Genet. Res., Camb. (1979), **34**, *pp.* 239–252
Printed in Great Britain

The production of heterozygous diploids and haploidization analysis in *Aspergillus amstelodami*

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(*Received* 27 *June* 1979)

SUMMARY

The steps in the parasexual cycle required for genetic analysis by mitotic haploidization were readily accomplished in *Aspergillus amstelodami*. Balanced heterokaryons were synthesized between strains carrying complementary auxotrophic mutations and heterozygous–diploid colonies were recovered from these heterokaryons at frequencies between 1×10^{-6} and 5×10^{-6} among conidia plated. When grown in the presence of *p*-fluorophenylalanine or benomyl, heterozygous–diploid strains produced discrete haploid segregants. Examination of the segregation of 12 mutations among haploid segregants from ten diploids indicated that these genes were located in six linkage groups, thereby increasing the number of linkage groups recognized in this species. This haploidization analysis provided a clear distinction between linked and unlinked genes and continuation of this approach should reveal the chromosome number of *A. amstelodami*. Linkage at meiosis was detected between only one of five pairs of mitotically linked genes tested, emphasising the value of mitotic-haploidization analysis for assigning markers to linkage groups. *A. amstelodami* is amenable to both mitotic and meiotic genetic analysis and further studies should permit extensive comparisons of the genetic and biochemical organization of this species with that of *A. nidulans*.

1. INTRODUCTION

Interest in the genetics of *Aspergillus amstelodami* was initiated by a report that natural white- and brown-spored strains belonging to the *A. glaucus* species group readily formed heterokaryons under non-selective conditions (Christensen, López & Benjamin, 1965). These strains were initially considered to constitute a new species, for which the binomial *A. heterocaryoticus* was proposed (Christensen *et al.* 1965), but subsequent genetical investigation indicated that they were natural conidial-colour mutants of *A. amstelodami* (Caten, 1979). Examination of other strains of *A. amstelodami* showed that ease of formation and stability under non-selective conditions were characteristic of heterokaryons between related strains of this species. This behaviour is atypical, as heterokaryons of many *Aspergillus* species are difficult to produce and are unstable unless forced by balanced deficiencies (Raper & Fennell, 1953; Pontecorvo *et al.* 1953; Clutterbuck, 1974). *A. amstelodami* therefore appeared to be a suitable organism for study of

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0016-6723/79/2828-7700 $01.00

heterokaryosis between independent isolates. The recognition in this work of specific heterokaryon incompatibility genes (Caten, Butcher & Croft, 1972) made it desirable to develop a genetic system in *A. amstelodami* to permit the mapping of the incompatibility genes.

Methods of genetic analysis based on both meiosis and mitosis have been developed in *A. nidulans* (Pontecorvo *et al.* 1953; Pontecorvo & Käfer, 1958; Clutterbuck, 1974; Cove, 1977). Because of the high level of meiotic recombination, the initial recognition of linkage groups and the allocation of new markers to these groups are most efficiently carried out by mitotic haploidization of somatic diploids (Pontecorvo & Käfer, 1958; Käfer, 1958; McCully & Forbes, 1965). Lewis & Barron (1964) reported the occurrence of the parasexual cycle in *A. amstelodami* and concluded that the pattern of the cycle was similar to that in *A. nidulans*. Subsequently, Lewis (1969) recognized two linkage groups and showed that the meiotic and mitotic maps were correlated. These previous studies demonstrated the suitability of *A. amstelodami* for genetic studies but did not describe the methods used in detail. We here report procedures for the production and haploidization of somatic heterozygous diploids of *A. amstelodami*. Using these parasexual techniques we have allocated twelve markers to six linkage groups.

2. MATERIALS AND METHODS

(i) *The organism*

A. amstelodami is a member of the *A. glaucus* group of the Aspergilli (Raper & Fennell, 1965) and has a life-cycle similar to that of *A. nidulans*. It is osmophilic and grows optimally around 30 °C on media containing a high percentage of sugar or salt (maximum radial growth rate = 5 mm/day). The conidia are produced in loosely radiate heads and are deep olive-green in colour. They may contain from one to four nuclei at maturity, with two being the most common. In the strains used in this work conidia with one, two, three or four nuclei occurred at frequencies of 23 %, 74 %, 2 % and 1 %, respectively. *A. amstelodami* is homothallic and the bright-yellow cleistothecia develop abundantly on media containing high concentrations of sugar but limited nitrogen. Mature asci containing eight, colourless ascospores are formed after 10 days incubation at 30 °C. With continued incubation ascospores are released into the cleistothecia which eventually rupture and free the ascospores. Germination of newly formed ascospores is poor but rises as they age. It may be enhanced up to fivefold by pre-incubation of plated ascospores at 45 °C for 4–6 days prior to shifting to 30 °C.

