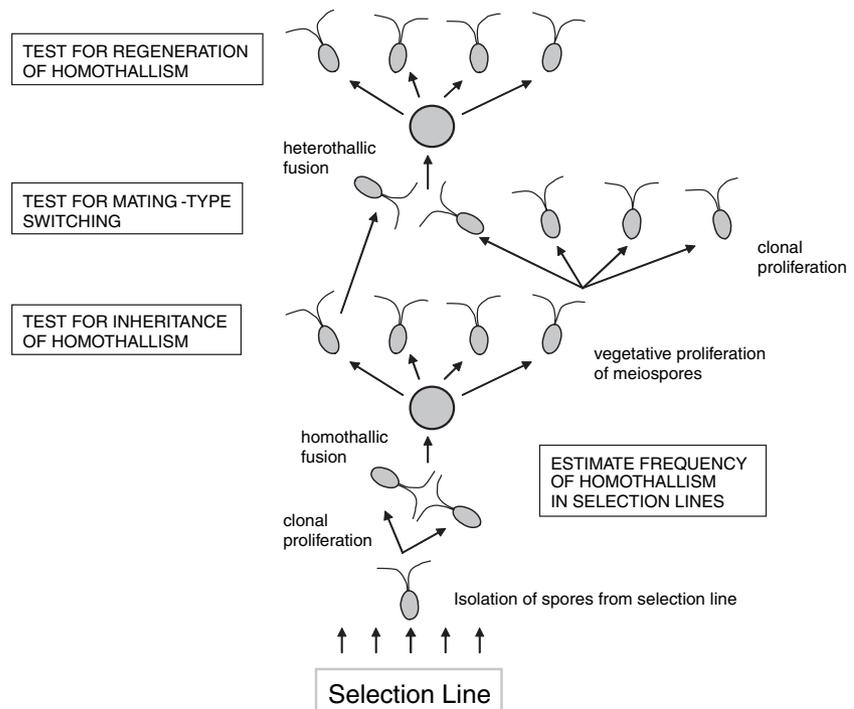


Fig. 6 Investigation of novel homothallism in the selection lines.

mate with one another, in contrast to 'heterothallic' systems where gametes mate only if they have different mating type and thus come from different clones). A series of experiments were devised to evaluate the frequency and the properties of the novel homothallic types (Fig. 6). Homothallism was discovered in the base population, where four of 120 (3.3%) spores tested showed weak homothallic reactions, with few zygotes produced. Homothallism could not be detected in the liquid and solid asexual lines (0 of 160 spores tested). Homothallism associated with strong clonal mating reactions and abundant zygote production was present at substantial frequencies in all sexual lines, solid and liquid. The overall frequencies of homothallic spores obtained were 51 of 160 (31.9%) in liquid mass-transfer; 87 of 160 (54.4%) in solid mass-transfer, 89 of 160 (55.6%) in liquid single-zygote and 106 of 160 (66.3%) in solid single-zygote lines, amounting to 333 of 640 (52.0%) in total.

Sexual phenotypes from homothallic matings

The transmission of homothallism was investigated by obtaining 20 zygotes from homothallic matings in one liquid mass-transfer and one solid single-zygote selection line. These zygotes were germinated and 20 random spores isolated and scored from each; some did not grow or did not mate. Seventy-nine of 393 from the liquid line and 71 of 358 from the solid line were homothallic. Thus, about 20% of the meiospores germinating from

homothallically produced zygotes are themselves homothallic, the remainder being a more or less equal mixture of *mt+* and *mt-* heterothallic spores.

Loss of homothallism during vegetative propagation

The spores germinating from homothallically produced zygotes were isolated and cultured clonally for a complete growth cycle of about 10 divisions, then re-typed in order to detect mating-type switching. All *mt+* spores (229 spores from 13 zygotes) and all *mt-* spores (236 spores from 13 zygotes) tested retained their gender as gametes. There was therefore no sign of switching of heterothallic mating type. Of the 232 initially homothallic spores tested, 47 remained homothallic, 115 were *mt+* and 70 were *mt-*. In 12 of 13 cases, the spores from a given homothallically produced zygote gave rise, after vegetative propagation, to a minority of homothallic spores and a majority of heterothallic spores belonging to the same mating type. The exception produced about equal numbers of both heterothallic mating types beside some homothallic spores.

Sexual phenotypes from derived heterothallic matings

A sample of the *mt+* and *mt-* heterothallic spores obtained from this scheme were then mated with one another. Sixteen crosses all produced a mixture of

homothallic, *mt+* heterothallic and *mt-* heterothallic progeny. The overall frequencies were 58 of 245 (23.6%) homothallic, 92 of 245 (37.4%) *mt+* and 95 of 245 (38.6%) *mt-*.

