SOURCES OF ERROR IN GENETIC ANALYSIS IN NEUROSPORA TETRASPERMA[1]

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ACCORDING to present understanding of normal ascus development in *Neurospora tetrasperma*, half of second division segregations are expected to produce asci with all four ascospores homokaryotic for one or the other member of an allelic pair (SANSOME 1946; CATCHESIDE 1951). Homokaryotic ascospore frequencies should therefore provide a convenient method of centromere-distance determination which is applicable either to tetrad or random-ascospore data (HOWE 1963). Since this method assumes, however, that homokaryotic ascospores arise from heterozygous crosses exclusively by crossing over, the occurrence of any other events yielding homokaryotic ascospores would bias centromere-distance determinations. At least three such events may be expected to be involved. First, the loss of either of the two nuclei initially present in heterokaryotic ascospores would produce ascospores homokaryotic for the surviving nuclear type. Secondly, dwarf ascospores which are uninucleate, and hence homokaryotic, occasionally occur and may be mistakenly isolated as normals. Thirdly, certain nuclear misarrangements occurring during ascus development may also lead to homokaryosis not attributable to crossing over.

Nuclear loss is strongly suggested in the random ascospore analysis of SEAVER (1937) although, as she pointed out, tetrad analysis would have provided more direct evidence. The occasional occurrence of dwarf ascospores was established in the very early work on this species (DODGE 1927). Genetic evidence concerning nuclear missarrangements during ascus development is especially scanty, because so few well marked tetrads have as yet been analyzed. The present paper evaluates both tetrad and random ascospore data bearing upon these three sources of error, estimates frequencies of occurrence, and considers means of experimental control.

MATERIALS AND METHODS

N. tetrasperma wild-type strain 87 and derived inbred strain 85, as well as mutants induced in these two strains (HOWE 1963), were used. Westergaard-Mitchell (1947) crossing medium, appropriately supplemented, was used both for making crosses and also as a routine culture medium to facilitate scoring of mating type heterokaryosis. All ascospore isolations were made directly from dissection plates to slants without recourse to sorbose plating in order to permit visual separation of normal ascospores from aberrant dwarfs.

N. tetrasperma ascospores from the 4-spored ascus are binucleate when cut out, are nearly

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always heterokaryotic for mating type, and consequently produce self-fertile mycelia. Such ascospores will survive if one of the two initial nuclei is lost, but the derived mycelia will be homokaryotic for mating type and phenotypically self-sterile. Self-sterility in some but not all four ascospores of an ascus provides presumptive evidence for nuclear loss. If, on the other hand, all four ascospores from an ascus should show self-sterility, then not only nuclear loss, but also nuclear misarrangments during ascus development, and legitimate second division segregation of the mating type locus may be implicated.

Errors in randomly isolated ascospores may be more difficult to detect and identify than in tetrads. Moreover, the accidental isolation of uninucleate dwarf ascospores represents an additional hazard. Since dwarfs are usually produced in asci having more than four ascospores, however, this source of error is virtually precluded in the analysis of 4-spored asci.

Only 4-spored asci having all ascospores viable were used for obtaining tetrad data. Such material provides the type of information sought in these experiments with the least ambiguity. The nature and frequency of events determined from tetrads were used to predict occurrences in randomly isolated ascospores, and then predicted and observed results for random isolates were compared statistically.

RESULTS

Nuclear loss: Figure 1 shows the ratios of self-fertile to self-sterile ascospores to be expected in the six possible types of asci having four viable ascospores. Ascus Types I and VI represent first and second division segregation of the mating type alleles, respectively, with no nuclear loss. Ascus Types II to V represent nuclear loss in one, two, three and four ascospores, respectively; first division segregation of the mating type locus is assumed, since second division segregation, as will be shown, is so rare that it may be ignored for present purposes. Alternatively, ascus Types III and VI may result from nuclear misarrangements during ascus development.

