GENETIC RECOMBINATION IN SEXUAL CROSSES OF PHYCOMYCES

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ABSTRACT

Sexual crosses between strains of *Phycomyces blakesleeanus*, involving three auxotrophic and one color marker and yielding a high proportion of zygospore germination, are described. Samples of 20–40 germ spores from 311 individual fertile germ sporangia originating from five two-factor and three three-factor crosses were characterized. The results show: (1) absence of any contribution of apogamic nuclei to the progeny, (2) confirmation of Burgeff's conjecture that the germ spores of any germ sporangium in most cases derive from one meiosis. In a cross involving two allelic markers the analysis of 175 pooled germ sporangia suggests an intragenic recombination frequency of 0.6%. All other factor combinations tested are unlinked. The bulk of the germ spores are homokaryotic. However, a small portion (4%) are heterokaryotic with respect to mating type.

PHYCOMYCES, in common with other members of the Mucoraceae, is heterothallic (BLAKESLEE 1904). It has two mating types, designated (+) and (−). The two types are indistinguishable except by the fact that sexual structures are produced only by their joint action. This joint action is initiated by the secretion of mating-type-specific substances (BULLOCK, DRAKE and WINSTANLEY 1972; MESLAND, HUISMAN and VAN DEN ENDE 1974; SUTTER 1975) which initiate a complex series of chemical and morphological changes culminating in the production of a zygospore (BERGMAN *et al.* 1969). The zygospore, after a very prolonged dormancy, germinates "directly", i.e. it produces a germ sporangiophore and germ sporangium without first producing a mycelium. The germ spores in the sporangium are multinucleate like those in the vegetative sporangium (3–4 per spore, a few less, a few more).

During the first decades of this century, first BLAKESLEE (1906) and then, in much greater detail, BURGEFF (1915, 1928) attempted to unravel the recombinational mechanisms involved in the sexual process. It was shown fairly convincingly that the nuclei of the mycelia were haploid and so were the mycelia produced by the germ spores. Morphological markers were used to show recombination. In all cases, the numbers of recombinants varied widely from germ sporangium to germ sporangium, reminiscent, from the present vantage point, of the situation in phage genetics, and adding to the complexity of the problem. In his last publication on this subject, BURGEFF (1928) surmised that, of the nu-

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merous haploid nuclei of both mating types that entered the zygospore, many fused in pairs to form diploid nuclei and that, in general, only one of these, with or without intervening replication in the diploid phase, underwent meiosis followed by a variable amount of postmeiotic mitosis. Thus the germ sporangium, just prior to the formation of the germ spores, should contain a mixed population of nuclei of various genotypes. Since most of the spores gave rise to homokaryotic mycelia, he conjectured that the germ spores are formed from "protospores", i.e. spore initials containing only one nucleus. This one nucleus then was assumed to undergo some mitotic divisions to bring the number of nuclei up to the known multinucleate state of these spores.

A few of the germ spores gave rise to a peculiar type, called "neutral" by BLAKESLEE because they do not react sexually with either (+) or (−) standard strains. They also produce in large numbers a strange new structure which he called a pseudophore. BLAKESLEE conjectured and BURGEFF (1914) proved that these neutral mycelia are in fact mating type heterokaryons and are reasonably explained by the assumption that a protospore occasionally encloses more than one nucleus and in some cases these nuclei may represent both mating types.

In the intervening decades several attempts were made to establish the karyological events by cytological means (BAIRD 1924; BURGEFF 1914, 1928; CUTTER 1942; KEENE 1919; LING-YOUNG 1930–1931; SJÖWALL 1946). Do the fusions between nuclei of different mating type take place in the early stages of zygospore formation or months later, shortly before germination, or still later? Do genuine meioses take place, again where and when? Do any haploid nuclei persist and contribute as such to the germ spores?

In recent years new genetic markers and improved methods for producing heterokaryons artificially become available and some attempts were made to use these advances to unravel the sexual mechanisms of Phycomyces. Both the cytological work and the sexual crosses have been reviewed by BERGMAN *et al.* (1969) and very recently by CERDÁ-OLMEDO (1974). Suffice it to say here that the cytological studies are inconclusive and that the recent genetic data have been too limited to settle some of the most elementary questions.

As this paper goes to press we have received a manuscript by CERDÁ-OLMEDO (submitted for publication) in which details of the experiments summarized in BERGMAN *et al.* (1969) and in CERDÁ-OLMEDO (1974) are given, as well as some new data. CERDÁ-OLMEDO's basic conclusions and ours, insofar as they overlap, are compatible.

Among the genetic markers that have become available recently are some auxotrophic ones obtained by mutagenization with nitrosoguanidine, some obtained in the laboratory of CERDÁ-OLMEDO in Sevilla and some during the summer Phycomyces workshops in Cold Spring Harbor in 1973 and 1974. Also a search, a few years ago, for a pair of strains giving a shorter dormancy yielded a (+) strain, UBC21, which in matings with NRRL1555(−), our standard strain in physiological research, gives a much shorter dormancy, less than 60 days, instead of the usual three to six months. We report in this paper the results obtained from nine crosses, including a first and second backcross to our standard (−)

strain, five two-factor crosses involving unlinked genes, two three-factor crosses involving unlinked genes, and one three-factor cross involving two different alleles of the same gene.

