EVIDENCE OF NON RANDOM DISTRIBUTION OF ASCUS CLASSES IN FRUITING BODIES OF NEUROSPORA CRASSA

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THE observation of morphologically identical twins among immature and degenerate asci (MITCHELL 1959) has suggested that unpredicted genetic relationships may exist between adjacent asci and may be reflected by similarities in phenotypes of strains obtained by serial isolations of ascospores from mature asci. Subsequent genetic analyses have indicated that adjacently formed asci may, rather regularly, show similar or identical patterns of segregation of known markers. This paper reports further indication of such a relationship provided by the results of analysis of individual perithecia from an exceptionally fertile cross.

A second motive for the work to be described stems from an interest in ratios of ascus classes. The observation of preferred recombinant frequencies among random spores (MITCHELL and MITCHELL 1954; MITCHELL 1958) leads to the prediction that preferred ratios of segregation classes among asci occur and, indeed, some indication of such preference may be seen in existing ascus data (MITCHELL 1960). However, larger numbers of asci per cross are needed, particularly if linked markers are involved.

A third point to be discussed, concerning the order of spore pairs within the ascus, is raised by the data presented and has been considered earlier by CATCHESIDE (1944) and WHITEHOUSE and HALDANE (1945).

METHODS

The cross: The two markers followed are *pyr-3a* (pyrimidine, 37301) and *cot* (colonial, temperature-sensitive, C 102), both assigned to linkage group IV. Parents of the cross considered are not standard strains but segregants from an ascus produced by an earlier cross of *cot* $\times$ *pyr*. This ascus came from the only exceptionally fertile perithecium among about 30 examined. The *pyr* parent (37301-3250-4A) of the earlier cross has been used as a standard strain for several years. The *cot* parent, on the other hand, is a recombinant from a cross between two very closely linked markers of group I, with one parent expressing *cot*. Three generations of inbreeding, with the latter three markers present, had preceded isolation of the *cot* recombinant but, since it is not known that inbreeding affected the results to be reported, details will not be given.

The cross, *cot*-8025-1a $\times$ *pyr*-8025-7A, was made on unsupplemented WESTERGAARD and MITCHELL medium, with *cot* as protoperithecial parent, and left to

mature at room temperature (about 24°C) for 22 days from the time of "fertilization".

Dissection of asci: Ascus dissections were done on shallow plates containing about 20 ml of nitrate minimal medium (STRAUSS 1951) solidified with four percent agar. In the center region of the plate an ascus cluster was removed from its perithecium by first pinching a hole in the perithecial wall with sharp forceps and then squeezing the contents into a drop of water. As those who dissect *Neurospora* asci are well aware, the walls of mature asci are fragile, and it is therefore somewhat difficult to free the asci from the perithecium without rupturing them. The rather unusual thinness and softness of the walls of perithecia from this cross aided in the attempt to obtain the ascus cluster as nearly intact as possible. The spores of individual asci were then separated by moving them outward, serially, towards the border of the plate. The intention was to dissect adjacent asci consecutively, so that the positions of dissected asci on the plate would bear a relationship as close as could be obtained to their positions in the ascus cluster. However, since the cluster is more like a filled circle than a ring, exact duplication could not be realized. Also it was estimated that, at best, not more than a third to a half of the normal-appearing asci per cluster were tested.

Classification of segregants: The plates of spores were heat-treated at 60°C for 30 minutes and then incubated at room temperature (about 24°C) overnight. After this, two classes of segregants were scored under a dissection microscope. Spores that had produced only very short germ tubes were classified as *pyr* and those that had given rise to small "colonies" consisting of several infrequently branched hyphae, as *pyr*⁺. If no germ tubes or hyphae had been produced the spore was, of course, scored as ungerminated. About 1 ml of a uridine solution (1 mg/ml) was then poured on the plate and spread over the "colonies" by tilting. This rarely disturbs the positions of the germinated spores. During the next five hours at 34°C water from the uridine solution was absorbed or evaporated; the *pyr* segregants grew in response to uridine and the expression of *cot* appeared. The four types of segregants then scored were characterized as follows: ++—hyphae continue to elongate at 34°C and branch infrequently; + *cot*—hyphae do not elongate at 34°C but produce frequent, regularly spaced, short side branches; *pyr* +—one or more of the short germ tubes elongate to form hyphae which branch infrequently; *pyr cot*—the short germ tubes branch laterally to form a tiny, compact "colony".

