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Parasexual Recombination in *Dictyostelium discoideum*: Selection of Stable Diploid Heterozygotes and Stable Haploid Segregants

(clones/temperature sensitive/ploidy/fruiting bodies/spore/slime mold)

E. R. KATZ* AND M. SUSSMAN†

Department of Biology, Brandeis University, Waltham, Massachusetts 02154

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ABSTRACT **Haploid strains of *Dictyostelium discoideum* bearing temperature-sensitive mutations have been used to select stable diploid, heterozygotic clones, which arise at low frequency (about 10^{-5}). Segregants arise from such diploids at low frequency (about 10^{-3}). The diploids were heterozygous for resistance to cycloheximide and were phenotypically sensitive to the drug. Growth of the diploid cells in the presence of cycloheximide automatically selected those segregants bearing the resistant allele, and facilitated examination of the assortment of unselected markers. The combination of the two selective methods provides a workable system of genetic analysis in this species. We have used this method to locate six markers on three different linkage groups.**

In *Dictyostelium discoideum*, ploidy is an inherited condition. Three kinds of strains have been isolated: stable haploid, stable diploid, and metastable. (All clones of the last type contained both haploid and diploid cells.) Each of the varieties could be isolated from the others (1, 2).

At least some diploid clones arose by cell fusion and karyogamy. Two haploid strains, marked with mutations affecting the color of the fruiting bodies, were grown in mixed culture and yielded at very-low frequency stable, diploid, doubly heterozygous clones. On continued passage the latter, in turn, yielded at very-low frequency stable haploid segregants, including all four of the expected parental and recombinant phenotypes. However, the low frequencies of karyogamy and segregation made the method impractical for routine use in the absence of selective techniques. The existence of parasexuality in this species has since been confirmed (3, 4). However, the conditions used yielded only unstable heterozygotes, which disappeared within a few generations and could not conveniently be used for segregation studies.

A first step in the direction of providing a practical routine system was taken by Loomis (5), who isolated temperature-sensitive mutants of *D. discoideum*. Pairs of mutants were mixed and incubated at the permissive temperature, and were then plated out on nutrient agar at the restrictive temperature. Clones of temperature-resistant recombinants were thereby selected. Unfortunately, only unstable diploid heterozygotes were encountered, and they could not be isolated and retained for quantitative study.

In this paper, we report a series of crosses from which stable diploid, heterozygotic clones were obtained. Temperature-sensitive haploid parents bearing additional outside markers were used. A method has been devised whereby the rare haploid segregants from such diploids are automatically selected. Together, the two selective methods provide the basis for a workable system of genetic analysis used here to follow the segregation and recombination of six markers. Three linkage groups have been identified thus far.

* Present address: Department of Biology, State University of New York at Stony Brook, Stony Brook, N.Y. 11790
† To whom reprint requests should be addressed.

MATERIALS AND METHODS

Isolation of Temperature-Sensitive Mutants. Strains derived from *D. discoideum* NC-4 (haploid) were grown on SM agar in association with *Aerobacter aerogenes* (6) at 22°C. The cells were mutagenized with nitrosoguanidine (Aldrich Chemical Co.) according to the method of Yanagisawa *et al.* (7). At a survival of 0.1–1%, about 6% of the clones displayed visible morphogenetic aberrations. Clones displaying normal fruiting bodies were picked with sterile toothpicks and reinoculated by stabbing each of two plates previously spread with an inoculum of *A. aerogenes*. One plate was incubated at 22°C and the other at 27°C. The wild type grows equally well at both temperatures. Mutants that grew at 22°C, but not at 27°C, were retained.

Parental Strains. M24 is a temperature-sensitive strain derived from the "brown" mutant br-1 of *D. discoideum*. The fruiting bodies of this mutant contain, in addition to the normal yellow pigment, a red-brown pigment that also seeps into and colors the agar substratum. Strain br-1 was one of the parent stocks used by Sussman and Sussman (2). M24 grew normally at 22°C, but not at all at 27°. If after several days at 27°C the plates were shifted to 22°C, growth resumed. Cells grown to the stationary phase at 22°C and then shifted to 27°C constructed fruiting bodies with normal morphology.