All strains of *A. amstelodami* used were derived from the naturally occurring, white and brown conidial-colour mutants A1 and A2 (Caten, 1979). Stock cultures were stored as dry conidia on anhydrous silica gel (Perkins, 1962; Roberts, 1969).

(ii) *Media and cultural conditions*

Minimal medium (M), complete medium (CM) and malt salt medium (MTS) were as previously described (Caten, 1979). Nutritional supplements were used at the

following final concentrations: lysine, 200 μg/ml; other amino acids, 100 μg/ml; riboflavin, 10 μg/ml; biotin, 1 μg/ml; other vitamins 2 μg/ml; adenine, 100 μg/ml. To restrict colony size when plating conidia or ascospores sodium deoxycholate (Koch-Light Laboratories, Colnbrook, Bucks. SL3 0BZ) was added at 0·4 mg/ml final concentration. Media containing sodium deoxycholate are indicated by the addition of D to the medium code, e.g. MD is minimal medium plus sodium deoxycholate. Minimal medium promoted development of the sexual stage and was used for sexual crosses as well as scoring auxotrophic mutations. Where rapid growth and conidial production were required MTS was used. Conidial and ascospore suspensions were prepared in a dilute solution (0·001 %, v/v) of tween 80. All cultures were grown at 30 °C.

(iii) *Origin of mutations*

The white (*wA1*) and brown (*bwA2*) conidial colour mutations were isolated from nature (Caten, 1979). All other mutations were induced by treatment with either far ultraviolet light (UV) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). For UV mutagenesis 10 ml of conidial suspension (10^6–10^7/ml) were irradiated to 5 % survival as described by de Bertoldi & Caten (1975). After irradiation the conidial suspension was diluted and plated on CMD or MTSD. A modification of the procedure described by Clutterbuck & Sinha (1966) for *A. nidulans* was used for NTG mutagenesis. Three mg of NTG (Ralph H. Emanuel, Wembley, U.K. HA0 1PY) were dissolved in 10 ml of 0·05 M-phosphate buffer (pH 6·0) by shaking gently in a water bath at 35 °C. To this solution 5 ml of conidial suspension (5×10^7/ml) pre-warmed to 35 °C were added to start the reaction and the mixture was incubated at 35 °C with gentle shaking. Samples of 2 ml were removed after 5 and 10 min, diluted with 3 ml tween 80 and washed three times by centrifugation and resuspension in tween 80. After the final wash, the conidial pellet was resuspended in 1 ml tween 80 and 0·1 ml aliquots from appropriate dilutions were plated on CMD. Under these conditions the 5 and 10 min incubations gave 18 % and 11 % survival respectively.

Morphological mutants were identified by screening the survivors from mutagenic treatment under a low-power dissecting microscope. Auxotrophic mutants were identified by the standard procedure and their nutritional requirements determined by auxanographic tests with individual amino acids and vitamins (Pontecorvo, 1949). Conidia from mutant strains were plated on MTSD and stock cultures established from single pure mutant colonies. The system of nomenclature used in *A. nidulans* (Clutterbuck, 1973) was adopted to denote genes and alleles. Wherever appropriate the same primary gene symbol was used as follows: *w*, white conidial colour; *bw*, brown conidial colour; *dil*, dilute conidial colour; *ad*, adenine requirement; *arg*, arginine requirement; *lys*, lysine requirement; *meth*, methionine requirement; *nia*, nitrate non-utilizing; *nic*, nicotinic acid requirement, *pro*, proline requirement (Clutterbuck, 1973, 1974). The mutation producing orange coloured cleistothecia has no equivalent in *A. nidulans* and was denoted *ocl*.

