GENES INFLUENCING SELECTIVE FERTILIZATION IN *NEUROSPORA CRASSA*

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ABSTRACT

The mutual attraction of conidia to protoperithecia of the opposite mating type was studied genetically in crosses where a mixture of conidia from two different strains, one of which was marked by an ascospore color mutant gene tan spore (*ts*), was applied to protoperithecia. Selective fertilization was measured as the frequency of perithecia fertilized by conidia from one strain in competition with conidia from another strain. Selective fertilization by a given strain varied throughout the range from 10 to 97% according to the strains of protoperithecial parent. The selective fertilization was revealed to be under the control of two or more loci, which appeared to have multiplicative action. No indication of a cytoplasmic effect on selective fertilization was obtained. The strength of the mutual attraction between conidia and protoperithecia decreased as genetic similarity increased.

ALTHOUGH *Neurospora crassa* is genetically one of the best known eucaryotic organisms, the detailed mechanisms of fertilization are not fully understood. Study of the mutual attraction between conidia and protoperithecia of opposite mating types had been hindered by lack of appropriate methods of analysis. A previous study (NAKAMURA and EGASHIRA 1961), using an ascospore color mutant marker, revealed that the majority of protoperithecia were each fertilized by a single conidium, and suggested that one genetic strain had a selective advantage over the other in successful fertilization of protoperithecia.

In this paper an ascospore color mutant is utilized: (1) to detect the presence of a genetic basis for selective fertilization of protoperithecia by conidia with different genetic backgrounds, and (2) to characterize the nature of this genetically controlled selective fertilization.

MATERIALS AND METHODS

Measurements of selective fertilization were based on frequencies of two types of perithecia by a cross, where a conidial suspension consisting of a mixture of two types of conidia, one from strain 4*A* (*ts*) and the other from various wild-type strains of mating type *A*, was applied to protoperithecia from a given wild-type strain of mating type *a*.

Strains: Strain 4*A* (*ts*) carries a mutant gene tan spore (*ts*, linkage group V) which is a spontaneous mutation in strain 4*A* and causes the ascospore to be tan instead of the normal black

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(NAKAMURA 1961). The wild-type strain, P2*a*, was derived, by Dr. D. D. PERKINS, from St. Lawrence wild types ST4*A* and ST73*a* by a series of inter- and back crosses (cf. NAKAMURA 1966). Wild-types KE1*A*, KE2*A*, KE3*A*, KN8*A* and KN48*A* are isolates from a cross between 4*A*(*ts*) and wild-type 8*a*. The 4*A* and 8*a* were originated by Dr. G. W. BEADLE and obtained from Nagao Institute, Tokyo (cf. A List of Cultures, maintained in the Japanese Type Culture Collection, Nagao Institute, Tokyo, 1950).

Crossing method: WESTERGAARD and MITCHELL's (1947) synthetic crossing medium was used. Fertilization was accomplished by applying conidial suspensions in sterile distilled water to protoperithecia on 7-day old cultures in Petri dishes. The numbers of protoperithecia of the *a* cultures were determined by sampling prior to the application of the conidial suspension. For preparation of the conidial suspension, cultures were harvested after 7 days incubation in test tubes containing BEADLE and TATUM's (1945) minimal medium, and filtered with a glass wool filter to remove hyphae. The number of conidia per unit volume was determined using the Thoma blood cell counter. The mixed suspension of conidia was made of approximately equal numbers of conidia from 4*A*(*ts*) culture and from a wild-type culture of mating type *A*. Throughout the paper, for convenience, such mixtures of conidia are represented by strain symbols connected by a plus (+), e.g., 4*A*(*ts*)+KE1*A*. Unless otherwise indicated, the ratio of numbers of conidia to protoperithecia was adjusted to approximately 100–110 : 1. Application of the suspension was carried out within 5 min after it was prepared to prevent possible formation of heterocaryons by germinating conidia. All cultures were incubated in darkness at 25°C.

Scoring method: The method of dissection of mature perithecia was as described by NAKAMURA (1966). Selective fertilization was measured by counting two types of perithecia, one type containing asci segregating for ascospore color, resulting from fertilization by conidia of strain 4*A*(*ts*), and the second type containing non-segregating asci, resulting from fertilization by wild-type conidia. All the perithecia examined had a single ostiole. Those with two or more ostioles due to the fusion of two separate trichogyne systems were discarded. Occasionally, perithecia were found to contain both segregating and non-segregating asci. These genetically mixed perithecia, due to fertilization by two or more conidia, were recorded but excluded from the tabulation of results. The frequency of such mixed perithecia, 0.5 — 2.0%, was in good agreement with that reported by NAKAMURA and EGASHIRA (1961).