Indirect response to sexual selection

The indirect response to sexual selection is reflected through zygote production by the solid selection lines mated in liquid medium, and by the liquid selection lines mated on solid medium. This is mainly of interest in evaluating sexual specificity. If the high frequency of within-line mating in the environment of selection were caused by the tendency of *mt-* gametes from a selection treatment to fuse preferentially with *mt+* gametes from the same selection treatment, rather than with the *mt+* tester, through sexual behaviour specific to the environment of selection, then it should not be expressed in the other environment. Consequently, sexual lines should map to the lower left of the specificity-productivity diagram. When the indirect response is plotted in this way, it clearly falsifies the hypothesis: liquid lines mating on solid medium, or solid lines mating in liquid medium, show the same pattern as the direct response (Fig. 7).

Screen for environment-specific mating

One thousand four hundred spores were isolated from the Liquid and Solid sexual lines and screened for failure to mate in the other environment. Those identified were tested in the environment of selection, to confirm that

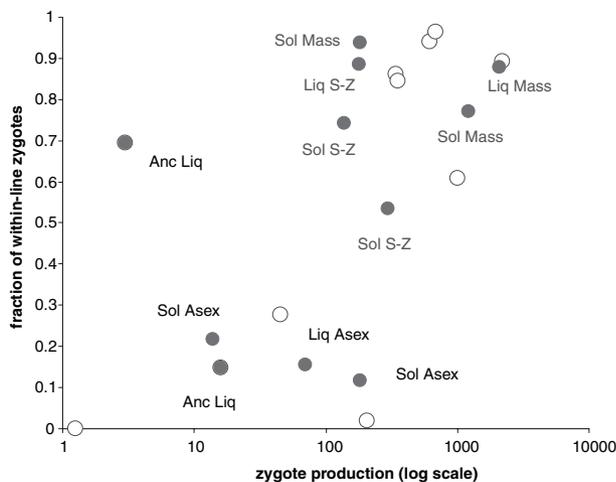


Fig. 7 The indirect response to sexual selection. Line totals for overall (total) zygote production and the fraction of zygotes produced by mating within the line, in the environment other than that of selection. The indirect response is shown by filled circles and can be compared with the direct response, shown as open circles as in Fig. 2.

they could still mate, and then re-screened. No isolates passed the second test; I could find no genotypes able to mate in the environment of selection but unable to mate in the other environment.

Discussion

The spore/gamete switch was altered by selection

The main switch point in the development of *Chlamydomonas* is the decision to differentiate as a zoospore or a gamete. The zoospore enters the vegetative cycle and is specialized for growth and reproduction; the gamete enters the sexual cycle and is specialized for fusion. This fundamental switch is normally regulated by nitrogen availability: nitrogen sufficiency leads to vegetative differentiation, nitrogen depletion to sexual differentiation. Under intense, long-term sexual selection this signal is reset or lost, and gamete fusion occurs spontaneously early in culture growth before any substantial nitrogen depletion has occurred. The most extreme liquid mass-transfer lines now have the shortest outcrossed sexual cycle of any known organism, about 5 days from zygote to zygote.

Spontaneous gamete development and fusion is associated with an increase in zygote production of about two orders of magnitude relative to the ancestor. Asexual lines generally produced somewhat more zygotes than the ancestor, perhaps through some degree of general adaptation to laboratory conditions of growth. They produce far fewer zygotes than the sexual lines, however, showing that enhanced mating was caused by sexual selection rather than by an indirect response to natural selection. During the course of the experiment, failure to mate was equally lethal in mass-transfer and single-zygote selection lines, but selection will be somewhat less effective in single-zygote lines because the random choice of a single zygote is expected to cause the accumulation of slightly deleterious mutations, including those that retard gamete development or reduce gamete performance. The response of the mass-transfer lines is therefore attributable to selection rather than to some physiological consequence of sexuality in preserving the vigour of progeny. The ranking of lines in terms of zygote production in the environment of selection as sexual mass-transfer > sexual single-zygote > asexual > ancestor can thus be reasonably interpreted as the outcome of sexual selection. The magnitude of the response that was obtained suggests that there is a large opportunity for sexual selection, with gamete fusion being regulated far below its maximal level, even in populations known to be sexually competent, such as the base population used here. The most likely reason for this is that sex greatly retards vegetative reproduction, simply because the sexual cycle occupies a considerable period of time during which reproduction cannot occur.