Table 1 gives the frequencies from various crosses of each ascus type observed among 512 asci having four viable ascospores. A greater frequency of Type III

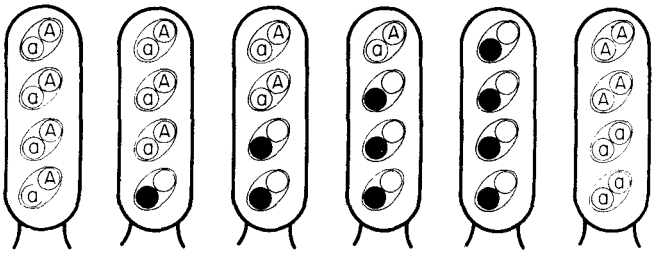

FIGURE 1.—Possible ascus types with respect to ratios of self-fertile and self-sterile ascospores in asci having all four ascospores viable. Solid nuclei, inviable; open nuclei, viable and, unless designated, containing whichever mating type allele survives by chance.

Types I and VI: Normal ratios produced by first and second division segregation of mating type locus, respectively; each ascospore with two viable nuclei. Type VI may also result from nuclear misarrangements during ascus development.

Types II to V: Aberrant ratios resulting from one or more ascospores having only one viable nucleus; first division segregation of mating type locus is assumed. Type III may also result from nuclear misarrangements during ascus development.

TABLE 1

Number of asci having various ratios of self-fertile to self-sterile ascospores in 512 unordered asci having all four ascospores viable

Strains crossed to wild types 85 or 87	Ascus type, and ratio self-fertile:self-sterile ascospores						Total asci
	I 4:0	II 3:1	III 2:2	IV 1:3	V 0:4	VI 0:4	
ad(101)	66	1(c)	1(g)	0	0	0	68
al(102)	56	2(a) 1(b)	0	0	0	0	59
arg(103)	53	2(b)	1(f)	0	0	0	56
arg(104)	32	3(a) 1(b)	1(e) 1(g)	0	0	0	38
col(105)	49	0	0	0	0	0	49
lys(106)	4	0	0	0	0	0	4
meth(107)	48	4(a) 2(b)	1(g)	0	0	2(h)	57
un(108)	61	1(a) 1(b)	2(e)	0	0	0	65
wild type	114	2(d)	0	0	0	0	116
Total asci	483	20	7	0	0	2	512
Percent	94.3	3.9	1.4	0	0	0.4	

The presumed origin of each ascus type is described in Figure 1.
Letters in parentheses refer to ascus groups in Table 2.

asci was observed than would have been expected on the basis of random nuclear loss alone. If loss in one ascospore were independent of loss in another ascospore of the same ascus, the expected frequency of Type III asci from random loss would be $(.039)^2 = 0.0015$. The observed frequency, however, was 0.014, or about nine-fold greater than expected. Both nonrandom loss, i.e., a tendency toward clustered occurrence, and nuclear misarrangements could account for the Type III ascus frequency observed, although these two events are not distinguishable from each other in the present data. No Type IV asci were found, and none would have been expected on the basis of random nuclear loss because of sample size. The cause of the apparent excess of Type III asci was evidently of insufficient magnitude to produce Type IV asci.

Ascus Types V and VI both have 0:4 ratios of self-fertile:self-sterile ascospores, but different origins. The only two asci found having a 0:4 ratio were arbitrarily classified as Type VI, since the observed frequency of nuclear loss or misarrangement and the total absence of Type IV asci would have made the occurrence of two Type V asci highly improbable. On this assumption, therefore, mating type postreduction (Type VI) occurred in not more than 0.4 percent of the asci, and even this low value may be an interpretive overestimate, since Type VI asci may also result from nuclear misarrangements during ascus development.