MATERIAL AND METHODS

Strains used: The strains used are listed in Table 1. These strains include some obtained as segregants in the present experiments.

Media: Two minimal media were used—GA, described previously (REAU 1972), and the very similar SI (SUTTER 1975), which contains 20 gm glucose, 1 gm monosodium glutamate, 0.5 gm $MgSO_4 \cdot 9\ H_2O$, 5 gm KH_2PO_4, 28 mg $CaCl_2$, 2 mg thiamine HCl, 1.5 mg $Fe(NO_3)_3 \cdot 9\ H_2O$, 1 mg $ZnSO_4 \cdot 7\ H_2O$, 0.3 mg $MnSO_4 \cdot H_2O$, 0.05 mg $NaMoO_4 \cdot 2\ H_2O$, 0.05 mg $CuSO_4 \cdot 5\ H_2O$, 2 mg citric acid$\cdot H_2O$, 15 g of agar and 1 l. distilled H_2O.

Complete media included 4% potato-dextrose agar plus 5 μg/ml thiamine HCl (PDA) and GA plus 0.1% yeast extract and 0.1% yeast extract and 0.1% Bacto-Casitone (GAYC) which was acidified to pH 3.2 with 1 N NCl (GAYCA) if colonial growth was desired.

These were supplemented with leucine at 100μg/ml (leu), nicotinic acid at 10 μg/ml (nic), and hypoxanthine at 100 μg/ml (HX). The media were solidified with 1.5–2% agar.

Mutagenesis: Vegetative spores at a concentration of 10^7 per ml were treated with 0.1 mg/ml of N-methyl-N′-nitro-N-nitrosoguanidine in 0.1 M citrate-phosphate buffer, pH 7, for 30 min at room temperature. The suspension was shaken occasionally. Survival ranged from 6% to 25%. The spores were washed three times by centrifugation and resuspension in buffer and once with water and seeded at a concentration of about 100 viable spores per plate on GAYCA medium. Colonies that grew were divided and tested for differential growth on minimal and complete medium. Putative auxotrophs were retested and screened for growth requirements by a successive approximation method (PONTECORVO *et al.* 1953) involving pools of nutritional substances (HOLLIDAY 1956).

TABLE 1

Strains used

Code*	Genotype†	Origin	Comments
NRRL1555	(—)	Wild type	from Northern Regional Research Laboratories, USDA, Peoria, Ill., USA
UBC21	(+)	Wild type	from R. J. BANDONI, Botany Department University British Columbia, Vancouver, BC, Canada
S102	*nicA101* (—)	from NRRL1555	by nitrosoguanidine mutagensis, by J. R. MEDINA, 1972
H1	*leu-51* (—)	from NRRL1555	by nitrosoguanidine mutagenesis, 1973
H4	*nicA51* (+)	from UBC21	by nitrosoguanidine mutagenesis, 1973
B-48–20	*nicA101* (+)	from UBC × S102	segregant from present experiments
J′-9–8	(+)	from B-48–20 × NRRL1555	segregant from present experiments
C169	*carA5* (+)	from UBC21 × C2	by S. TRUBATCH, 1971
C170	*carA5* (+)	from UBC21 × C2	by S. TRUBATCH, 1971

* C, H, and S refer to strain collections at the California Institute of Technology, Cold Spring Harbor Laboratory, and the University of Sevilla. B and J′ refer to the crosses listed in Table 3a with the numbers after the code letter for the cross indicating the germ sporangium and the isolate from that sporangium.

† *car* designates genes involved in β-carotene synthesis, not a nutritional marker.

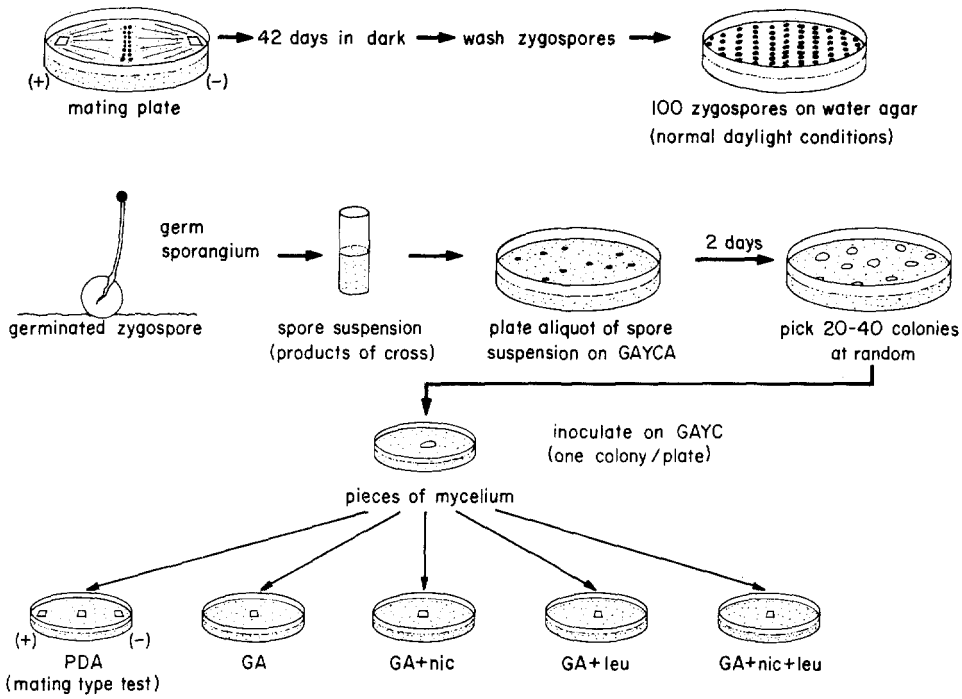

FIGURE 1.—Procedure for sexual crosses and analysis of the genotypes of the contents of a single germ sporangium, example of cross D (*nicA51*(+) × *leu-51*(−)). For explanation see text.