Asexual lines become sexually sterilized through mating type fixation

The asexual and the unselected lines each became fixed for a single mating type quite early in the history of the experiment. This is very unlikely to have been the result of drift or sampling, as the number of cells transferred is in excess of 10^5 . It was not caused by an inherent competitive advantage of one mating type, as that mating type would have been fixed in all lines. It is most plausibly attributable to a selective sweep by some mutation conferring greater fitness in the conditions of culture, arising on an arbitrary mating type background and thereby carrying this mating type with it as it spread through the population. The fixation of one mating type, of course, precludes mating in a heterothallic population. This process of sexual sterilization, which seems to occur so rapidly and predictably in laboratory cultures, might be an important constraint on the evolution of heterothallic sexual systems in natural populations.

The gender system evolved from heterothallic to homothallic

The most dramatic outcome of sexual selection was the replacement of the classical heterothallic system of sex determination in *C. reinhardtii* by a largely homothallic system. Traces of homothallism can sometimes be found in *C. reinhardtii* cultures. Even in the original CC-1010 *mt+* wild-type, prolonged observation will reveal occasional homothallic fusions, although these are often irregular and I have never succeeded in obtaining zygotes from them. Other species of *Chlamydomonas* are perennially homothallic.

Several genera of ascomycetes contain both homothallic and heterothallic species. In many cases homothallism is associated with loss of one of the two mating type loci in bipolar species, but in *Neurospora terricola* both mating types loci are present in the same mycelium (Glass *et al.*, 1990). Homothallism arises from heterothallism in *Cochliobolus* by an unequal cross-over that effectively fuses the two mating-type idiomorphs (Turgeon, 1998). In these cases, homothallism is stable and may result in sexual isolation.

The simplest explanation for the spread of homothallism through sexual selection is that homothallism increases the rate of fusion because any other gamete is a potential fusion partner. This does not seem very plausible, however: in liquid culture the cells are highly motile and each will encounter dozens of others within a few minutes. Another possible mechanism of selection is that excess gametes are lethal unless homothallic. In the population as a whole, or in the small clump of gametes formed by agglutination, one mating type is likely to be somewhat more frequent than the other. After all mating pairs have been formed, therefore, there will be a

residuum of unmated gametes of the majority mating type. In principle, this effect could be substantial. If heterothallic gametes always agglutinate in groups of four, half the clumps will include two unmateable *mt+* or *mt-* gametes, assuming that the only constraint is that each clump must include at least one member of each mating type. The probability that a random heterothallic gamete will succeed in fusing is then only two-thirds the corresponding probability for a homothallic gamete. In larger clumps, or in the population as a whole, the advantage of homothallism is likely to be much smaller, however.

In a strictly heterothallic population, a mutation that removed the regulation of gametogenesis by nitrogen would necessarily occur in a cell of one or the other mating type. It would fail to lead to spontaneous mating, because no gametes of the other mating type would be available, and it would not tend to spread. The same mutation occurring in a homothallic cell, however, would create a sexually compatible clone that would mate spontaneously. Strong selection for zygote production early in culture growth would in this way create positively frequency-dependent selection for a homothallic type, which would spread rapidly. This seems to provide a straightforward mechanism for the evolution of homothallism as the indirect response to sexual selection for spontaneous mating.

The overall frequency of homothallism was greater in solid than in liquid selection lines, and also more frequent in single-zygote than in mass-transfer lines. Thus, homothallism might evolve more readily when there are spatial or genetic constraints on mating. It is possible to imagine simple mechanisms that would act in this way. For example, heterothallic colonies growing on solid medium will be unable to mate if their neighbours happen to be of the same mating type, whereas cells in liquid medium can swim until they encounter a sexually compatible partner. The experiment was not set up to investigate this hypothesis, however, which must remain speculative until a more systematic attempt to test it has been made.

Homothallism is genetically unstable

The sexual phenotypes appearing in the progeny of matings are at first sight difficult to interpret. Homothallism is an elusive phenotype that is continuously lost from vegetative cultures, yet can be restored by heterothallic mating. U. Goodenough (personal communication) has suggested that this behaviour is strongly reminiscent of strain CC-421 of *C. reinhardtii*, described by Ferris & Goodenough (1997), in which a copy of the *mid* gene has been transposed to an autosome. When this copy is transferred to a *mt+* background by mating, it creates a genotype that mates as *minus*. A clone of this genotype will be rendered unstable by excision of the unknown '421 element', designated *ele*, responsible for

transposition. Complete excision of *ele* and the associated copy of the *mid* gene restores a stable *plus* line. Incomplete excision that removes part of *ele* but retains *mid* yields a stable *minus* line. A growing culture will in this way come to contain both *plus* and *minus* gametes, and will therefore express homothallic mating. Crosses between *plus* and *minus* strains isolated from the culture will show an apparently regular segregation of mating type. Homothallic mating, between a *minus* gamete bearing *ele* and a *plus* gamete from which *ele* has been excised, will often yield zygotes homozygous for *ele* through replicative transposition during meiosis. The progeny will be a mixture of gametic phenotypes: *plus*, if excision occurs early in culture growth; *minus*, if no excision occurs; and homothallic if excision occurs during colony growth to produce a mixture of gametes bearing and lacking *ele*.