DISCUSSION OF RESULTS

Tabulation of data: The numbers of asci of each class from each of the nine perithecia tested are shown in Table 1. The phenotypes are designated as follows: *r* = *pyr* +; *s* = + *cot*; *rs* = *pyr cot*; + = + +. All asci are included from which as many as five of the eight spores germinated and could be scored. This insures that at least one member of each of three of the four spore pairs of an ascus will be represented. Actually, germination was exceptionally good. From 282 asci scored only about nine percent of the spores failed to germinate; thus few had as many as three ungerminated spores. The classifications made are, there-

TABLE 1

Number of asci of each class from each of nine perithecia from + cot × pyr +. Phenotype designations are: r = pyr +; s = + cot; rs = pyr cot; + = + +

Ascus classes	Phenotypes of spore pairs				Ascus classes per perithecium									Totals
	1, 2	3, 4	5, 6	7, 8	1	2	3	4	5	6	7	8	9	
PD-1	1	r	r	s	s	3	8	8	9	9	7	10	12	78
	2	s	s	r	r		7	9	7	13	9	13	7	79
PD-2	1	r	s	r	s		1	1	3		4	1	2	14
	2	s	r	s	r	1		1	1	3	2	2	2	15
	3	r	s	s	r	2		1	2	2	1		2	12
	4	s	r	r	s	1			2	2	2	1	1	11
T-1	1	r	rs	+	s			4	1			1		7
	2	rs	r	s	+			1	1	1	1	2	1	9
	3	r	rs	s	+				1		1	1		4
	4	rs	r	+	s					1	1	1		4
	5	+	s	r	rs					3	1	1		6
	6	s	+	rs	r			2	2	1	1	1		8
	7	+	s	rs	r			1	3	2	1	1		12
	8	s	+	r	rs			2		1	1	1	2	9
T-2	1	s	rs	+	r									
	2	rs	s	r	+									
	3	s	rs	r	+									
	4	rs	s	+	r									
	5	+	r	s	rs				1		1	2	1	5
	6	r	+	rs	s									
	7	+	r	rs	s								1	1
	8	r	+	s	rs									
T-3	1	r	s	rs	+									
	2	s	r	+	rs				1					1
	3	r	s	+	rs					1		1		3
	4	s	r	rs	+	2								2
	5	rs	+	r	s									
	6	+	rs	s	r							1		1
	7	rs	+	s	r					1				1
	8	+	rs	r	s									

fore, thought to be adequate for the present purpose, but the frequency of failure of 1:1 segregation will not be considered. The occasional irregular asci have been placed in the classes they most nearly resemble.

In Table 2 the asci, designated by the classes shown in Table 1, are listed in the order in which they were isolated. Those which could not be scored are designated by NS.

Twin ascus phenotypes: Admittedly the method employed to detect twin phenotypes is a crude one. Observation of immature asci at magnifications high enough to allow detection of twin morphologies has shown that when the ascus

TABLE 2

The order in which asci were isolated from each perithecium. The ascus class designations used are those shown in Table 1. NS = not scored