M60 is a temperature-sensitive strain derived from the "white" mutant wh-1 of *D. discoideum*. The fruiting bodies do not contain the normal yellow pigment. Strain wh-1 was the other parent stock used by Sussman and Sussman (2). The performance of M60 at 22°C and 27°C with respect to growth and morphogenesis was similar to that of M24.

M28 is a temperature-sensitive, brown mutant isolated from br-1. In this strain the spores are round instead of elliptical, as they are in the wild type. The round and temperature-sensitive characters are probably independent. In diploid heterozygotes, the round character is dominant and temperature sensitivity is recessive. It should, however, be noted that in attempts to obtain independent revertants,

TABLE 1. *Mean diameters of spores from haploid parent stocks and from diploid, heterozygotic derivatives*

	Haploids			Diploids	
Strain	M24	M60	M46	M24 x M60	M24 x M46
Mean diameter (μm)	8.9	9.2	8.0	12.3	12.3

Spore-diameter measurements were made with an ocular micrometer. At least 200 randomly chosen individuals in each category were examined.

only partially temperature-resistant revertants were encountered. Its growth and morphogenetic properties are like those of M24.

M46 is a temperature-sensitive, white mutant with round spores, isolated from wh-1.

TS12 is a temperature-sensitive, white, elliptical spored strain that is resistant to the antibiotic cycloheximide at a concentration of 300 μg/ml of agar. The wild type and all of the other mutants described above failed to grow on cycloheximide agar when plated at low densities.

Crosses. The parent stocks were harvested from growth plates and were washed by repeated centrifugations. The cells were mixed in equal numbers, and aliquots containing 10^8 cells were deposited on 42-mm Whatman no. 50 filters resting inside 60-mm petri dishes on pads saturated with a buffer–salts–streptomycin solution (6). Control filters supporting 10^8 cells of either parental type were also prepared. Under these conditions, about 1000 multicellular aggregates are formed and fruiting bodies are constructed synchronously over a 24-hr period at 22°C. The developing aggregates were harvested in sterile water at about 17 hr and were disaggregated by repeated passage through the tip of a 10-ml pipette, followed by vibration on a Vortex mixer. Samples of 10^5–10^6 dissociated cells were spread on plates with bacteria and incubated at 27°C. After 3 days, temperature-resistant plaques appeared within the bacterial lawn. These were incubated further to permit the cells within the plaques to construct fruiting bodies, and were then purified by one clonal passage and examined for ploidy, temperature resistance, fruit pigmentation, and other properties.

RESULTS

Selection of stable, diploid, heterozygotes

With the procedure described in *Methods*, the following crosses were attempted: M24 x M46; M24 x M60; M28 x TS12. In each case, a temperature-sensitive haploid that formed white fruiting bodies was crossed with a temperature-sensitive haploid that formed yellow-brown fruiting bodies. The resulting stable heterozygotic clones, which appeared with a frequency of 1×10^{-5}, were temperature resistant and constructed fruiting bodies with the yellow pigment characteristic of the wild type, rather than either the white or the brown plus yellow fruiting bodies characteristic of the two haploid parents. The diploids grew normally at 27°C and were quite stable at both 27°C and 22°C, showing a reversion frequency of 3×10^{-4} (see below).

Crosses M24 x M46 and M24 x M60. In both these crosses the mean spore diameters of the presumed diploids were significantly larger than those of the haploid parents (Table 1). The values are consistent with the spore-size distributions reported for diploid clones by Sussman and Sussman (1, 2), and confirmed by their examination of several hundred metaphase figures. In this study, one presumed diploid was spot-checked and yielded metaphase figures with 14 chromosomes. Inspection of the temperature-resistant clones for fruit pigmentation and spore size eliminated leaky parental haploids and segregants from unstable heterozygotes. It should be noted that since the M24 x M46 diploid produces an oblong spore, the round-spore marker in M46 behaves as a recessive.

TS12 x M28. The diploid clones produced by this cross formed nearly round spores, considerably larger and slightly more ovoid than the M28 haploid parent, but they clearly resemble M28 much more than they do the TS12 parent. It thus appears that the round marker in M28 is dominant to the elliptical and is, therefore, different from the round marker in M46.