(iv) *Isolation of heterokaryons and heterozygous diploids*

The isolation of heterozygous diploids was based upon the method established for *A. nidulans* by Roper (1952). The two strains to be crossed carried complementary auxotrophic mutations and were of different conidial colour (generally white and brown). They were inoculated 1 mm apart on MTS to form a heterokaryon. After 4 days incubation heterokaryotic sectors could be recognized at the interface by the presence of mixed conidial heads (Gossop, Yuill & Yuill, 1940). These heterokaryons were subcultured to M by transferring small mycelial blocks (1–2 mm^2) from the edge of the sectors. The majority of such transfers produced balanced heterokaryons which grew at near wild-type rates on M and could be subcultured indefinitely. Conidial colour was determined autonomously (Pontecorvo, 1946) and balanced heterokaryons appeared as a fine mosaic of contrasting conidial colour, with up to 50% of individual conidial heads being mixed.

For diploid isolation, four mass hyphal inocula of a balanced heterokaryon were placed on M within a single petri dish. After 4 days incubation conidia were washed into suspension by flooding the plate with tween 80. The conidial suspension was mixed on a Rotamixer, filtered through sterile glass wool to remove mycelial fragments and cleistothecia and then plated on MD at 1×10^5 to 5×10^5 conidia per plate in a soft agar overlay (MD solidified with 0·8% (w/v) Oxoid agar No. 3). These plates were examined for prototrophic colonies after 6 days incubation.

(v) *Haploidization analysis*

Haploidization of heterozygous diploid strains was induced with *p*-fluorophenylalanine (FPA) (Lhoas, 1961; McCully & Forbes, 1965). A 0·1% (w/v) solution of FPA, prepared by the method of Day & Jones (1969), was added to MTS to give a final concentration of 80 μg/ml and a dilute conidial suspension of the diploid plated to give approximately 50 colonies per plate. At 80 μg FPA/ml conidial viability was not reduced but the growth and conidiation of the resulting colonies were inhibited. After 7–14 days incubation a number of colonies produced more vigorously growing sectors bearing white or brown or green conidial heads (the parental diploids had green conidia). Conidia were picked from these sectors with a fine glass needle and plated on MTSD. Most samples were taken from sectors with white or brown conidia but a few green sectors were also sampled. Not more than one sector was isolated from any single colony. The ploidy of the segregants was determined by comparison of their conidial diameter with that of standard haploid and diploid strains. For genotype classification the haploid segregants were replicated onto appropriate differential media from MTSD master plates.

The fungicide, benomyl, induces haploidization of heterozygous diploids of *A. nidulans* (Hastie, 1970; Kappas, Georgopoulos & Hastie, 1974). Recent tests have shown that benomyl is preferable to FPA as a haploidizing agent for *A. amstelodami*, in that haploid segregants appear more rapidly and are more conspicuous. A commercial grade of benomyl ('benlate', Du Pont) was used at a

final concentration of 0·2 μg/ml. The benlate powder was incorporated into molten MTS as a sterile aqueous suspension and the diploid to be haploidized was transferred with a 3 × 3 needle replicator from a lawn on MTSD onto the benlate plates. Segregant sectors appeared in the diploid mycelium after 5–10 days incubation and were isolated and classified as for the FPA induced segregants. Of the diploids analysed in this paper, only A1/38 + A79 (Table 3) was haploidized with benlate.

(vi) *Meiotic analysis*

Sexual crosses were set up either by the procedure described by Caten (1979) or by transferring a balanced heterokaryon of the two strains to be crossed to a fresh plate of M. Mass cleistothecia were sampled after 12 days incubation and progeny originating from hybrid asci were isolated by recombinant selection (Caten, 1979).

3. RESULTS

(i) *Mutants*

Following mutagenesis, eight auxotrophic and two morphological mutants were isolated (Table 1*b*). With the exception of two adenine requiring strains (A1/9 and A79) the auxotrophs had different nutritional requirements and each was designated by the appropriate phenotypic symbol. One of the morphological mutants produced orange cleistothecia (phenotype Ocl) as opposed to the wild-type yellow and the second had conidia which were a paler green than the wild-type (phenotype Dil).