P-1	P-3 cont.	P-4	P-4 cont.	P-5 cont.	P-6 cont.	P-7	P-7 cont.	P-8 cont.	P-9 cont.
2T-3-4	NS	2NS	PD-1-2	2PD-1-1	PD-1-1	PD-1-1	PD-1-1	3PD-1-1	PD-1-1
PD-2-2	PD-1-2	T-1-7	T-1-6	PD-2-3	PD-2-3	T-1-3	T-1-2	T-1-2	PD-1-2
PD-2-4	PD-1-1	T-1-3	T-3-2	T-1-4	PD-1-1	2PD-1-1	PD-1-2	T-1-4	T-1-3
2PD-1-1	PD-1-2	T-1-1	2PD-2-1	PD-1-1	PD-1-2	PD-2-1	PD-1-1	PD-1-1	T-1-7
2PD-2-3	PD-2-2	PD-1-1		NS	NS	T-3-3	T-1-4	T-1-6	2PD-1-1
PD-1-1	NS	NS	P-5	PD-2-2	T-1-2	PD-2-4		3PD-1-1	T-1-8
	2T-1-1	2PD-2-3	T-2-5	PD-1-2	PD-2-2	NS	P-8	PD-2-2	T-2-7
P-2	PD-1-1	2PD-1-2	PD-2-4	PD-1-1	T-1-3	T-1-1	PD-1-1	PD-1-1	PD-2-3
3PD-1-1	2T-1-1	PD-1-1	PD-1-2	PD-1-2	PD-1-2	3PD-1-2	PD-1-2	PD-2-1	PD-1-1
PD-1-2	PD-1-2	T-1-7	NS	NS	T-1-4	NS	T-3-3	T-1-2	T-1-1
PD-2-1	NS	PD-1-2	PD-1-2	2PD-1-2	PD-1-1	PD-1-1	T-1-8	PD-2-1	PD-1-2
PD-1-1	2PD-1-1	T-1-2	PD-2-2	2T-1-7	T-1-7	PD-1-2	T-2-5	PD-2-2	2PD-1-1
PD-1-2	2PD-1-2	PD-1-1	2PD-1-1	PD-1-2	PD-2-1	T-1-6	PD-1-1		PD-2-3
PD-1-1	PD-1-1	PD-2-4	PD-2-2	T-1-5	PD-1-2	PD-1-2	PD-1-2	P-9	T-2-5
2PD-1-2	T-1-6	PD-1-1	T-1-8	NS	T-1-5	2PD-1-1	PD-1-1	PD-2-2	T-1-7
2PD-1-1	T-1-2	PD-2-4	PD-2-4	PD-1-2	PD-1-2	T-1-7	PD-1-2	PD-1-1	PD-2-4
PD-1-2	T-1-6	PD-2-2	2PD-1-2	PD-2-3	T-1-6	T-1-8	T-3-6	2PD-1-2	NS
PD-1-1	PD-1-2	PD-1-1	PD-1-1	PD-1-1	PD-1-1	4PD-1-2	PD-1-2	T-1-7	PD-2-2
2PD-1-2	PD-2-3	2PD-1-2	NS	NS	PD-1-2	PD-2-2	PD-2-4	PD-1-2	PD-1-1
	T-1-7	NS	T-1-6	T-1-2	PD-1-1	PD-1-2	PD-1-1	T-1-2	PD-2-4
P-3	2NS	PD-2-1	T-1-5	PD-1-2	2PD-1-2	PD-1-1	T-1-8	6PD-1-2	PD-2-1
T-1-8	PD-1-2	PD-1-1	PD-1-2		2PD-2-1	PD-1-2	PD-1-2	PD-2-3	3PD-1-2
PD-1-2	PD-2-1	PD-1-2	PD-1-1	P-6	PD-2-4	T-1-5	T-2-5	2PD-1-1	PD-1-1
NS		2PD-1-1	NS	PD-1-1	PD-2-1	PD-2-2	PD-2-3	T-1-8	PD-2-1
T-1-8		NS	T-3-3	PD-1-2	T-1-8	PD-1-2	PD-1-2	NS	
PD-1-2		PD-1-1	T-1-5	PD-1-1	PD-2-4	PD-1-1	PD-2-3	T-1-7	
3NS		T-1-6	NS	PD-1-2	T-3-7	T-1-2	PD-1-2	PD-1-1	
3PD-1-1		T-1-7	PD-1-2	PD-2-2		T-2-5	NS	T-1-5	