RESULTS

Effect of density of the conidial suspension on selective fertilization: One would expect that selective fertilization, if any, would be more pronounced as the density of the conidial mixture per protoperithecium increases. To test this, a mixed suspension of approximately equal numbers of conidia from strain 4*A*(*ts*), which carries a mutant gene tan spore (*ts*), and from strain KE1*A* was diluted to make three different densities of conidia. These suspensions were used to fertilize protoperithecia from P2*a* cultures.

The frequencies of perithecia resulting from fertilization by conidia of strain 4*A*(*ts*) which competed with KE1*A* conidia were determined by examination of about 100 perithecia from each of ten replicate plates for each density of conidia. Although the two kinds of conidia were present in equal numbers at all densities, strain 4*A*(*ts*) conidia showed much greater success in fertilization of protoperithecia than did strain KE1*A* conidia. The proportion of successful fertilizations by strain 4*A*(*ts*) were 72.8%, 79.6%, and 88.5% at densities of 5, 20, and 75 conidia per protoperithecium respectively. The values increased significantly ($P < 0.001$) for each increased density. There was no significant difference (at 95% confidence limits) among the replicates for each conidial density.

Nuclear control of selective fertilization: To determine the nature of possible genetic control of the selective fertilization, a suspension containing strain 4*A*(*ts*) and strain KE2*A* conidia was used to fertilize protoperithecia from (1) ten replicates of P2*a*, (2) ten random spore isolates, of mating type *a*, from a cross P2*a* (protoperithecial parent) × KE2*A* (conidial parent), and (3) ten random spore isolates, of mating type *a*, from a cross KE2*A* (protoperithecal parent) × P2*a* (conidial parent). This also provided the means to examine the possibility of extrachromosomal control of the selective fertilization by comparing the frequencies of offspring obtained from the reciprocal crosses fertilized by the strain 4*A*(*ts*) conidia (crosses 2 and 3).

The results, expressed as percentages of perithecia resulting from fertilization by 4*A*(*ts*) conidia, are given in Table 1a. Although there was restricted variation in the frequencies among the ten replicates of P2*a* × 4*A*(*ts*)+KE2*A*, the frequencies in the remaining two sets of crosses were significantly heterogeneous. This would strongly suggest that the segregation and reassortment of some inherent factor(s) influencing the selective fertilization occurred when the reciprocal crosses were made.

To substantiate this hypothesis, three of the isolates from each of the reciprocal crosses (P2*a* × KE2*A* and KE2*A* × P2*a*) were crossed with the 4*A*(*ts*)+KE2*A* conidial mixture. The results (Table 1b) confirm the restricted variation in frequencies among ten replicates from any given protoperithecial parent, although the three values from each set of reciprocal crosses were significantly different from each other ($P < 0.001$ except for comparisons between cross 5 and 6 where $P < 0.01$).

The lack of significant difference in mean frequencies between the two sets of crosses (2 and 3) and the similarity in distribution of the frequencies among the isolates of the two sets indicate that cytoplasmic effects, if any, do not play a significant role in determining selective fertilization. Therefore, we assume that selective fertilization is under the control of nuclear gene(s), although this does not mean that cytoplasmic effects should be completely ruled out on the basis of this one experiment.

Multilocus control of selective fertilization: If the selective fertilization is controlled by a single pair of alternate alleles or multiple alleles at a single locus, a bimodal distribution of resulting perithecial frequencies would be expected in the type of crosses shown in Table 1a (crosses 2 and 3). The frequencies, however, show at least three significantly different values, among each set of reciprocal crosses as indicated in the previous section (see Table 1), which suggests the alternative hypothesis that the frequency could be affected by genes at several different loci.

To confirm the latter hypothesis a mixture of conidia from 4*A*(*ts*) and KN8*A* cultures was applied to protoperithecia from 50 cultures of random spore isolates from a cross of KN8*A* × P2*a*, and, as a control, from 24 replicate cultures of strain P2*a*. The frequency of perithecia which were fertilized by 4*A*(*ts*) conidia was determined (Figure 1). The random isolates from KN8*A* × P2*a* gave heterogeneous frequencies ranging from 23.7% to 97.0%, with a single mode about 30–

TABLE 1

The effect of different strains of protoperithecial parents on selective fertilization

