HETEROCARYOSIS AND THE PARASEXUAL CYCLE IN ASPERGILLUS FUMIGATUS[1]

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THE parasexual cycle in filamentous fungi is initiated by the formation of a heterocaryon, usually involving haploid strains differing in nutritional requirements and, if possible, in color or colonial morphology. Consequently, genetically dissimilar nuclei are intermingled in a common cytoplasm. A "fusion" of such nuclei may occur to give a heterozygous nucleus which usually may be captured by plating conidia from the heterocaryon on a defined medium. A non-sectoring prototrophic colony with the color or morphology of the wild-type haploid strain is diploid. During the vegetative multiplication of nuclei in a diploid strain, somatic crossing over or nondisjunction leading to haploidization or both may occur so that conidia eventually may be obtained which have either a diploid nucleus homozygous for certain gene markers or a haploid nucleus containing new combinations of gene markers. Disomic nuclei may also be involved either in somatic crossing over or in haploidization or both. The discovery of the parasexual cycle and its successful exploitation for genetic studies in *Aspergillus nidulans* (PONTECORVO, ROPER, HEMMONS, MACDONALD and BUFTON 1953) provided the methodology for similar investigations in other species of filamentous fungi, particularly those lacking a perfect stage.

A preliminary study of the genetics of color mutants with different nutritional deficiencies in four diploid strains of *A. fumigatus* by means of the parasexual cycle indicated that the frequency of haploid segregants with parental genotypes was generally not greater than that of haploid segregants with recombinant nuclei (BERG and GARBER 1962). One linkage group involving three genes was detected. This paper presents additional data obtained from 17 diploid strains which suggest that an unequivocal demonstration of linkage groups in this species by means of the parasexual cycle may not be possible. Data are also presented to illustrate the possible technical difficulties which may attend each step in demonstrating the parasexual cycle in *A. fumigatus*.

MATERIALS AND METHODS

A colony from a single condium of strain 188 of *A. fumigatus* Fres. provided the initial stock culture used in this investigation. We are indebted to DR. W. R.

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MARTIN, Department of Microbiology, University of Chicago, for this strain. Cultures exhibited excellent growth and produced numerous green uninucleate conidia (YUILL 1950) on both defined and complex media. The composition of the media, cultural conditions, and procedures for obtaining suspensions of conidia have been presented by BERG and GARBER (1962) who also described methods for isolating nutritionally deficient and both prototrophic and auxotrophic-color mutants by irradiating conidia with ultraviolet light. Additional color mutants were obtained from Strain 10, dilute green conidia requiring leucine. Nutritional mutants were isolated from prototrophic-color mutant strains by the cotton-velveteen method (LEDERBERG and LEDERBERG 1952). The additional mutant strains listed in Table 1 provided material for 51 heterocaryons.

Heterocaryons were readily obtained by the frontier method (BERG and GARBER 1962). Turbid suspensions of conidia were obtained from heterocaryons growing either on defined or complex medium. Plates of defined medium were seeded with 0.1 ml of the original or of a tenfold dilution of the suspensions. Prototrophic

TABLE 1

Mutant haploid strains involved in heterocaryons

Number	Origin	Color*	Genotype†
10	wild type	dil. green (NA)	*leu*
10a	10	albino (NA)	*al-lys leu*
10–21	10	yellow (NA)	*ye-3 leu*
10–32	10	ecru (A)	*ec leu*
10–36	10	tan (A)	*tn leu*
10–39	10	white (A)	*wh-2 leu*
11a	11	buff (A)	*bu ade-1*
11d–11	11	mustard (A)	*bu^{mu} pan-1*
2021	20	blue (A)	*bl-ex cys*
2111	21	chartreuse (A)	*ch ade-2*
2121	21	chartreuse (A)	*ch met-1*
2122	21	chartreuse (A)	*ch lys-1*
2123	21	chartreuse (A)	*ch pab-1*
2211	22	creamy (A)	*cr pan-2*
2221	22	creamy (A)	*cr ade-3*
2223	22	creamy (A)	*cr arg*
2311	23	taupe (NA)	*ta met-1*
2321	23	taupe (NA)	*ta phe*
2413	24	cinnamon (NA)	*ci thi*
2521	25	brown (NA)	*ta^{br} pro*
2522	25	brown (NA)	*ta^{br} val*
2641	26	white (NA)	*wh-1 lys-2*
R17213	Dip 17	cinnamon (NA)	*ci thi met-1*
R24317	Dip 24	creamy (A)	*cr pab-1 ade-3*
R32111	Dip 32	creamy (A)	*(cr bl) ex arg*
R25115	Dip 25	yellow	*(bl ch) ex pab-1 cys*
R517	Dip 5	white	*(bl wh-1) ex lys-2*