cluster is placed on a flat surface the members of a twin pair may, perhaps more often than not, be separated by several other asci and be adjacent only at their attached ends. However, the coincidences, shown in Table 2, of the same T-1 or PD-2 subclass are thought to indicate twinning. In these two main classes of intermediate frequency there are 30 cases in which more than one ascus of a subclass was obtained from the same perithecium and in 21 of these cases two of the same subclass were isolated consecutively or were separated by one, two, three or four asci of another type. Also, if the two T-3-4 asci are not twins then their consecutive isolation from the same perithecium would appear to represent an unlikely event.

This indication of adjacently located twin phenotypes, coupled with the frequent observation of degenerate twins, both showing the same symptoms of degeneracy, leads to the conclusion that the phenotypes of twins must be determined simultaneously for both members by the same events. Such a conclusion

conflicts, of course, with current hypotheses which attribute the determination of ascus phenotype to segregations occurring at meioses assumed to take place in individual asci which, prior to meiosis, represent zygotes, each consisting of a single cell with a single diploid nucleus. If this were the situation, the segregation pattern of any ascus would be independent of that of its neighbors in the ascus cluster. The regular observation of twin asci seems, therefore, to be irreconcilable with the hypothesis that meiosis occurs in preformed asci.

Other observations concerning the structure of immature asci also make this hypothesis appear unlikely (MITCHELL 1960). When seen with phase contrast, the ascus content appears to consist of several hyphae which have associated intimately with one another to form the spores and their matrices. Their proximity during development suggests that a twin pair may be formed by the same group of hyphae, the twin phenotypes being determined by the genetic constitutions of these hyphae.

Spore pair order: Although indications of non randomness have been reported (CATCHESIDE 1944; WHITEHOUSE and HALDANE 1945) the various subclasses within a main ascus class, for example PD-1-1 and PD-1-2, have usually been assumed to be equally probable, reflecting merely the various possible positions of the four nuclei following meiosis in the ascus. However, from the results with respect to the tetatype-2 class it seems rather unlikely that, in this cross, the eight T-2 subclasses were equally frequent. If this were so, then the probability of getting the result observed, that is, five of the six T-2 asci in one subclass and one in another would be $1/6250$. A hitherto unrecognized significance of spore pair order seems, rather, to be suggested.

The observation that four of the T-2 asci were isolated consecutively or second consecutively with T-1-8 or T-1-7 has given rise to the speculation that the T-2 and T-3 asci do not constitute separate segregation classes but, rather, spore-pair rearrangements of T-1. This speculation has not led to a simple explanation of the improbable distribution of the T-2 subclasses since pursuit of it in this direction seems to require that significance be attached to the suggested deficiency of T-1-3 and T-1-4 asci. However, it also seems necessary to assume that a particular type of spore-pair rearrangement is more likely and this possibility is regarded as interesting because it might reflect unknown details of ascus construction. Nonlinear asci are not uncommon, particularly in some crosses. Such asci can be observed to attain linearity through rearrangement and the relationship of the spores to their matrices may be such as to favor pair-wise rearrangement. The possibility that the T-2 and T-3 asci arose by rearrangement is, of course, also interesting in connection with the conventional interpretation of them as segregation patterns produced by double crossovers.

Ratios of ascus classes: The relative frequencies of the ascus classes shown in Table 1, parental ditypes 1 and 2, tetratypes 1, 2 and 3 (BARRATT, NEWMAYER, PERKINS, and GARNJOBST 1954) have formerly been interpreted as reflecting the frequencies of crossing over between the loci of the two markers, if they show linkage, and between these loci and the centromere of their chromosome. Such an interpretation of the data presented would place *cot* about 24 map units from the

centromere with *pyr* almost equidistant both from the centromere and from *col*. However, the ratio of parental ditypes to tetratypes, 209:73, is very nearly 3:1 while the PD-1 to PD-2 ratio, 157:52, is almost precisely 3:1. This result is viewed with considerable interest because the sample is larger and was more uniformly classifiable than average and because ratios suggested by other data have already led to the suspicion that such a result may be indicative of something other than a very tidy spacing of genes along the chromosome (MITCHELL 1960).