Table 2 gives further information about the 29 asci that had one or more self-sterile ascospores. The data may be used to determine whether nuclear loss was allele-associated, except for the mating type locus, since no sex tests of the self-

TABLE 2

Allelic loss in the 29 asci in Table 1 having self-sterile ascospores

Group	Number of asci	Ascospore phenotype 1	2	3	4	Total alleles lost +	*m*
a	10	s-f	s-f	s-f	s-s	0	10
		+	+	+	+		
b	7	s-f	s-f	s-f	s-s	7	0
		+	+	+	m		
c	1	s-f	s-f	s-f	s-s	1	0
		m	m	+	+		
d	2	s-f	s-f	s-f	s-s	–	–
e*	3	s-f	s-f	s-s	s-s	0	6
		+	+	+	+		
f*	1	s-f	s-f	s-s	s-s	1	1
		+	+	+	m		
g*	3	s-f	s-f	s-s	s-s	†	
		+	+	m	m		
h	2	s-s	s-s	s-s	s-s	‡	
		+	+	+	+		
Total	29					9	17

* Attributable to nuclear misarrangement as well as nuclear loss.
† Undeterminable.
‡ Interpreted as mating type postreduction, not nuclear loss.
Mating type was the only marker segregating in Group d. s-f (A,a) = self-fertile; s-s (A,A or a,a) = self-sterile; + (+,+ or +, m) = wild type; m (m, m) = mutant.

sterile isolates were made. (The only sex tests made were those for 59 self-sterile randoms in Table 3; nuclear loss was not associated with either mating type allele). In Group a of Table 2, for example, the single self-sterile ascospore indicates nuclear loss, while its wild phenotype shows that the nucleus which was lost contained the mutant allele. Since Group a has ten asci, a loss of ten mutant alleles is represented. Although there was a tendency toward greater loss of mutant alleles in the total data, the 9:17 ratio does not differ significantly from the 1:1 chance expectation ($P = 0.12$), which indicates that nuclear loss in these experiments was not associated with differential allelic viability. If the four Type III asci in Groups e and f are attributed to nuclear misarrangement rather than to nuclear loss, the ratio becomes 8:10, which also does not differ significantly from a 1:1 ratio ($P = 0.65$).

Randomly isolated ascospores of normal size from several experiments were scored for mating type heterokaryosis. Observed ratios of self-fertile to self-sterile randoms were compared with ratios calculated in two ways from tetrad data in order to account for the origin of the self-sterility. Calculated ratio I (Table 3) assumes that the self-sterile randoms owed exclusively to mating type postreduction, an event with an observed frequency in tetrads of 0.004 (Table 1). Calculated ratio II assumes that nuclear loss or nuclear misarrangment was also operative and is derived as follows:

Frequency s-s randoms = ¼ Type II + ½ Type III + ¾ Type IV + Types V and VI.

Substituting the observed tetrad frequencies from Table 1, we have:

Frequency s-s randoms = ¼ (0.039) + ½ (0.014) + 0.004 = 0.021

TABLE 3

Randomly isolated ascospores of normal size from wild × wild and various mutant × wild crosses compared with calculated values from tetrad data

Crosses	s-f:s-s ascospores			Chi-square tests comparing ratios	
	Observed ratio	Calculated ratio I	Calculated ratio II	Observed and I	Observed and II
+ × +	3495:59*	3540:14	3480:74	146	3.1
				P<.01	P>.05
m × +	1353:30	1377:6	1354:29	96	0.03
				P<.01	P>.05

* Sex tests revealed 27A:32a; not significantly different from 1:1 ratio (P>.05).
Calculated ratio I assumes exclusive mating type postreductional origin of s-s ascospores; calculated ratio II assumes nuclear loss or misarrangement is also operative.

Table 3 shows that the postreduction hypothesis (ratio I) is unable to account for the amount of self-sterility obtained in the random isolates, since the difference between observed and expected ratios is highly significant. When allowance is also made for the occurrence of nuclear loss or misarrangement (ratio II) at a frequency predicted by the tetrad data, however, observed and expected ratios do not differ significantly. Nuclear loss or misarrangement, less easily identified in randoms than in whole asci, is nevertheless demonstrable in the randoms by comparative means.