Selection of haploid segregants

In the cross M28 x TS12, the first parent was sensitive to cycloheximide at a concentration of 300 μg/ml of agar. The second parent had been selected for resistance to the drug. All of the diploid heterozygotes turned out to be sensitive. Thus, the cycloheximide-resistant marker appears to be recessive, a conclusion already reached (Yanagisawa and Sussman, unpublished data) by study of the segregation of diploid, cycloheximide-resistant mutants that had been obtained by mutagenesis of the original diploid, heterozygotic strains isolated by Sussman and Sussman (2). It was expected that by plating spores from the diploid heterozygotes from the M28 x TS12 cross on cycloheximide agar we would select 50% of the haploid segregants, i.e., those bearing the cycloheximide-resistant (cy^r) allele. It was further expected that the other markers would assort randomly, unless linked with the cycloheximide locus. In fact, diploids from the cross M28 x TS12 did yield cycloheximide-resistant, haploid segregants, at a frequency of about 3×10^{-4}. Sussman and Sussman (2) reported that diploid clones yield spontaneous haploid segregants of all classes at a frequency of about 10^{-3}.

Analysis of the cycloheximide resistant, haploid segregants

The assortment of outside markers among the cycloheximide-resistant, haploid segregants was decidedly nonrandom. Only four segregant classes were encountered among 191 independently occurring, cycloheximide-resistant, haploid clones. These were: white, brown, temperature-sensitives; white, temperature-sensitives; yellow, temperature-resistants; yel-

TABLE 2. *Distribution of phenotypes among cycloheximide-resistant, haploid segregants from cross M28 x TS12*

	White, temperature-sensitive	Yellow, temperature-resistant	Total
Brown	35	61	96
Not brown	41	54	95
Total	76	115	191

low, brown, temperature-resistants. All formed elliptical spores with size distributions characteristic of the haplophase. The relative proportions of these segregants are shown in Table 2.

These data suggest the following linkage relationships of our markers:

1. wh^- is linked to t_1^s, both having been contributed by the TS-12 parent. Conversely, wh^+ is always associated with t_1^r.

2. The linkage group containing wh^- and t_1^s is probably independent of the cy^r linkage group. The ratio of white temperature-sensitives to yellow temperature-resistants (*tr*) was 76:115, slightly less than 1:1, but perhaps understandable on the grounds of selective growth since the $wh^-t_1^s$ contains two mutant alleles, where the $wh^+t_1^r$ has two wild-type alleles. In one experiment it was possible to enrich for white, temperature-sensitives by deliberately selecting the smallest, slowest growing plaques.

3. The linkage group containing br^+ is independent of both the linkage group containing wh^- and t_1^s and the linkage group containing cy^r. Among the cycloheximide-resistant segregants, the ratio of brown to not brown was 95:96. Among the white, temperature-sensitive segregants, the brown to not brown ratio was 35:41, and among the yellow temperature-resistant segregants, the ratio was 61:54.

4. t_2^s and sp^R (round spores) are located in the same linkage group as cy^s. No round segregants were observed nor were any yellow temperature-sensitives found, as would be expected if sp^R and t_2^s were segregating.

The putative linkage groups shown in Table 3 lead to the prediction that, among unselected haploid segregants, one would expect to find cycloheximide-sensitives. All of them should also be temperature-sensitive and should form round spores; the fruit pigmentation markers should assort at random. The diploid heterozygote was plated clonally on SM agar without cycloheximide. Several thousand clones were examined with respect to fruiting-body color. Six haploid clones were isolated, two white–brown and four yellow–brown. Both of the white–browns were temperature-sensitive, cycloheximide-sensitive, and produced round spores. Of the yellow–browns, three were temperature-resistant, cycloheximide-resistant, and oblong. The fourth was temperature-sensitive, cycloheximide-sensitive, and produced round spores.

An exceptional segregant class

In addition to the above, some diploid heterozygotes from the cross M28 x TS12 yielded an exceptional class of segregants. When these heterozygotes were plated on cycloheximide agar, at least 10% of the cy^r clones produced elliptical spores, but of a size range characteristic of the diplophase.

TABLE 3. *Three putative linkage groups in D. discoideum*

Strain	Genotype		
TS12	$wh^- t_1^s$	br^+	$cy^r t_2^r sp^E$
M28	$wh^+ t_1^r$	br^-	$cy^s t_2^s sp^R$

Symbols: t_1^s and t_2^s are temperature-sensitive, t_1^r and t_2^r are temperature-resistant; wh^- is white, wh^+ is yellow; br^- is brown, br^+ is not brown; sp^R is round spore, sp^E is elliptical spore; cy^r is cycloheximide-resistant, cy^s is cycloheximide-sensitive.