Each of the new mutants was crossed sexually to a strain wild-type for the character in question and around 200 progeny examined for segregation of the mutant phenotype. With the exception of the Pro (strain A1/14) and Meth (strain A1/15) mutants, the crosses gave a 1:1 segregation confirming that the mutant phenotype was determined by a single mutation. The two exceptional crosses produced an excess of wild-type progeny (114 Pro^+:86 Pro, 128 Met^+: 68 Met) which was attributed to the segregation of a single gene with reduced viability of the mutant allele. A tendency for there to be a deficiency of the mutant allele was also observed with the other auxotrophic mutations at high plating densities (> 200 colonies per plate). The mutation in each mutant was denoted by the appropriate primary gene symbol followed by the locus specific letter (generally *A*) and the mutant isolation number (Table 1*b*). The two adenine mutations complemented in a balanced heterokaryon and were designated *adA1* (strain A1/9) and *adB2* (strain A79). Strain A53 was originally isolated as having a requirement for ammonia or various amino acids. Growth tests indicated that this mutant was unable to utilize nitrate but could grow when nitrite or hypoxanthine was provided as nitrogen source. This phenotype is characteristic of mutations in the nitrate reductase structural gene, denoted *niaD* in *A. nidulans* (Pateman *et al.* 1964; Cove, 1976), and this gene symbol was accordingly adopted for the mutation in strain A53. Meiotic crosses of the pale conidial colour mutant to white (*wA1*) and brown (*bwA2*) conidial-colour strains showed that this mutation was not

expressed in white strains but produced pale brown conidia when together with *bwA2*. This phenotype is similar to the *dilA* gene in *A. nidulans* and the same gene symbol was adopted. The mutation responsible for the orange-cleistothecial phenotype (*oclA1*) was both expressed and inherited independently of conidial colour.

Table 1. *Genotype and origin of strains*

Strain number	Genotype*				Origin
(*a*) Ancestral strains					
A1	*wA1*	—	—	—	Natural isolate; see Caten (1979)
A2	*bwA2*	—	—	—	Natural isolate; see Caten (1979)
(*b*) Induced mutants†					
A1/38	*wA1*	*lysA38*	—	—	Induced in A1 by NTG‡
A1/57	*wA1*	*nicA57*	—	—	Induced in A1 by NTG
A16	*bwA2*	*oclA1*	—	—	Induced in A2 by UV‡
A1/9	*wA1*	*lysA38*	*adA1*	—	Induced in A1/38 by NTG
A1/14	*wA1*	*lysA38*	*proA1*	—	Induced in A1/38 by NTG
A1/15	*wA1*	*lysA38*	*methA1*	—	Induced in A1/38 by NTG
A53	*bwA2*	*nicA57*	*oclA1*	*niaD1*	Induced in A24 by NTG
A55	*bwA2*	*nicA57*	*oclA1*	*argA1*	Induced in A24 by NTG
A79	*bwA2*	*nicA57*	*oclA1*	*adB2*	Induced in A24 by UV
A49	*nicA57*	*dilA1*	—	—	Induced in A25 by UV
(*c*) Recombinants					
A24	*bwA2*	*nicA57*	*oclA1*	—	Sexual cross: A1/57 × A16
A25	*nicA57*	—	—	—	Sexual cross: A1/57 × A16
A68	*bwA2*	*lysA38*	*oclA1*	*proA1*	Parasexual cross: A1/14 × A24
A78	*bwA2*	*nicA57*	*oclA1*	*dilA1*	Parasexual cross: A49 × A68

* See text for gene symbols.
† The last mutation in each genotype was newly induced in that strain.
‡ NTG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV = far ultraviolet light.

(ii) *Isolation of heterozygous diploids*

Two types of colony generally developed from the plating of conidia from balanced heterokaryons on MD. The majority of colonies were heterokaryons as shown by their mosaic appearance with respect to conidial colour. The other colonies were of uniform conidial colour and were presumed to be diploids. Where, as with most crosses, the parent haploids were of genotypes *wA bwA*$^+$ and *wA*$^+$ *bwA*, heterokaryons and diploids were readily distinguished by their mottled white/brown and pale green colours, respectively. Diploid colonies were recovered at frequencies between 1×10^{-6} and 5×10^{-6}. Heterokaryons occurred at frequencies between 10^{-4} and 10^{-5} and presumably originated primarily from heterokaryotic conidia. Considering the high frequency of mixed conidial heads in the parental balanced heterokaryons and that 77% of conidia contain two or more nuclei, this frequency of heterokaryotic conidia is remarkably low and suggests that the distribution of nuclei into conidia is non-random.