Dwarf ascospores: Some *N. tetrasperma* ascospores are smaller than usual and are believed to arise primarily in asci containing more than four ascospores by the cutting out of two or more of the eight ascus nuclei singly rather than pairwise during ascosporogenesis (DODGE 1927). DODGE (1932) verified genetically the uninucleate condition of dwarfs and the binucleate condition of normals by showing that in 5-spored asci (the most common supernumerary type) the two dwarfs produce self-sterile mycelia, whereas the three normals usually produce self-fertile mycelia. The eight ascus nuclei may also be accounted for in 6, 7, and 8-spored asci (Table 4).

Determining dwarf ascospore frequency microscopically in a random ascospore population is somewhat difficult, because size ranges overlap to some extent, as shown by the following measurements (in microns) made at 950× on ascospores from the cross 85A × 85a:

	25 dwarfs from 25 5-spored asci	25 normals from 25 4-spored asci
Mean and SE	13.6 ± 0.3 × 23.5 ± 0.3	16.3 ± 0.4 × 31.2 ± 0.3
Range	11.3 – 15.5 × 21.2 – 25.4	12.7 – 19.8 × 28.2 – 32.5

Table 4 shows an estimate of dwarf ascospore frequency made not by scoring randoms but rather by scoring a large population of asci for number of ascospores per ascus and then calculating numbers of normals and dwarfs by using the ratio of the two kinds expected in each ascus category. The estimate obtained in this manner is 8.2 percent.

Figures 2 and 3 show 4-, 5-, 6-, 7-, and 8-spored asci collected from the cross 85A × 85a after extensive searching. The 5-, 6-, and 7-spored asci are oriented

TABLE 4

Determination of dwarf ascospore frequency by microscopic examination of all asci obtained intact from each of 20 perithecia. Cross: 85A × 85a

Observed number of ascospores per ascus	Observed number of asci	Percent	Expected number and type of ascospores per ascus		Calculated number and type of ascospores present		
			Normal	Dwarf	Normal	Dwarf	Total
4	807	87.3	4	0	3228	0	3228
5	80	8.7	3	2	240	160	400
6	32	3.5	2	4	64	128	192
7	5	0.5	1	6	5	30	35
8	0	0	0	8	0	0	0
Total	924				3537	318	3855
Percent normal ascospores					91.8		
Percent dwarf ascospores						8.2	

so that the dwarf ascospores in each ascus are uppermost in the photograph. The 5-spored ascus at higher magnification (Figure 4) shows more clearly the difference between ascospore types, as well as variation within a type (cf. uppermost normal ascospore with the two smaller normal ones beneath it). The rarity of the