Sexual crosses: Figure 1 shows the procedure for sexual crosses and analysis of the genotypes of the progeny. All crosses were made by inoculating pieces of mycelium of the strains to be mated at opposite margins of 10-cm-diameter PDA plates in some cases fortified with hypoxanthine and/or leucine. The plates were incubated for 42 days in the dark either at 17° or 22°. At that time mature zygospores including suspensors were picked up individually, washed several times with sterile H_2O, and set out on water agar plates at 22° in normal daylight conditions. These plates were inspected every day. Ripe sporangia from germinated zygospores were picked up in a drop of water between the tips of tweezers and put into 1 ml of sterile water. Samples of various sizes from such a suspension were plated out on GAYCA medium. Of the colonies formed after two or three days, a sample of 20 or 40 were picked at random and transferred to 5-cm GAYC plates. Each isolate was then tested for mating type on PDA or PDA + leu plates, and for auxotrophy on a set of suitably supplemented miminal medium plates. Where applicable, color was tested both on the original acid and on the supplemented plates.

In the case of the three-factor cross, E (see Table 3a), where two allelic markers are involved, the tests of 31 individual germ sporangia did not show any recombinants. In this case a pool of germ sporangia was analyzed also (see RESULTS).

RESULTS

(1) *Effect of environmental and intrinsic conditions on dormancy and germination percentages of the zygospores*

Three kinds of germination are involved in the analysis of sexual crosses:

a) fraction of zygospores germinating to form a germ sporangium;

b) fraction of germ sporangia yielding any viable spores;

c) fraction of spores germinating from a germ sporangium yielding any viable spores.

Seven crosses were set up to test the effect of extrinsic conditions on the duration of dormancy and the fertility of the zygospores. These are listed in Table 2. Each cross was set up at 22° and 17° on four different media, two complete (PDA and PDA + HX) and two minimal (SI + nic and SI + nic + HX). Dormancy was similar for all four media, with PDA + HX being slightly, but significantly, better than the others. Table 2 is therefore limited to data obtained with this medium.

At 22° the shortest dormancy is between 56 and 58 days, while at 17° it is 20 to 40 days longer, confirming the finding of SCHWARTZ (1926) that 22° gives a shorter dormancy. The percentage of zygotes germinated one month after the first generation varied from 59% to 100% at 22° and from 31% to 92% at 17°. The finding that it is consistently higher at 22° than at 17° and that it is 100% in several cases is limited to the medium of choice (PDA + HX). The spread in dormancy is defined as the number of days after the first germination at which 50% of the germinating zygospores have germinated. No striking differences are found between different media and between the two temperatures. 22° and PDA + HX favor both short dormancy and high germination of zygotes.

The effect of backcrossing on dormancy and germination rate was tested in a series of 29 backcrosses between (+) segregants of the first series of crosses and and NRRL1555, our standard (−) parent, at 22° and 17° on PDA + HX and SI + nic + HX. Results on PDA + HX are given in Figure 2. Many backcrosses yielded no germinations at 17° even two months after the first germination at 22°. Clearly the dormancy also involves intrinsic factors, possibly genetically determined. All but four of the backcrosses showed shortest dormancies longer than any of the shortest dormancies of the parental crosses (longer than 60 days), and the whole set shows a wide spread in shortest dormancies (Figure 2). We

TABLE 2

Germination of zygospores

Mated pair (+)	Mated pair (−)	Shortest dormancy (days) 22°	Shortest dormancy (days) 17°	Percent zygospores germinated 22°	Percent zygospores germinated 17°	Spread in dormancy (days) 22°	Spread in dormancy (days) 17°
UBC21	S102	56	91	79	80	5	3
H4	NRRL1555	57	91	100	44	5	9
C170	NRRL1555	56	85	100	92	6	6
C169	NRRL1555	56	74	59	58	7	13
C169	S102	56	74	83	31	4	3
C170	S102	57	81	90	78	6	12
UBC21	NRRL1555	58	91	100	63	5	8
Avg.				87		5.4	7.7

All mating plates were PDA + HX. These were kept at the temperatures indicated for 42 days. Then 100 zygotes from each cross were set out on water agar at 22°. Dormancy is counted from the day at which mating plates were inoculated to the germination of the zygote.