	Cross(es)		Percentage of perithecia fertilized by strain 4A(*ts*)											
			Plates*											
	Protoperithecial parent	Conidial parent	1	2	3	4	5	6	7	8	9	10	Mean	Homogeneity P
a†														
	1. P2*a*	4A(*ts*)+KE2A	68	64	64	63	63	61	60	57	53	53	60.6	>0.05
	2. 10 isolates from a cross P2*a* × KE2A	4A(*ts*)+KE2A	90	84	81 (#1)	75 (#2)	74	67	56 (#3)	55	51	48	68.1	<0.001
	3. 10 isolates from a cross KE2A × P2*a*	4A(*ts*)+KE2A	87	84 (#4)	82	80	72	71 (#5)	71	63	51	51 (#6)	71.2	<0.001
b‡														
	4. P2*a*	4A(*ts*)+KE2A	72	71	70	67	67	64	63	62	58	56	65.0	>0.05
	5. #1	4A(*ts*)+KE2A	83	81	80	78	77	74	74	72	71	70	76.0	>0.05
	6. #2	4A(*ts*)+KE2A	80	74	72	69	69	68	67	67	66	66	69.8	>0.05
	7. #3	4A(*ts*)+KE2A	70	67	66	65	65	63	61	60	57	52	62.6	>0.05
	8. #4	4A(*ts*)+KE2A	90	88	87	87	87	87	86	85	83	81	86.1	>0.05
	9. #5	4A(*ts*)+KE2A	87	84	83	81	80	78	77	76	74	74	79.4	>0.05
	10. #6	4A(*ts*)+KE2A	68	67	67	64	62	61	59	58	52	50	60.8	>0.05

* Crosses were made in 10 replicates except for crosses 2 and 3 where the 10 plates represent 10 different crosses involving 10 different isolates. For convenience, the results are arranged from high to low percentages, although collection of the data was random. The percentage was based on examination of about 100 perithecia per plate.

† #1–#6 stand for isolates utilized in the crosses in (b).

‡ The total frequency in each cross is not significantly different (at 95% confidence limits) from that in the corresponding crosses in (a), except for cross 10 where P is approximately 0.05.