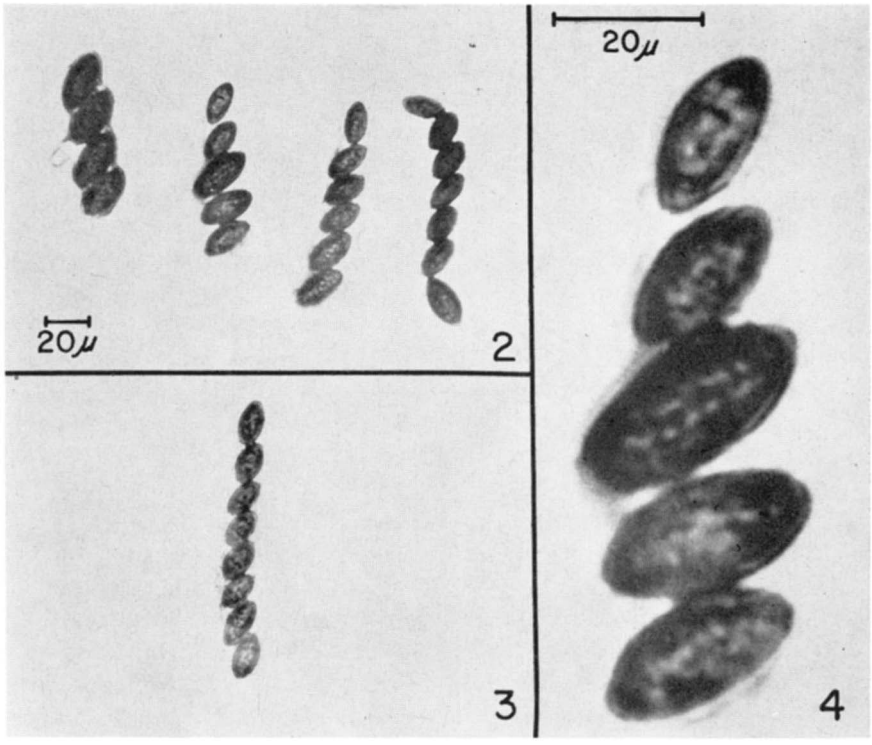

FIGURE 2.—Left to right, 4-, 5-, 6-, and 7-spored asci in one field; dwarf ascospores uppermost. FIGURE 3.—An 8-spored ascus, rarest supernumerary type. Same scale as Figure 2. FIGURE 4.—Enlargement of the 5-spored ascus in Figure 2. All asci from the cross 85A × 85a.

8-spored type is attested by its absence from Table 4. That this 8-spored ascus is not an *N. crassa* contaminant or a species-hybrid was confirmed by the fact that the eight derived mycelia produced abundant four-spored asci with *N. tetrasperma* tester strains, but showed poor fertility with *N. crassa* testers. Similar crossing tests with mycelia from the 5-, 6-, and 7-spored asci also verified their specific purity.

Dwarf ascospore production was found to be clustered rather than random in occurrence. The observed frequency of 5-spored asci was 0.087 (Table 4). Six-spored asci, resulting from two rather than one failure of pairwise delimitation of nuclei in an ascus, would be expected on a random basis to have a frequency of $(0.087)^2 = 0.0076$. Similarly, 7-spored asci would be expected with a frequency of $(0.087)^3 = 0.00066$. The observed frequencies of 6- and 7-spored asci, however, were repectively about five and eight times as great as expected.

Nuclear misarrangements: According to cytological observations of DODGE (1927) and COLSON (1934), nuclear passing normally occurs at both the second and third divisions in the developing ascus. Nuclear misarrangements, i.e., failure of passing to occur normally at either or both of these two divisions, however, might lead to homokaryosis interpreted as crossing over. Only two asci showed mating-type homokaryosis for all four ascospores (Table 1) that suggests possible nuclear loss, mating type postreduction, or nuclear misarrangements. Reasons have already been given as to why these two asci should probably not be interpreted as nuclear loss, but no decision can as yet be made about the relative likelihood of the other two events until additional data become available. Some or all of the apparent excess of Type III asci could have been caused by nuclear misarrangements, or by nonrandom nuclear loss, as pointed out previously.

DISCUSSION

SEAVER's (1937) study employed random ascospores exclusively but nevertheless provides good evidence for nuclear loss on statistical grounds. She found in a sample of 400 randoms of normal size that 22 of a total of 51 self-sterile isolates were homokaryotic for the marker *l*; this finding was unexpected, since *l* had shown homokaryosis only once in 900 self-fertile randoms in another experiment. The fact that *l* became homokaryotic frequently in self-sterile cultures but rarely in self-fertile cultures suggests that most of the homokaryosis for *l*, as well as the self-sterility, owed to nuclear loss. SEAVER suggested as possible causes of loss either the heat-shock used to induce ascospore germination or failure of nuclear division, which would eliminate one nuclear type from the complement. Causes, as well as the exact time of loss, are still undetermined.