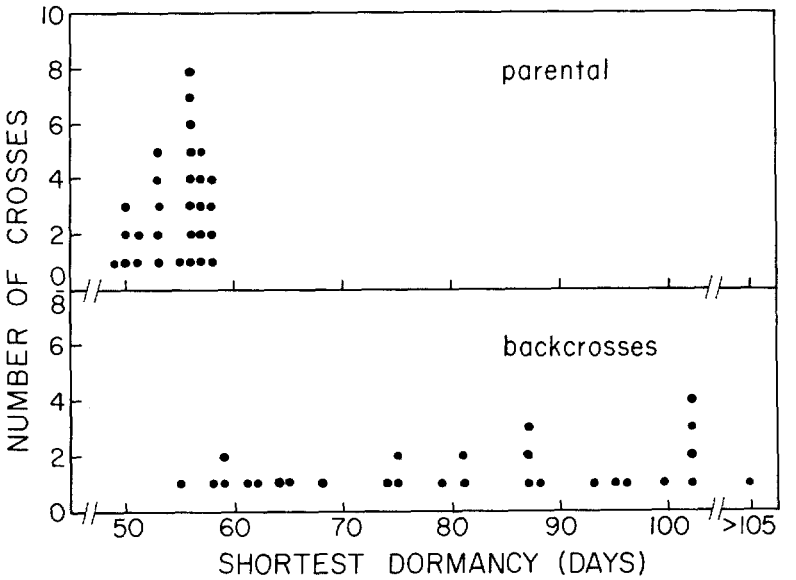

FIGURE 2.—Shortest dormancies in 28 parental and 29 backcrosses. Segregants with (+) mating type from crosses A, B, C, and D were backcrossed to the standard, wild-type (—) strain.

conclude that the short dormancy of our standard pair is determined polygenically.

(2) *Recombinants in sexual crosses*

(A) *Data for samples from single germ sporangia:* The sexual crosses for which individual germ sporangia were analyzed are listed in Table 3a. The samples taken from some germ sporangia yielded mycelia all of the same genotype. We call this class of sporangia one-type (1T). The genotype may be either parental (class 1TP) or recombinant (1TR). In a two-factor cross there are two possible 1TR's; in a three-factor cross there are six.

The samples from some germ sporangia yielded two genotypes. We call this class of sporangia two-type (2T). The two genotypes may be either a reciprocal pair, parental or recombinant (class $2T_{recip}$), or they may *not* constitute a pair of reciprocals (class $2T_{mixed}$).

Some germ sporangia yielded three or four, or in the case of three-factor crosses, more than four genotypes in the sample tested. In the case of three-factor crosses yielding more than four genotypes it is clear that these could not have been produced by one meiosis (class >4T). In the case of three or four genotypes these may be compatible with one meiosis (classes 3T and 4T) or they may not—namely, in those cases where more than two of the types found involve a particular allele of a gene. Such germ sporangia will be classified as pseudo3T and pseudo4T types.

This classification scheme ignores the occurrence of mating type heterokaryons and other heterokaryons in the germ sporangia in the samples taken.

TABLE 3a

Crosses analyzed

Code	Type	Cross	Genotypes	Number of sporangia analyzed	Sample size for each sporangium	Average shortest dormancy	Percent zygotes germinated	Percent sporangia fertile	Average spores per sporangium
E	1 factor	H4 × S102	*nicA51*(+) × *nicA101*(—)	31	20	53	83	54	9000
A	2 factor	H4 × NRRL1555	*nicA51*(+) × (—)	22	20,40	54	78	48	6500
B	2 factor	UBC21 × S102	(+) × *nicA101*(—)	39	20	52	79	35	7800
C	2 factor	UBC21 × H1	(+) × *leu-51*(—)	16	20	57	72	37	6500
J′	2 factor	F_1(B) × NRRL1555	*nicA101*(+) × (—)	51	40	59	75	74	9500
J′S	2 factor	$F_1(F_1(B \times NRRL1555)) \times S102$	(+) × *nicA101*(—)	53	40	52	70	60	9000
D	3 factor	H4 × H1	*nicA51*(+) × *leu-51*(—)	36	40	55	52	30	5000
X	3 factor	C169 × S102	*carA5*(+) × *nicA101*(—)	63	40	58	68	41	——
Y	3 factor	C170 × S102							
Avg.						55	72	46	7600

TABLE 3b

Progeny from the three-factor cross H4 × H1 (nicA51(+) × leu-51(—) ≡ (— + +) × (+ — —)*

Class	size†	Number of segregants of genotype								mt het‡
		leu-51 (—) + — —	*nicA51* (+) — + +	(—) + + —	*nicA51 leu-51*(+) — — +	(+) + + +	*nicA51 leu-51*(—) — — —	*nicA51* (—) — + —	*leu-51* (+) + — +	
1 TP	4		4							
	300		39							1
	60	40								
	800	40								
	128		15							
1 TR	50					38				2
	500						40			
	500							40		
	200				11					24
	75						15			
	400					40				
	500			40						
	500						39			
	2			2						
	34				27					
	80			40						
	200			40						
	300			40						
	150				40					
	300			45						
	700								40	
	120						35			
$2T_{recip}$	300			20	20					
	64					32	7			
	250					20	20			
$2T_{mixed}$	600			11			29			
	65		39			1				
	150			33					5	2
	50	30							2	8§
	200	2		37						
	700					24			16	
	300					8			25	7
3T	500			1		23	3			13
	20	1	9			2				
p4T¶	200	1		2		28	1			8
>4T	55	2	3	6		4			19	4
No. of germ sporangia containing the genotype		7	6	13	4	11	9	1	6	9

* Each line represents the isolates from a single germ sporangium.
† Total viable spores in the germ sporangium.
‡ Mating type heterokaryons.
§ All mating type heterokaryons were prototrophic, except this set, which was auxotrophic for leucine.
¶ Pseudo4T.