The possibility in mind is that the apparent linkage is due, not to physical linkage between the loci of two single-factor characters but rather, to the presence of a larger number of independently segregating factors which affect expression of the phenotypes scored. It may be seen that the frequency of wild segregants from the asci tabulated was very nearly 6.25 percent, the expected frequency of wilds from a cross involving four unlinked markers, if meiosis of a fusion nucleus precedes ascospore formation. It is also the expected frequency, in the absence of linkage, of the double recessive class in the F_2 of a dihybrid cross in diploid organisms. Other observations commonly made also suggest involvement of a larger number of factors. Often, within a phenotype class there may be seen differences in the degree of expression such as to suggest that the phenotype is arrived at in several different ways. Also, as might be expected if this is true, recombinant frequencies from crosses involving different isolates of the same markers often vary, and there are indications that the variation is discontinuous (MITCHELL 1958). Crosses of segregants from the asci described here are being studied in order to learn more about shifts in ascus class ratios.

Inheritance of perithecial characters: It is commonly observed that perithecia from the same cross vary with respect to such details as size, thickness of walls, morphology of asci and spores, proportions of normal and degenerate asci, and viability of spores. Observations made in connection with the cross described here indicate that at least some of these traits are heritable, although not in an obviously straightforward way. As mentioned above, the parents were obtained from the only highly fertile perithecium among about 30 from the first cross. Also, germination of spores from the exceptional perithecium was much more nearly complete than that of spores from a few of the less fertile ones. In the daughter cross the exceptional fertility and spore viability remained, but differences between perithecia with respect to ascus morphology were seen. Intra-ascus crosses of segregants from perithecia 8 and 9, which showed different ascus morphologies, have now been made and, in each case, characteristics associated with the parent perithecium were again observed. This may be useful since it suggests the possibility of selecting, for a given cross, desired attributes of perithecium and ascus morphology. It is also interesting because it suggests segregations of genetic factors prior to perithecium formation. This recalls characteristics of vegetative growth of certain strains discussed earlier (MITCHELL 1959). These characteristics include regularly occurring and heritable shifts in phenotypic expression and general occurrence of sectoring in colonies from single conidia or ascospores.

In data from other crosses from which individual perithecia were analyzed

indications have been seen that the perithecia of one cross may differ with respect to the relative frequencies of ascus classes. In the data given here there is little to suggest such differences. Perithecium 2 may have been exceptional but the sample is too small to allow a decision. However, it seems possible that in crosses between less closely related strains the occurrence of differences of this sort may tend to blur both progeny and ascus class ratios.

SUMMARY

Asci from an exceptionally fertile cross were analyzed in a way which is thought to indicate strongly that pairs of asci formed adjacently in the perithecium may be regularly identical with respect to patterns of segregation of known markers. This result indicates a need for revision of current hypotheses attributing the determination of ascus phenotypes to segregations occurring at meiosis in preformed asci.

The occurrence of certain ascus classes in almost precise 3:1 ratios suggests, along with other observations briefly discussed, that recombinant frequencies of less than 50 percent from two-marker crosses may not necessarily indicate physical linkage. Even though the progeny can be placed in the expected four main phenotypic classes, the possibility of several independently segregating factors having related and interacting effects on phenotypic expression seems an interesting one to consider.

The observation of a rather striking preference for one particular spore pair order among eight supposedly possible ones suggests that the spore pair order may have significance with which it is not ordinarily credited. The more probable orders within twin ascus pairs appear to be identical or exactly reversed. This is thought, perhaps, to reflect some detail in the mechanism of ascus formation but to be complicated by spore pair rearrangements within the ascus.

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