The estimated dwarf ascospore fraction, 8.2 percent, emphasizes the advisability of employing a means of excluding either dwarfs or normals from any genetic analysis purported to be based upon only one or the other. A rapid method for selective ascospore screening has unfortunately not yet been devised. Stereomicroscopic selection, although slow, was evidently quite effective in avoiding dwarfs, since the frequency of self-sterility obtained was accounted for by nuclear loss or misarrangement (Table 3). Selection of over 1000 dwarfs in another study

was about 97 percent efficient in excluding normals, as judged by the fact that only about 3 percent self-fertility was obtained (HOWE 1964). In both instances the use of 60× magnification without an eyepiece micrometer was satisfactory.

In another study (HOWE 1963a) involving two markers which segregated as though unlinked to each other but as though close to their respective centromeres, one ascus in a sample of 11 had all four ascospores homokaryotic for both markers. The probability of this occurring by second division segregation of both loci simultaneously is very small owing to the proximity of both loci to their respective centromeres, and this ascus was therefore interpreted as resulting from nuclear misarrangements. Alternatively, the cause could have been nuclear loss, although loss of a nucleus in each of the four ascospores would have had to occur, and the present investigation has shown how unlikely such losses are. Studies on larger samples of asci are needed to determine nuclear misarrangement frequencies more accurately.

The best marker for minimizing errors by genetic means is the mating-type locus, because this marker is present in all stocks, is usually heterokaryotic in ascospores of normal size, and the loss of either allele may be detected visibly without conidial plating because of the resultant self-sterility. If tetrad analysis is restricted to the use of 4-spored asci, all of whose ascospores show self-fertility, then nuclear loss and uninucleate dwarfness would be precluded. This preclusion also applies to randoms. Since self-sterility in all members of an ascus may occasionally occur by legitimate mating type postreduction and perhaps also by nuclear misarrangements, however, it would be preferable in critical studies to use additional genetic markers unlinked to each other and to mating type but, like mating type, showing a high frequency of first division segregation. Asci with all ascospores self-sterile but heterokaryotic for one or more of the other markers would be validated; those showing self-sterility as well as homokaryosis for the other markers could be excluded. The present investigation suggests that few such exclusions would be necesssary.

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SUMMARY

Tetrad analysis in *N. tetrasperma* has revealed, with varying degrees of certainty, exceptional events which would, if undetected, bias genetic studies by simulating crossing over when none had actually occurred. These events are nuclear loss, presumably owing to nuclear lethality; production of dwarf ascospores which are uninucleate instead of binucleate; and nuclear misarrangements during ascus development. Nuclear loss and dwarf ascospore production may be excluded from experimental data by restricting analyses to ascospores whose derived mycelia show self-fertility. Nuclear misarrangements should be detectable in critical studies, if desired, by use of the mating type locus and additional centromere markers.

LITERATURE CITED

CATCHESIDE, D. G., 1951 *The Genetics of Microorganisms.* Pitman, London, England.

COLSON, B., 1934 The cytology and morphology of *Neurospora tetrasperma* Dodge. Ann. Botany **48**: 211–224.

DODGE, B. O., 1927 Nuclear phenomena associated with heterothallism and homothallism in the ascomycete, Neurospora. J. Agr. Res. **35**: 289–305. —— 1932 Crossing hermaphroditic races of Neurospora. Mycologia **24**: 7–13.

HOWE, H. B., JR., 1963 Markers and centromere distances in *Neurospora tetrasperma.* Genetics **48**: 121–131. —— 1963a Measuring fertility by genetically controlled ascospore yield in *Neurospora tetrasperma.* Can. J. Genet. Cytol. **5**: 180–188 —— 1964 Vegetative traits associated with mating type in *Neurospora tetrasperma.* Mycologia (in press).

SANSOME, E. R., 1946 Maintenance of heterozygosity in a homothallic species of the *Neurospora tetrasperma* type. Nature **157**: 484–485.

SEAVER, B., 1937 Additional data on sex reactions in monospore races of *Neurospora tetrasperma.* Mycologia **29**: 258–265.

WESTERGAARD, M., and H. K. MITCHELL, 1947 Neurospora. V. A synthetic medium favoring sexual reproduction. Am. J. Botany **34**: 573–577.