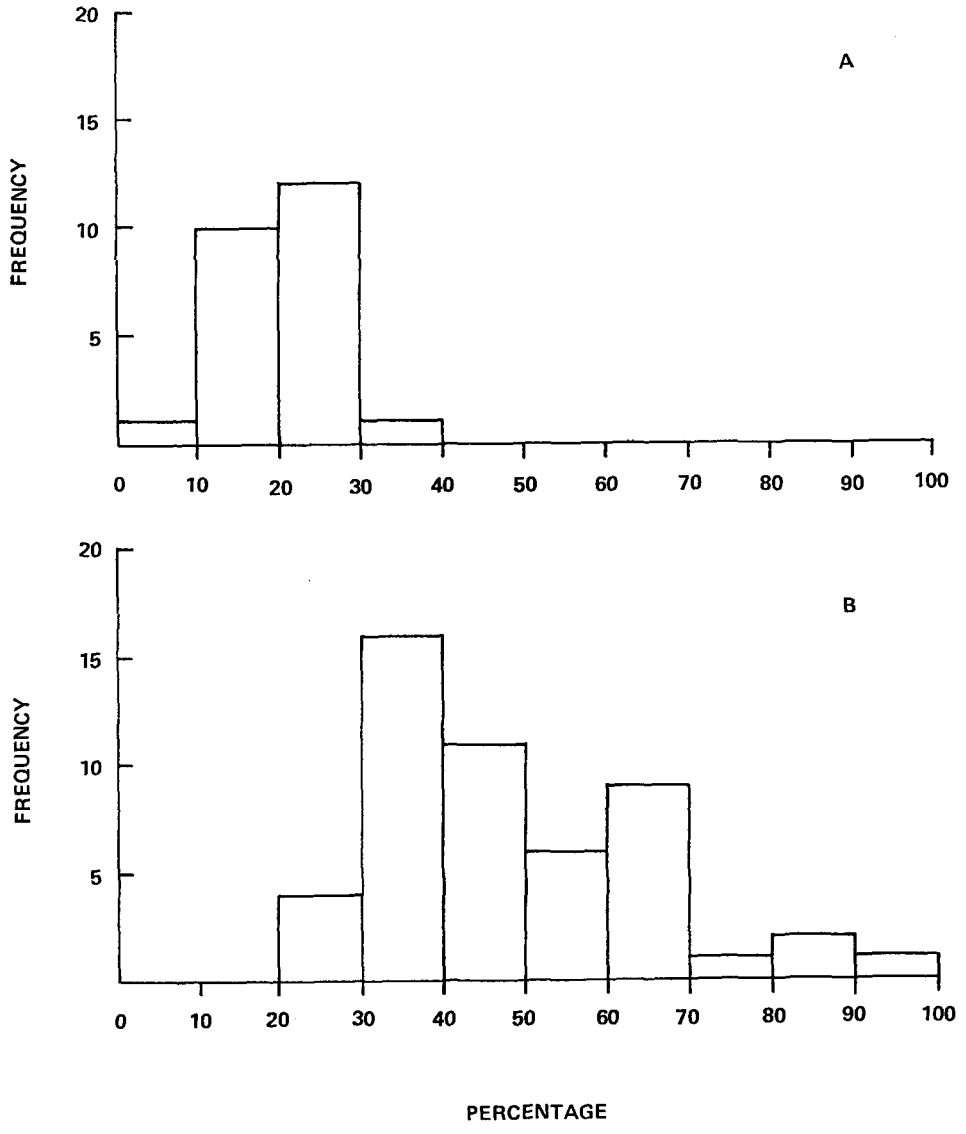

FIGURE 1.—Percentage distribution of perithecia fertilized by 4*A*(*ts*) conidia in competition with KN8*A* conidia, using (A) 24 replicate plates of a P2a culture and (B) 50 plates of different random isolate cultures from a cross KN8*A* × P2*a*, as protoperithecial parents. The percentage was based on examination of about 100 perithecia per plate.

40% and a mean of 47.6%, while the control P2*a* cultures gave a homogeneous distribution in frequency, ranging from 9.5% to 32.3% with a mode about 20% and a mean of 20.9%. This does not support the hypothesis of single locus control, which would predict two modes, one of which would be at the frequency of the control cross.

The mean frequency of successful fertilization by KN8*A* conidia was signifi-

TABLE 2

Effect of genetic similarity on selective fertilization

	Cross(es)		Percentage of perithecia fertilized by strain 4A(*ts*)											
			Plates*											
	Protoperithecial parent	Conidial parent	1	2	3	4	5	6	7	8	9	10	Mean	Homogeneity P
a														
1.	P2*a*	4A(*ts*)+KE3A	35	32	32	30	29	28	23	21	21	20	27.1	>0.05
2.	10 isolates from a cross P2*a* × 4A(*ts*)	4A(*ts*)+KE3A	23	23	19	12	11	7	5	1	1	0	10.2	<0.001
3.	10 isolates from a cross P2*a* × KE3A	4A(*ts*)+KE3A	74	58	57	57	44	41	36	28	24	16	43.5	<0.001
b														
4.	P2*a*	4A(*ts*)+KN48A	58	56	54	53	48	46	46	44	44	42	49.1	>0.05
5.	10 isolates from a cross P2*a* × 4A(*ts*)	4A(*ts*)+KN48A	51	41	39	36	35	33	32	29	16	—	34.7	<0.001
6.	10 isolates from a cross P2*a* × KN48A	4A(*ts*)+KN48A	73	72	69	60	57	57	57	56	52	51	60.4	<0.01

* For crosses 1 and 4 each cross was made in 10 replicates and in the remaining crosses the 10 plates represent 10 different crosses involving 10 different isolates. For convenience, the results are arranged from high to low percentages, although collection of the data was random. The percentage was based on examination of about 100 perithecia per plate.

cantly lower in the backcross (52.4%) than in the control (79.1%), suggesting a decrease in attraction between conidia and protoperithecia as genetic similarity increased. Similar results can be seen in Table 1a.

Effect of genetic similarity on selective fertilization: The effect of genetic similarity on the selective fertilization was further tested by crossing 4*A*(*ts*)+KE3*A* conidia with protoperithecia from (1) ten replicates of P2*a* as a control, (2) ten random spore isolates, of mating type *a*, from a P2*a* × 4*A*(*ts*) cross, and (3) ten random isolates, of mating type *a*, from a P2*a* × KE3*A* cross. The isolates used for the backcross involving P2*a* × 4*A*(*ts*) were limited to those carrying ts^+, since ascospores carrying the *ts* were inviable (NAKAMURA 1961).

The results, expressed as percentages of perithecia resulting from fertilization by 4*A*(*ts*) conidia, are given in Table 2a. In comparison to the control (27.1%), the isolates from the P2*a* × 4*A*(*ts*) cross showed a significant ($P < 0.001$) decrease in mean frequency (to 10.2%), while the isolates from the P2*a* × KE3*A* cross showed a significant ($P < 0.001$) increase in mean frequency (to 43.5%) of protoperithecia fertilized by 4*A*(*ts*) conidia. In one isolate (cross 2) the 4*A*(*ts*) conidia were completely excluded from fertilization of protoperithecia.