As a sample, Table 3b gives the results for all the germ sporangia of the three-factor cross D. The germ sporangia are arranged according to the classification outlined above, starting with the class 1TP and ending with the class >4T. For each germ sporangium the numbers of segregants in each genotypic class are given.

(B) *The allelism of* nicA51 *and* nicA101 *(cross E):* These two auxotrophs for nicotinic acid were obtained in independent mutagenizations of the two mating types. The following evidence indicates allelism:

a) A mating type heterokaryon obtained by grafting *nicA51* (+) and *nicA101* (−) does not complement.

b) A heterokaryon obtained by grafting *nicA51 leu* (−) and *nicA101* (−) does not complement.

c) Mating type heterokaryons were found in cross E in 12 of 31 germ sporangia (Table 3a), and these were invariably auxotrophic for nicotinic acid.

d) Biochemical tests show that both mutants are blocked in the step between quinolinic acid and nicotinic acid.

These two mutations were assigned to the same gene, *nicA*. No prototrophic recombinants were found among 620 segregants tested (20 from each of 31 germ sporangia). To test for rarer recombinants by a less laborious method the spores from 175 germ sporangia were pooled in 1 ml H_2O, assayed for total spores, for viability on GAYCA medium, and for number of prototrophs. The results are shown in Table 4.

The table shows that about 0.33% of the viable progeny are prototrophic. This is not an unreasonable value for intragenic recombination or gene conversion and is consistent with the absence of prototrophs in the 31 single-germ sporangia tested, since each germ sporangium represents clones of only one or a few segregants. Cross E, therefore, is in effect a one-factor cross (mating type), and the individual germ sporangia fall into two classes: 1T or 2T. The majority, 24 out of 31, are 1T, but statistically the only parental types are equally represented. The presence in the germ sporangia of the missing mating type is clearly suggested by the fact that half of the 1T germ sporangia (12 out of 24) did yield one or more mating type heterokaryons in the sample tested.

(C) *The disproportion of the segregants (preliminary discussion) and proportion of mating type heterokaryons:* A striking feature, noted by all observers since BLAKESLEE, is the great and erratic disproportion in the numbers of segre-

TABLE 4

Recombination between nicA51 (+) *and* nicA101 (—)
Tests on a pool of 175 germ sporangia

	Number	Percent of spores	Percent colonies of GAYCA
Total spores	8×10^5	100	—
Colonies on GAYCA	1.2×10^5	15	100
Colonies on GA	404	.05	0.33

Percent recombination = $0.33 \times 2 = 0.66$.

gants of the various genotypes found. In fact, most germ sporangia yield no representative of one or more of the expected genotypes or may even yield no viable spores at all. Before trying to identify the causes of these disproportions we must analyze the *average* aspects of the recombination mechanisms. Two kinds of scoring could be done:

a) determine the frequencies of alleles and recombinants averaged over many germ sporangia from one cross. To do this we would add the numbers of segregants of each genotype found in all the germ sporangia. Here the aim might be to identify the contribution of apogamics, if any, and to assess linkage, if any.

b) determine the frequencies of 1TP, 1TR, $2T_{recip}$, $2T_{mixed}$, 3T, 4T, and >4T germ sporangia. If we wish to decide whether the primary mechanism involved is meiotic, and if so, how many meioses occur per germ sporangium, and what fraction are ditype, or tetratype, we should not count the total numbers of the respective genotypes, but the numbers of germ sporangia in which the genotypes are present. In scoring procedure (a), a germ sporangium showing 20 or 40 of a particular genotype is given a very much higher weight than one in which the genotype is found only once, even though in both cases only one meiosis may be involved. In scoring procedure (b), the effects of the secondary mechanisms causing the disproportions are largely eliminated. We therefore preferred procedure (b) which considers only the *presence* or *absence* of certain classes in the tested sample. Procedure (a) corresponds more nearly to the analysis obtainable from pooled germ sporangia and permits pooling many germ sporangia. This gain is offset by the inability to separate out the disproportioning mechanisms. We have used procedure (a) to assess the proportion of mating type heterokaryons in our crosses. In the parental crosses it is 7% (233 of 3359). It is 3.5% (135 of 3873) and 0.2% (3 of 1846) in the first and second backcrosses, respectively. We prefer to await the results of further backcrosses before drawing conclusions from the trend in these numbers.