* A-autonomous, NA-nonautonomous.
† *leu*-leucine, *lys*-lysine, *pan*-pantothenic acid, *cys*-cysteine, *ade*-adenine, *met*-methionine, *pab*-paraminobenzoic acid, *arg*-arginine, *phe*-phenylalanine, *thi*-thiamine, *pro*-proline, *val*-valine, *ex*-exudate.

colonies on this medium were diploid; spontaneous heterocaryons were easily detected.

Appropriate dilutions of suspensions of conidia harvested from diploid strains were added to the surface of complex medium containing 0.013 percent DL-para-fluoro-phenylalanine (FPA) to detect haploid sectors in the restricted diploid colonies (MORPURGO, in LHOAS 1961). Preliminary experiments indicated that haploid mycelium exhibited markedly more rapid growth than diploid mycelium on complex medium containing FPA. Conidia from one haploid sector of a diploid colony were streaked on complex medium containing FPA to restrict mycelial growth from contaminating diploid conidia. The conidia from haploid sectors in such streaks were transferred to complex medium; the color and nutritional requirements of the haploid colonies were then determined by procedures presented by PONTECORVO *et al.* (1953).

RESULTS

Mutant strains: Nutritionally deficient mutants of the prototrophic-color strains retained their original color. The four color mutants obtained from Strain 10 (dilute green, leucine-requiring) retained their requirement for leucine. Haploid diauxotrophic segregants from certain diploid strains were also used for certain heterocaryons. The constant appearance of numerous droplets on the surface of colonies of the blue strain (*bl-ex*) made possible the detection of this gene marker in those segregants which exhibited the droplets but not the blue color.

The albino strain (*al-lys*) had been classified as an auxotrophic-color mutant in that the altered color was assumed to be associated with the requirement for lysine (BERG and GARBER 1962). Approximately 3×10^8 conidia from strain 10a were irradiated with ultraviolet light to obtain approximately 0.1 percent survival. The entire suspension was divided into aliquots of 0.1 ml which were seeded on defined medium supplemented with leucine; 19 dilute green colonies which required leucine were obtained. This observation indicated that the loss of the requirement for lysine resulted in the restoration of color, which in this case was the dilute green associated with the requirement for leucine. Two other mutants requiring lysine were obtained from prototrophic-color strains but these mutants retained their color.

Allelism: Nutritionally deficient mutants with the same requirement and exhibiting complementation were assumed to be nonallelic and color mutants yielding green conidia at their frontier were nonallelic; such mutants were assigned the same symbol but different numbers.

Diploid strains were needed to demonstrate that two autonomous or two non-autonomous color mutants with similar phenotypes were nonallelic. If a diploid strain had green conidia, the color mutants were nonallelic; if a diploid strain had conidia with a color either similar to one component strain or intermediate between the color of each component strain, the mutants were allelic and were assigned superscripts. The taupe and brown strains were allelic in that neither their frontier nor the diploid strain exhibited green conidia. Diploids involving the other color strains listed in Table 1 displayed green conidia.