This model would explain why homothallism is neither fixed through selection nor eliminated through loss during vegetative proliferation. If it should be confirmed by molecular techniques, it will provide a striking example of how transposable elements are entrained in the evolution of sexual processes. This would underline the contribution of transposable elements to the structure of the mating type region in *Chlamydomonas* emphasized by Ferris & Goodenough (1997), and lend more credibility to the fundamental role of transposable elements in the evolution of the sexual cycle (Hickey & Rose, 1988; Bell, 1993).

Sexual isolation did not evolve

After about 3 years of selection, the evolution of spontaneous mating in the lines had allowed the Liquid lines to be propagated as strictly aquatic and the Solid lines as strictly terrestrial organisms. This might be expected to have created pronounced differences both in conditions of growth and in conditions of mating, entraining divergent natural and sexual selection. There was nevertheless no indication that heterothallic gametes in the mass-transfer lines had evolved any substantial degree of sexual specificity. Moreover, the single-zygote lines failed to show the idiosyncratic sexual responses expected from processes such as founder-flush speciation (Powell, 1978). A screen for isolates able to mate only in the environment of selection was unsuccessful. It remains possible that there was some degree of population-specific isolation, independent of the environmental conditions for mating. This would be consistent with the decline in within-line mating through time (Fig. 5). In selection experiments with the dung fly *Sepsis*, an appreciable degree of sexual isolation caused by a tendency to mate with individuals from the same population evolved within 35 generations (Martin & Hosken, 2003). My experiment, however, may not have been long enough, nor extensive enough, for there to be any appreciable

chance of observing speciation. The experiment would probably have detected speciation had it occurred, under the conditions of divergent natural and sexual selection that I employed, at an aggregate rate of 10^{-3} per sexual cycle or more. The actual rate of speciation that can be achieved in the laboratory may be much lower than this, or perhaps more discriminatory protocols are necessary to cause divergence within a reasonable period of time. It is also possible, however, that mating type genes may not be capable of evolving in the gradual, sequential manner that genes affecting vegetative functions normally display.

The genes that affect sexual fusion often evolve very rapidly. Genes involved in mate or gamete recognition have elevated rates of evolution, relative to genes encoding vegetative function, in arthropods (Nurminsky *et al.*, 1998; Ting *et al.*, 1998; Tsauro *et al.*, 1998), molluscs (Lee *et al.*, 1995; Swanson & Vacquier, 1995), echinoderms (Metz & Palumbi, 1996) and mammals (Sutton & Wilkinson, 1997), including humans (Wyckoff *et al.*, 2000). Within the basidiomycete genus *Ustilago*, a bipolar heterothallic system evolves from a tetrapolar system through linkage between the locus controlling fusion through pheromone production and reception, and a locus regulating the development of an infectious dikaryon, to form a complex region, somewhat reminiscent of the *Chlamydomonas* mating type region, in which recombination is suppressed (Bakkeren & Kronstad, 1994). The mating-type region of *Chlamydomonas* also evolves rapidly. No homologues of *mid* or *fus1* could be detected by low-stringency Southern blots in other Volvocales, including other species of *Chlamydomonas* (Ferris *et al.*, 1997). This family of green algae has undergone extensive divergence in the last 75 My, with the species of *Chlamydomonas* surveyed diverging in the last 5 My or less (Liss *et al.*, 1997). The sole exception was a version of *mid* detected in *C. incerta*, the nearest known relative of *C. reinhardtii*. This version directs development as the *minus* gamete in *C. reinhardtii* but is nonetheless a very different sequence, with 35 of 148 nonsynonymous and 32 of 148 synonymous substitutions (Ferris *et al.*, 1997). In contrast, *mid* and *fus1* are almost identical in *C. reinhardtii* isolates from localities thousands of kilometres apart (Ferris *et al.*, 1997). This suggests that mating type genes evolve in an episodic or saltatory fashion, with new sexually isolated lineages appearing abruptly as the result of major changes affecting a large portion of a mating type gene or genes. This is consistent with the failure of selection experiments such as the one reported here, and also with the failure of mutational screens to identify new mating types in genetically well-known microbes such as *Saccharomyces*. The genetic mechanism responsible for inducing extensive changes that result in sexual isolation in eukaryotic microbes remains unknown, but it may not be unreasonable to speculate that transposable elements are again involved.

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