(D) *Are the alleles used nonselective?*: Are any of the genetic markers used in some way selected in our crosses? Such a selection could be inherent in the sexual and recombinational processes, or it could be caused by the assay procedure. Table 5 lists for each allele the number of occurrences of genotypes

TABLE 5

Occurrence of parental alleles in the genotypes of the progeny

Cross	*nic*	*nic+*	(+)	(—)	*leu*	*leu+*	*carA*	*carA+*
A	19	15	19	15				
B	28	32	31	29				
J'S	55	55	58	52				
J'	55	60	55	60				
D	20	37	27	30	26	31		
X & Y	59	74	69	64			67	66
C			14	14	12	16		
Total	236	273	269	268	38	47	67	66

TABLE 6a

Linkage tests for the markers used

Cross	*nicA* & mt*		*leu* & mt		*nicA* & *leu*		*carA* & *nicA*		*carA* & mt	
	P	R	P	R	P	R	P	R	P	R
A	16	18								
B	33	27								
J'S	53	57								
J'	59	56								
D	30	27	33	24	20	37				
X & Y	70	63					56	77	69	64
C			12	16						
Total	261	268	45	40	20	37	56	77	69	64

Total for all pairs of markers $\frac{P : R}{451 : 466}$

For each pair of markers the number of germ sporangia are listed in which the pair was found to be present in the parental (P) or recombinant (R) form in the sample tested.
* Mating type.

containing that allele. It shows that there is no bias for any allele, with the possible exception of *nic*+ (273 *nic*+ : 236 *nic*). This bias, while not statistically significant, may be due to a small contribution from heterokaryons in the progeny. It is similar to the bias that we would have obtained for a mating type allele if in the mating type heterokaryons one or the other of the alleles were dominant.

(E) *Linkage tests for the markers used. Do apogamic nuclei contribute to the progeny?*: Table 6a lists, for each pair of markers, the number of germ sporangia in which the pair was found in the parental (P) or recombinant (R) genotypic form in the sample tested. Each of the six possible pairs of the four markers involved was tested except *carA* and *leu*. None showed evidence for linkage. In one case (*nic* and *leu*) there was a deficit of germ sporangia containing the parental types that may be related to the presence of a small fraction of germ sporangia containing heterokaryons which in this cross would obscure the scoring of both parental classes.

TABLE 6b

Parental (P) and Recombinant (R) monotypic germ sporangia

Two-factor crosses:	P	R	Three-factor crosses:	P	R
A	5	6	D	5	17
B	14	10	X & Y	4	15
J'S	10	8			
J'	9	5			
C	2	5			
Total	40	34		9	32

The tests rule out a significant contribution from apogamic nuclei to the progeny, since these would appear as a bias in favor of the parental combinations. For all marker pairs and crosses combined we have the ratio P:R = 451:466.

Apogamic progeny might occur among 1T germ sporangia since they could arise without recombination's taking place. This was tested by classifying 1T germ sporangia as parental (P) or recombinant (R) (Table 6b). We expect, in the absence of linkage in two-factor crosses: P:R = 1 (found 40:34); in three-factor crosses: P:R = 1:3 (found 9:32). These results clearly indicate that apogamic nuclei do not contribute significantly to the 1T germ sporangia.

(F) *Do recombinants arise by a classical type of meiosis, and if so, how many meioses contribute to the progeny in any one germ sporangium?*: For a cross involving two or more markers a classical meiosis produces four haploid cells representing zero or two pairs of reciprocal genotypes. If recombinants arise by some other process of haploidization, as in the parasexual cycle of diploid Aspergillus mycelia (FINCHAM and DAY 1971) where it is ascribed to random losses of chromosomes, then there will be neither a preference for reciprocal pairs nor a limitation to two reciprocal pairs from any germ sporangium. Also, if there are many meioses contributing to any one germ sporangium, there will be no bias in favor of reciprocal types and no limitation to four types.

In Table 7 the numbers of germ sporangia in the various crosses are listed according to the classes they represent. For three-factor crosses 9 of the 99 germ sporangia analyzed belong to classes requiring more than one meiosis (pseudo3T,

TABLE 7

Numbers of germ sporangia belonging to various classes

Cross	1T	$2T_{recip}$	$2T_{mixed}$†	3T‡	4T§	Pseudo-3T¶	Pseudo-4T¶	>4T¶	mt H‖	Total
	Two-factor									
A	11(2)*	2(0)	5(3)	3(2)	0				1	22(8)
B	24(2)	5(0)	5(1)	4(2)	1(0)					39(5)
C	7(0)	0(0)	2(0)	3(2)	2(1)				2	16(5)
J'S	18(0)	10(1)	10(0)	8(2)	7(0)					53(3)
J'	14(1)	11(8)	7(3)	11(7)	8(5)					51(24)
Total	74(5)	28(9)	29(7)	29(15)	18(6)				3	181(45)
	Three-factor									
D	22(3)	3(0)	7(3)	2(1)	0	0	1(1)	1(1)		36(9)
X & Y	19(3)	7(2)	17(6)	7(3)	5(1)	4(1)	2(0)	1(0)	1	63(17)
Total	41(6)	10(2)	24(9)	9(4)	5(1)	4(1)	3(1)	2(1)	1	99(26)
E	24(12)	6(0)							1	31(13)

* In parentheses: numbers of germ sporangia in each class with at least one mating type heterokaryon in the sample tested.
† Two nonreciprocal types.
‡ Two reciprocal pairs with one type missing.
§ Two reciprocal pairs.
¶ Three or more types representing more than two of a particular allele of a gene.
‖ Number of germ sporangia yielding mating type heterokaryons only.

pseudo4T, >4T), giving us a lower limit of 0.09 for the fraction of germ sporangia involving more than one meiosis.

On the other hand, the recombinational mechanism clearly gives rise to a preponderance of reciprocal pairs. Consider the ratio of $2T_{recip}$:$2T_{mixed}$. If the two types were picked at random, we would expect:

in two-factor crosses = 1:2 (found 28:29)

in three-factor crosses = 1:6 (found 10:24).