Diploid strains: Suspensions containing from 1×10^7 to 1×10^8 conidia per ml were harvested from nine heterocaryons grown on defined and on complex medium and plated on defined medium to determine the frequency of diploid conidia (Table 2). Heterocaryons grown on defined medium usually yielded more diploid conidia than heterocaryons grown on complex medium. In three cases, diploid conidia were obtained from a heterocaryon grown on one but not on the other medium.

Conidia from 51 heterocaryons were seeded on defined medium to obtain diploid colonies. Whereas 36 heterocaryons yielded diploid colonies, the remaining 15 did not. In the latter cases, the seeded plates were either clear or completely overgrown with mycelium. Spontaneous heterocaryons were usually detected on the overgrown plates.

Diploid colonies from 35 heterocaryons were grown on complex medium lacking FPA and examined, using a dissecting microscope, to detect sectors exhibiting conidia with a color other than green. These segregant conidia usually displayed the color of one or the other component strain. Of the 35 green diploid strains, 25 yielded segregant conidia and were termed unstable diploids. Conidia of five stable diploid strains were plated on complex medium containing FPA; segregant sectors were either very uncommon or could not be detected in the diploid colonies. This observation suggested that FPA could not induce haploidization in the stable diploid strains.

Although the unstable diploid strains clearly differed with respect to their frequency of segregant sectors on FPA-medium, no satisfactory and practical procedure could be devised to express these differences. The number of haploid segregants from the 17 diploid strains listed in Table 3 reflects the relative frequency of segregants since all detectable sectors from colonies on ten plates were picked for further analysis.

Conidia from diploid and from haploid strains were seeded on complex medium containing FPA. Diploid colonies were restricted and obviously smaller than haploid colonies. The diameter of colonies was not obviously different when

TABLE 2

Frequency of diploid colonies obtained by plating conidia from nine heterocaryons grown on defined or complex medium

Heterocaryon	Frequency $\times 10^{-8}$	
	Defined medium	Complex medium
16	25	35
17	no data	510
20	670	0
21	0	5.7
22	870	6
23	78,000	0
27	620	44
28	27	no data
29	26	0.4

haploid colonies on complex medium lacking or containing FPA were compared.

Haploid segregants: Sectors in diploid colonies grown on complex medium containing FPA were detected by their conidial color. Not all possible haploid genotypes were recovered from any diploid strain. Except for three strains, both parental genotypes were isolated from the haploid segregants. Two diploids involving strain 10a (*al-lys leu*) did not yield segregants with the genotype of this strain; neither parental genotype was present among segregants from Diploid 58 which involved two diauxotrophic strains. A summary of the color and nutritional requirements of haploid segregants from 17 diploid strains is presented in Table 3.

Only diploid strains involving the blue-exudate (*bl-ex*) strain gave haploid segregants which appeared to include both color markers. These segregants exhibited the exudate but not the blue color. For example, Diploid 25 involved blue-exudate and chartreuse and yielded yellow segregants with an exudate. Segregants from Diploid 62 (*cr ex arg/bu ade-1*) furnished evidence indicating that the component haploid strain with an exudate (R32111) had both the creamy (*cr*) and blue-exudate (*bl-ex*) markers. The following phenotypes were found among the segregants from Diploid 62: creamy, creamy with exudate, buff, light buff, and light buff with exudate. Whereas creamy appeared to be epistatic to blue, the exudate associated with blue was expressed. The buff color was diluted in the presence of creamy or blue.

Linkage: A rational analysis of the frequency of haploid segregants from a diploid strain to detect linkages would be difficult if the parental genotypes constituted a minority of the haploid segregants or if one or two genotypes represented a majority of the segregants. In 14 of the 17 diploid strains, less than 50 percent of the haploid segregants had parental genotypes. The ratio of haploid segregants with the color of one or the other component strains in the diploid was usually significantly different from unity. For example, haploid segregants with the blue-exudate marker were in the minority among segregants from diploids involving a component strain with this gene marker; the one exception was found in Diploid 26. Segregants with the creamy marker were usually in the majority among segregants from diploids involving a component strain with this marker. Consequently, linkages other than the *al-lys leu bu* group (BERG and GARBER 1962) could not be detected with certainty.