TABLE 4. *Distribution of segregants from aberrant diploids*

Yellow temperature-resistant brown cycloheximide-resistant elliptical	41
White temperature-sensitive brown cycloheximide-resistant elliptical	15
White temperature-sensitive not brown cycloheximide-resistant elliptical	10

Most aceto orcein-stained metaphase nuclei (8) in cells taken from these clones were clearly diploid. (The others showed at least 13 or 12 chromosomes, but because of the angle of viewing or staining irregularities they could not be diagnosed unequivocally as either diploid or aneuploid.) We suppose that this class of segregants arose by the loss of the chromosome bearing the sp^R allele, followed by the preferential duplication of the sister chromosome to reattain the diplophase. Parasexual diploid segregants of this type have been encountered in *Aspergillus* (9). This is the simplest, though by no means the only possible explanation. For example, the odd segregants could also be the result of somatic nonreciprocal recombination leading to homozygosity of one part of the chromosome.

We have isolated spontaneous segregants of this aberrant variety, as well as segregants induced by parafluorophenylalanine (Katz, E. R., unpublished data). Since the distributions were the same, the results were combined in Table 4. It can be seen that both the $wh^-t_1^s$ and the br^- linkage-groups appear to be segregating. It should be noted that the segregants were recognized by fruiting body color, which renders the fourth expected class (yellow–not brown) undetectible. This also accounts for the large excess of browns, since brown is much easier to detect than white. Most importantly, it can be seen that the $cy^rt_2^sp^R$ linkage group is not segregating. This is consistent with our hypothesis that the chromosome bearing this group has been reduplicated and is present in the homozygous condition.

DISCUSSION

In this paper, we have confirmed the results of Loomis (5), i.e., that in attempted crosses the use of haploid parental stocks of *D. discoideum* that bear temperature-sensitive mutations permits one to select relatively rare recombinants with high efficiency. Each of our own parental stocks also bore a recessive mutation affecting fruiting-body pigmentation. Stable diploid heterozygote clones arising from these crosses could first be selected by growth at the restrictive temperature, and then be confirmed by the wild-type pigmentation of their fruiting bodies and by the spore-size characteristic of the diplophase. Thus, leaky haploid parental clones or segregants of unstable heterozygotes could be eliminated by inspection. The stable diploid heterozygotes could be preserved and carried as clonal stocks for detailed segregation analyses.

The results reported here also offer a routine method for selection of the haploid segregants that arise during growth of the diploid population. Diploids arising from the cross M28 x TS12 are heterozygous for cycloheximide-resistance and are phenotypically sensitive. Growth in the presence of cycloheximide automatically selected the class of segregants bearing the cy^r allele, and permitted us to study the assortment of

all markers not linked with it. In this way we have shown linkage in the slime molds for the first time and have defined three linkage groups. In principle, it should be possible to construct diploids heterozygous for recessive drug-resistance markers located on each of the seven chromosome pairs, and by plating the diploid on agar containing the appropriate drug(s) to select any desired segregant class.

The mechanisms of karyogamy and of haploidization still remain obscure. We are presently unable to explain why the procedures followed by Loomis (5), Loomis and Ashworth (3), and by Sinha and Ashworth (4) should have preferentially selected very unstable heterozygotes, whereas our own conditions favor the selection of stable ones similar to those originally demonstrated by Sussman and Sussman (2). It is clear that ploidal stability is an inherited property, but its physiological and genetic bases are unknown. As yet, there is no definitive information bearing on the mechanism of haploidization. Our own results and those published in the earlier studies indicate that segregants haploid for any one marker are haploid for all others. Thus, if haploidization proceeds via progressive losses of chromosomes as observed in *Aspergillus* (9) and suggested for *Dictyostelium* (4), the aneuploid derivatives must be quite unstable. The exceptional class of diploid segregants encountered in the cross M28 x TS12 does seem to indicate that differential chromosome losses do occur and that aneuploidal instability can result in reattainment of the diplophase rather than further loss of chromosomes.

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