Furthermore, for the three-factor crosses, the 3T:pseudo3T and 4T:pseudo4T cases if the genotypes were picked at random, we would expect:

3T:pseudo3T = 3:4 (found 5:3)

4T:pseudo4T = 3:32 (found 9:4).

There is a strong bias in favor of reciprocal pairs, indicative of a meiotic mechanism.

(G) *The loss of genotypes*: Any one genotype generated by meiosis appears to have about a 50% chance of appearing in the samples taken from the germ sporangia. This fact becomes evident from a comparison of 1T with 2T germ sporangia. The 1T must have started out at least with two types, and yet both two- and three-factor crosses show more 1T than 2T:

two-factor crosses 1T:2T = 74:57

three-factor crosses 1T:2T = 41:34.

We infer that the chance of finding any one meiotic product in the sample taken is roughly ½. This estimate is compatible with finding about twice as many 3T as 4T and with finding, in both two- and three-factor crosses, only 5–10% 4T.

(H) *Ditype and tetratype meioses*: A somewhat sharper inference can be drawn by comparing $2T_{recip}$ and $2T_{mixed}$. If there is only one meiosis, its four products will represent either two or four types, the ratio between these two depending on closeness to the centromeres and on the number of markers involved. If any event tetratype meioses will tend to be more frequent in three-factor than in two-factor crosses.

Ditype meiosis will not contribute to the class of $2T_{mixed}$ germ sporangia, but can contribute to the $2T_{recip}$ class, even with the loss of one or two of the primary meiotic products. Tetratype meioses can contribute to the 2T classes only if two types are lost and will then contribute ⅓ to $2T_{recip}$, ⅔ to $2T_{mixed}$ (Table 8). For

TABLE 8

Contributions of ditype and tetratype meioses to the classes of 2T germ sporangia

Type of meiosis	Meiotic products lost	Relative contribution to	
		$2T_{recip}$	$2T_{mixed}$
Ditype	0	1	
	1	1	
	2	2/3	
Tetratype	2	1/3	2/3
Found	2-factor	28	29
	3-factor	10	24

the three-factor crosses the data suggest that tetratype meioses predominate, since the observed ratio of the 2T classes (10:24) is close to the predicted value (1:2). On the other hand, in the two-factor crosses ditype meioses contribute substantially, perhaps about half the cases, reducing the observed ration of 2T classes from 1:2 to about 1:1.

(I) *The postmeiotic mitoses*: The evidence cited suggests strongly the occurrence of regular meioses in the production of recombinants, generally not more than one meiosis per germ sporangium, and the absence of a contribution from apogamic nuclei. Since the germ sporangia produce about 10^4 spores, of which about 10^3 are viable, and these viable spores represent few genotypes, there must be many postmeiotic mitoses. An indication of their synchrony can be obtained by comparing the disproportion of the two genotypes in the $2T_{recip}$ and $2T_{mixed}$ classes. Insofar as these derive from one meiosis the pair correlations should be alike (approaching 50%). However, these classes may also arise from two meioses and more often so for the $2T_{mixed}$ than for the $2T_{recip}$ class. This admixture should show less correlation between the members of the pair than the contribution from germ sporangia with only one meiosis. Figure 3 gives data for these two classes of 2T sporangia. The $2T_{recip}$ are slightly better correlated. A large part of the disproportion found is, of course, a pure sampling effect.

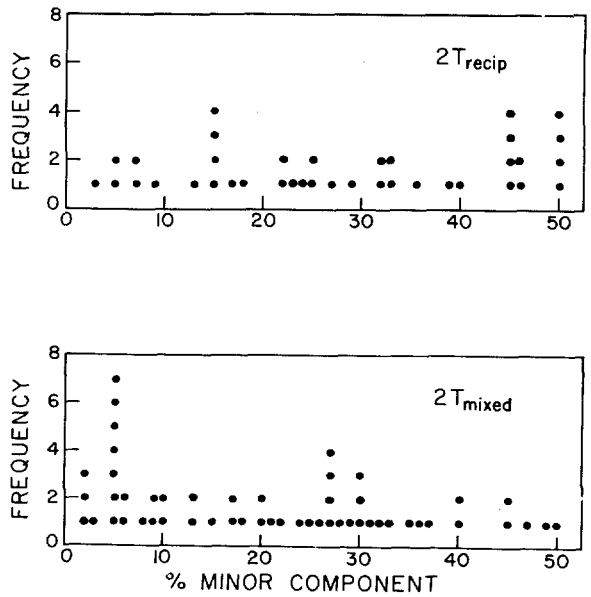

FIGURE 3.—Disproportion of the two genotypes in the $2T_{recip}$ and the $2T_{mixed}$ classes for all two- and three-factor crosses. For one meiosis the precent minor component should approach 50%. The disproportion (deviation from 50%) is due to (1) sampling error, (2) unequal proliferation of the progeny of a single meiosis, (3) unequal proliferation of progeny from *different* meioses. The third cause will contribute preferentially to the $2T_{mixed}$ pairs and may be the cause of the somewhat greater disproportion in this class of germ sporangia.