DISCUSSION

Color mutants have been invaluable markers in demonstrating the parasexual cycle in *A. fumigatus* (BERG and GARBER 1962). Each mutant, however, must be identified as autonomous or nonautonomous. The concept of autonomous and nonautonomous gene action originated in studies on eye-color mutants of *Drosophila melanogaster*. STURTEVANT (1920) reported the first case in a gynandromorph female fly which was heterozygous for vermilion, a sex-linked mutation, and which did not exhibit a vermilion eye on the male portion of the head. BEADLE and EPHRUSSI (1936) investigated the differentiation of the pigment of

TABLE 3

Haploid segregants from 17 diploid strains

Diploids			Segregants	
			Frequency and genotypes	
Strain	Genotype	Total	Parental	Recombinant
5	*bl-ex cys/wh-1 lys-2*	91	21-*bl-ex cys*, 11-*wh lys*	6-*bl-ex* +, 34-*wh* +, 7-*wh ex* +, 5-*wh ex lys*, 7-*bl-ex cys lys*
12	*cr arg/ta met-2*	59	4-*cr arg*, 18-*ta met*	1-*cr* +, 36-*ta* +
14	*wh-2 leu/bl-ex cys*	28	11-*wh leu*, 13-*bl-ex cys*	2-*wh* +, 1-*bl-ex* +, 1-*wh ex* +
16	*ci thi/bl-ex cys*	44	25-*ci thi*, 6-*bl-ex cys*	8-*ci* +, 1-*bl-ex* +, 4-yellow *ex* +
17	*ci thi/ch met-1*	76	7-*ci thi*, 2-*ch met*	27-*ci* +, 35-*ch* +, 5-*ci thi met*
22	*ta met-2/cr ade-3*	45	1-*ta met*, 19-*cr ade*	19-*ta* +, 2-*cr* +, 4-*cr ade met*
24	*ch pab-1/cr ade-3*	153	29-*ch pab*, 43-*cr ade*	46-*ch* +, 15-*cr* +, 2-dk. *cr* +, 18-*cr pab ade*
25	*ch pab-1/bl-ex cys*	55	4-*ch pab*, 1-*bl-ex cys*	25-*ch* +, 5-*bl-ex pab cys*, 7-yellow *ex* +, 5-yellow *ex pab*, 4-yellow *ex cys*, 4-yellow *ex pab cys*
26	*al-lys leu/bl-ex cys*	79	76-*bl-ex cys*, 1-*al-lys leu*	2-*bl-ex* +
27	*al-lys leu/ch ade-2*	24	8-*ch ade*	15-*ch* +, 1-*al-lys leu ade*
28	*al-lys leu/cr pan-2*	102	34-*cr pan*	66-*cr* +, 2-*al-lys leu pan*
32	*cr arg/bl-ex cys*	27	5-*cr arg*, 12-*bl-ex cys*	4-*cr* +, 5-*bl-ex* +, 1-*cr ex arg*
33	*ye-3 leu/cr pan-2*	65	6-*ye leu*, 11-*cr pan*	1-*ye* +, 46-*cr* +, 1-*ye leu pan*
39	*bu^{mu} pan-1/ec leu*	79	26-*bu^{mu} pan*, 13-*ec leu*	33-*bu^{mu}* +, 7-*ec leu pan*
58	*ci thi met-1/cr pab-1 ade-3*	45	. . . . .	18-*ci* +, 19-*cr* +, 5-*cr ade*, 1-*cr thi*, 1-*ci thi*, 1-*cr ade met*
59	*bu ade-1/ye-3 leu*	145	98-*bu ade*, 9-*ye leu*	35-*bu* +, 3-*ye* +
62	*cr ex arg/bu ade-1*	67	1-*cr ex arg*, 15-*bu ade*	3-*cr* +, 3-*cr ex* +, 1-*cr arg*, 2-*cr ade*, 5-*cr ex ade*, 23-*bu* +, 2-lt. *bu* +, 8-lt. *bu ex* +, 3-lt. *bu ade*, 1-lt. *bu ex ade*

a number of eye-color mutants of *D. melanogaster* by transplanting eye disks from mutant larvae into other mutant or wild-type larvae. The vermilion and cinnabar mutants were nonautonomous and the remaining mutants, autonomous; alleles at the white locus were autonomous. GREEN (1952) has reported that alleles at the vermilion locus were nonautonomous.