A similar experiment, using strain KN48*A* instead of strain KE3*A* confirmed the above results (Table 2b). These results support the hypothesis that attraction between conidia and protoperithecia decreases as genetic similarity increases.

DISCUSSION

The crossing experiments reported in this paper establish the occurrence of selective fertilization of protoperithecia by conidia from different strains in *Neurospora crassa*.

If the cause of the selective fertilization were due to decreased function of the conidia of one strain, which predetermines the competition, one would expect a uniform reduction in the frequency of fertilization by that strain in all crosses. The variation in selective fertilization due to the different protoperithecial parents (Tables 1 and 2; Figure 1) makes this possibility highly unlikely. The possibility that disproportionately larger average number of nuclei per conidium in one strain would predetermine the competition has been ruled out for the same reason, and from a previous observation (NAKAMURA and EGASHIRA 1961) that selective fertilization occurred when similar average numbers of nuclei per conidium in each of the two components of a conidial suspension was used. Alternatively, it appears that the selective fertilization is a consequence of a genetically based mutual attraction between conidia and protoperithecia. The physiological mechanisms of the observed selective fertilization are unknown.

The exact number of genes responsible for the selective fertilization cannot be estimated from the present data. However, the presence of many separate genes is suggested from the overall distribution of the frequencies of selective fertilization. In the set of crosses shown in Figure 1, these frequencies occurred throughout the range from 10 to 97% of the perithecia, depending upon the protoperithecial strains.

A skewed distribution of the frequencies shown in Figure 1 can be converted to a normal distribution by transforming the data to a logarithmic scale. This would suggest that the action of the genes influencing the selective fertilization is multiplicative rather than additive.

As to the multilocus control of the selective fertilization, it is possible that certain homoallelic combinations of genes between conidia and protoperithecia suppress the attraction between two types of gametes. This could account for the decrease in selective fertilization of protoperithecia by conidia of a genetically similar strain (Tables 1a and 2; Figure 1). Unfortunately, no data involving successive backcrosses are available in the present study. It is interesting to note studies on crossing-over frequencies in *N. crassa*, where the frequencies are under control of multiple loci and are enhanced as genetic similarity increases (STADLER and TOWE 1962; NAKAMURA 1966).

Although the location of the genes affecting the selective fertilization is unknown, it seems probable that at least most of the genes which in a homoallelic combination suppress the attraction between gametes are not closely linked to the mating type locus. All of the isolates from KN8*A* × P2*a*, when crossed with a 4*A*(*ts*)+KN8*A*, conidial mixture, gave frequencies of perithecia fertilized by 4*A*(*ts*) higher than that of the control parental cross, i.e. P2*a*, crossed with a 4*A*(*ts*)+KN8*A* conidial mixture (Figure 1). If the genetic region closely linked to the mating-type locus were responsible for changes in selective fertilization, then the results for most of the isolates (Figure 1B) would have shown the parental frequency (Figure 1A), since the isolates were selected for mating type *a*. Similarly, it is not likely that the genes involved in selective fertilization are closely linked to the *ts* locus. Otherwise, one would expect the mode in frequency distribution of the isolates from a cross P2*a* × 4*A*(*ts*), fertilized by the 4*A*(*ts*)+KE3*A* or 4*A*(*ts*)+KN48*A* conidial mixtures (Table 2), to be at the parental mode (P2*a* × 4*A*(*ts*)+KE3*A* or P2*a* × 4*A*(*ts*)+KN48*A*), since the isolates were selected for ts^+ as well as mating type *a*.

The genes governing selective fertilization resemble the mating-type alleles in that differences promote crossing and that this would tend to promote outbreeding and discourage inbreeding in a competitive situation. This is the opposite of the action of vegetative incompatibility alleles with respect to heterocaryon formation, where genetic similarity promotes stable hyphal fusions in *N. crassa* (WILSON and GARNJOBST 1966). Stable heterocaryons are not a prerequisite to crossing in this species. Thus, as BUTCHER (1968) states, the sexual cycle may actually be favoured by the fact that the heterocaryon is at a disadvantage.

The observed correlation between genetic similarity and lower value of selective fertilization might provide a simple method to determine genetic relationships among strains of different origins, provided they produce ascospores with normal pigmentation in crosses with standard *N. crassa* wild types.

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