DISCUSSION

We have described a series of two- and three-factor crosses between strains of Phycomyces in which the zygotes germinate after a highly reproducible dormancy period, around 55 days, counting this period (for convenience) from the day of setting up the mating plates. Actual dormancy (counted from the maturation of the zygospore) is about a week shorter. In backcrosses the dormancy varies from this minimum to much longer periods, suggesting a polygenic determination. However, by appropriate selection of segregants in the successive crosses short dormancy has been maintained (presently through the third backcross).

In each of these crosses a high proportion of zygotes (70%) germinate, but the germination of the germ spores tends to be low 5–10%, with marginal improvement in successive backcrosses. The low germinability is probably responsible for three other aspects: (1) a high disproportion between the genotypes recovered, (2) total absence of some expected genotypes, and (3) total sterility of some germ sporangia. At present we cannot adduce evidence whether or not these three aspects are closely related. They might result solely from genotypic differences between the segregants because of non-isogenicity of the original pair.

Our tests for intragenic recombination between two alleles of the *nicA* gene are preliminary, since reciprocal recombinants were not recovered. Such a recovery has to await the construction of strains with closely linked flanking markers. The recombination rate is similar to values found for intragenic recombination in yeast, though much larger than corresponding values in Neurospora.

Both the two- and three-factor crosses indicate that the parental haploid nuclei which enter the zygospore do not reappear in the germ spores. In addition, they give evidence for a meiotic process operating in the generation of recombinants, and about one meiosis per germ sporangium. This evidence derives essentially from two features: (1) In the 2T germ sporangia there is a strong statistical preference for $2T_{recip}$ as against $2T_{mixed}$, and (2) in the germ sporangia of the three-factor crosses there is a strong preference for the 3T and 4T, compatible with a single meiosis, as against the pseudo3T and pseudo4T germ sporangia. However, the three-factor crosses also show clear evidence for the *occasional* occurrence of more than one meiosis, in *at least* 9% of the germ sporangia (9 out of 99). This number is probably close to the true value as seen from the following argument: In a three-factor cross with three unlinked markers (our case) the chances (a) that two independent meioses give the same tetratype is 1 in 6; (b) that they partially overlap is 4 in 6; (c) that they do not overlap is 1 in 6. In case (a) the second meiosis will not be detected; in case (c) it will almost certainly be detected; in case (b) the chance of detection is about one-half, since only one member of each of three reciprocal pairs needs to come through to permit detection. Altogether, then, the chance of not detecting a second meiosis is 1/6 and $1/2 \times 4/6 = 1/2$. Thus, the secon meiosis actually detected (9%) probably represent 1/2 of their true occurrence.

In conclusion, then, our data validate BURGEFF's (1928) conjecture with the germ spores of any one germ sporangium in most cases are derived from a single meiosis.

We are thus confronted in the sexual cycle of Phycomyces with a cycle which starts with thousands of haploid nuclei in the young zygospore and ends with thousands of nuclei in another structure, the young germ sporangium, and passes through an intermediate stage with effectively only one diploid cell. This cycle is somewhat reminiscent of that of the macrocysts of Dictyostelium species (ERODOS, NICKERSON and RAPER 1972): a number of haploid cells aggregate; one pair fuses to form a diploid; this cell engulfs and partially digests the others, undergoes meiosis, mitotic proliferation, and germination of the cyst. Here, too, genetic evidence for true meioses, and probably a single meiosis per macrocyst, has recently been presented (MACINNES and FRANCIS 1974). Cytological evidence for meiosis had been presented much earlier (WILSON 1953; WILSON and Ross 1957). It is a very reasonable guess that in Phycomyces, as in Dictyostelium, the original haploids are eliminated before the meiotic products appear on the scene, to obviate the need to distinguish between the two types of haploid nuclei.

Which mechanism limits the sexual structure to one meiosis? Is only one fusion (diploid) nucleus formed (not proliferating by mitoses), the appearance of which stops the occurrence of other fusions? If so, when and where does this key event occur? The fusion which does occur must be limited to nuclei of opposite mating type, because if it were not, and diploids homozygous for mating type could contribute to the progeny (a homothallic situation), such progeny would generate an excess of zygospores yielding parental genotypes. This is in conflict with our findings.

A complementary question arises in connection with the mating type heterokaryons, both those arising naturally in our crosses and those made artificially. In the mycelia of these heterokaryons nuclei of opposite mating type occur in great numbers and closely intermingled. Is fusion necessarily limited to the sexual structures or can it also occur in vegetative hyphae, followed by recombination and segregation? In other words, do the nuclei, in order to recognize each other as of opposite mating type, require for fusion some special physiological environment (zygospore) or will any environment do? Further, if fusion at one point in a hypha does occur will it be the signal to stop other fusions? Will a diploid nucleus, once formed, be capable of mitotic diversions? BURGEFF (1915) reported observations on germinating zygospores that had been forced to shift into the development of a mycelium (by submerging them) and claimed that mycelia thus formed were at first diploid (thick hyphae, large nuclei). This interesting observation may have been correctly interpreted and raises the possibility of similar phenomena in artificial mating type heterokaryons. These questions obviously can be attacked by analyzing suitable marked mating type heterokaryons, and such experiments are in progress. These experiments, on which we hope to report later, do show that new genotypes are generated in mating type heterokaryons (as shown by analysis of progeny spores) and that vegetative segregation of mycelial areas displayed abnormal characteristics, including invariability, does take place.

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