PONTECORVO (1946) applied the terminology of autonomy and nonautonomy to spore-color mutants of filamentous fungi. In an autonomous mutant, the color of uninucleate conidia from a heterocaryon is determined *solely* by the genotype of each conidium; in a nonautonomous mutant, spore color is determined by the genotypes included in the heterocaryon. Two multiple allelic series for spore color have been found in *A. fumigatus*. In one series (*bu* and bu^{mu}) both mutants were autonomous and in the other (*ta* and ta^{br}), nonautonomous. These observations in *A. fumigatus*, therefore, paralleled those in *D. melanogaster*. Autonomy or nonautonomy may be a characteristic of a locus and may be related to the type of compound determined by the locus in a biosynthetic pathway, that is, diffusible or non-diffusible.

Except for *Colletotrichum lagenarium* (DUTTA and GARBER 1960) and *Neurospora crassa* (personal communications), a review of the published literature did not reveal unsuccessful attempts to demonstrate the parasexual cycle in a species of filamentous fungi. Although the parasexual cycle was demonstrated in *A. fumigatus*, not all heterocaryons in this species yielded diploid conidia. Plates of defined medium seeded with conidia from approximately 28 percent of the heterocaryons did not yield prototrophic (diploid) colonies. The plates were either clear or were so overgrown with mycelium that prototrophic colonies could not be detected. Furthermore, not all diploid strains gave haploid segregants. Consequently, evidence for the parasexual cycle could not be obtained for approximately half of the heterocaryons in *A. fumigatus*. Future studies on the genetics of this species by means of the parasexual cycle will require a relatively large number of different heterocaryons to obtain those unstable diploid strains which will yield a reasonable number of haploid segregants.

Attempts to demonstrate linkage and to construct linkage groups for 26 genes in *A. fumigatus* by means of the parasexual cycle have not been satisfactory. BERG and GARBER (1962) found one linkage group involving three loci. Although color markers were assigned to 12 loci, segregants with both color markers were not usually detected. Epistasis may have been responsible for this observation. Certain recombinant genotypes involving nutritional markers were not found among haploid segregants from diploid strains which may be expected to yield segregants with parental and recombinant genotypes. The absence of certain recombinants might be explained on the basis of coupling or repulsion. Such an assumption presumes linkage for certain loci and independent segregation for other loci. Attempts to manipulate data from 17 diploid strains to produce a coherent picture of linkage or of independent segregation were frustrated by contradictory evidence from different diploid strains involving a common component strain.

A survey of the published literature in other species of filamentous fungi was

undertaken to determine whether the results obtained by means of the parasexual cycle in *A. fumigatus* were unusual. Linkage groups have been reported in *A. nidulans* (KÄFER 1958), *Penicillium expansum* (BARRON 1962), and *Verticillium albo-atrum* (HASTIE 1962). It is interesting to note that the four nutritional markers which were used in *V. albo-atrum* were considered to be in one linkage group. Linkage was suggested for certain loci in *A. niger* (PONTECORVO, ROPER, and FORBES 1953). No linkages were detected in *A. sojae* (ISHITANI, IKEDA, and SAKAGUCHI 1956), *Fusarium oxysporum* f. *pisi* (BUXTON 1956; TUVESON and GARBER 1959), *F. oxysporum* f. *cubense* (BUXTON 1962), *Cephalosporium mycophilum* (TUVESON and COY 1961), *Cochliobolus sativum* (TINLINE 1962), or *P. chrysogenum* (PONTECORVO and SERMONTI 1954). Finally, not all possible recombinant genotypes were recovered from diploid strains of *A. sojae*, *F. oxysporum* f. *pisi*, *C. mycophilum*, and *C. sativum*.

Several explanations may be offered for the failure to detect more than one linkage group of three genes for the 26 markers which were studied by the parasexual cycle in *A. fumigatus*. The possibility that this species has 24 chromosomes and that the 23 unlinkable genes are each on a different chromosome presents certain problems related to probability and luck. Although haploid strains have uninucleate conidia, the number of nuclei in conidia from diploid strains was not determined. Granting the possibility that a number of conidia from diploid strains may have at least two nuclei, it is difficult to propose a feasible explanation for the apparent lack of linkage on the basis of such conidia. Colonies from binucleate conidia would be heterocaryotic from the start and relatively large sectors with segregant genotypes would be anticipated. The observed sectors were relatively small and usually occurred late in the growth of the colony. KÄFER (1961) has presented a thorough analysis of the processes of spontaneous recombination in vegetative diploid nuclei of *A. nidulans* in which diploid, haploid, and aneuploid segregants were obtained. All aneuploids in this species were unstable and easily recognized. Furthermore, they would not upset the ratio of markers and linkage relationships in haploid nuclei. It is difficult to consider chromosomal aberrations at this time as a significant factor. The possible effect of selection on the frequency of haploid nuclei in a diploid mycelium offers an attractive explanation for the apparent absence of linkage for 23 genes involved in 17 diploid strains of *A. fumigatus*.

Somatic crossing over in diploid strains would yield diploid-diploid heterocaryons; haploidization in such strains would yield haploid-diploid heterocaryons. Diploid nuclei may first be involved in somatic crossing over and then undergo haploidization. Haploid segregants from diploid strains of *A. fumigatus* were detected as sectors bearing conidia with the color of one of the component strains. If haploid nuclei with different genotypes were subjected to selection during their multiplication in haploid-diploid heterocaryons, the frequency of haploid conidia with different genotypes could not be predicted on the basis of independent segregation or of linkage. Linkage would be detected for those genes so closely linked that somatic crossing over between them either would not occur or only rarely occurred prior to haploidization. Although any one diploid strain

of *A. fumigatus* might yield haploid segregants with a restricted number of genotypes to indicate that certain genes were in repulsion or coupling, a number of diploid strains with a common haploid component would be required to support such a conclusion. The detection of linkage by meiotic recombination in *A. nidulans* provides the necessary control in determining linkages by means of the parasexual cycle. Linkage studies by the parasexual cycle in imperfect fungi must be viewed with caution when relatively few data are available.

TUVESON and GARBER (1961) demonstrated experimentally induced alterations in nuclear ratios detectable by the genotypes of conidia harvested from haploid-haploid heterocaryons of *F. oxysporum* f. *pisi* grown on defined medium supplemented with graded concentrations of required compounds. It may be possible to alter the frequency of haploid segregant conidia from diploid colonies of *A. fumigatus* by this technique.

SUMMARY

Twenty-seven haploid strains differing in color and nutritional requirements of the imperfect fungus *Aspergillus fumigatus* were involved in 51 heterocaryons. Color mutants were assigned to 12 loci; two allelic series, each with two mutants, were detected. The medium on which heterocaryons were grown and the combination of haploid strains appeared to determine the frequency of diploid conidia. Thirty-seven diploid strains were obtained and, of 36 strains, 25 were unstable in that they yielded haploid segregants.

Although parental and certain recombinant genotypes usually occurred among haploid segregants from 17 diploid strains, the frequency of parental genotypes was generally lower than that of prototrophic recombinants. Twenty-three of 26 genetic markers could not be assigned to linkage groups. The absence of detectable linkages was attributed to nuclear selection of haploid segregants with certain genotypes in the haploid-diploid mycelium. Three alternate explanations were briefly discussed. A survey of published data on the frequency of haploid segregants from diploid strains of 11 taxa of filamentous fungi indicated that linkage was not detected in six taxa.

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