The ploidy of the presumed diploids was confirmed by comparisons of conidial diameter. Measurement of 200 conidia from the initial diploid A1/38+A24 and its two haploid components indicated that conidia from diploids and haploids had mean diameters of $5{\cdot}09 \pm 0{\cdot}02\,\mu m$ and $4{\cdot}01 \pm 0{\cdot}01\,\mu m$ respectively. For subsequent suspected diploids, measurement of 20 conidia was sufficient to confirm or refute diploidy. As previously noted in *A. nidulans* (McCully & Forbes, 1965) and *A. amstelodami* (Lewis, 1969) diploid colonies heterozygous for *wA* and *bwA* could be distinguished from green haploids by their lighter colour. Furthermore, the conidial heads of diploid strains were smaller then those of haploids. These morphological differences provided convenient criteria for the preliminary recognition of diploids and subsequently for the identification of green haploid segregants from diploids.

When the conidial suspensions were prepared from heterokaryotic cultures that were more than 6 days old a third type of colony was recovered on the diploid isolation plates. Like the diploids, these colonies were prototrophic and homokaryotic but were of brown, white or green conidial colour. Measurements of conidial size showed them to be haploid. While reversion of the auxotrophic mutations may account for some of these colonies this cannot explain the presence of recombinant phenotypes for the unselected morphological markers. It is likely that these prototrophic haploids originated from contamination of the conidial suspension with ascospores since cleistothecia develop rapidly in this fungus.

(iii) *Haploidization analysis*

The conidial diameter of all FPA-induced segregants from the first three diploids analysed was compared with that of standard haploid and diploid strains. All of 152 white and brown segregants were haploid and measurement of conidial diameter was discontinued for such segregants from further diploids. However, the green segregants included both haploid and diploid strains and the ploidy of such segregants from all diploids was ascertained from measurements of conidial size. Similar results were obtained with the benlate-induced segregants of diploid A1/38+A79.

The first diploid haploidized involved the parental strains A1/38 and A24 and was heterozygous for five markers: *bwA*, *wA*, *lysA*, *nicA*, *oclA*. One hundred and nine brown, white and green haploid segregants were isolated from two independently synthesized cultures of this diploid and their genotypes determined. The results from the two cultures were homogenous and have been pooled (Table 2). Because of the epistatic nature of *wA* the allele present at the *bwA* locus in white segregants could not be ascertained. (Although superficially similar to the *yA* gene in *A. nidulans*, the *bwA* gene does not determine a comparable *p*-diphenol oxidase (Caten, 1979) and hence cannot be scored by a colour reaction with *N*,*N*-dimethyl-*p*-phenylenediamine (Clutterbuck, 1972).) Of the three unselected markers, *lysA* segregated in a 1:1 ratio while *nicA* and *oclA* showed an excess of wild-type and mutant alleles respectively (Table 2).

All ten possible pairwise combinations of the five markers showed reassortment,

although the four genotypes were seldom recovered at equal frequency (Table 2). These deviations from independent assortment, however, arose primarily from the unequal number of wild-type and mutant alleles for the individual markers and many recombinant genotypes were recovered in each case. Reassortment between *wA* and *bwA* was difficult to detect because of the use of these mutations as selective markers and the epistatic nature of *wA*. The small number of green wA^+ bwA^+ recombinants recovered may be attributed to the difficulty of detecting these among the diploid mycelium. To confirm the independent assortment of these two mutations, 16 white conidial segregants were assayed for the presence of the bwA^+ or *bwA* allele by sexual crosses to a tester of genotype wA^+ *bwA*. Nine of the segregants proved to be of the recombinant *wA bwA* genotype. It was concluded therefore that *bwA*, *wA*, *lysA*, *nicA* and *oclA* are located in five different linkage groups, designated I, II, III, IV and V.

Table 2. *Reassortment of markers among haploid segregants from the first diploid*

Diploid $\frac{\text{A1/38}}{\text{A24}} = \frac{wA\ +\ lysA\ +\ +}{+\ bwA\ +\ nicA\ oclA}$.

		w		*lys*		*nic*		*ocl*		Selected markers
		+	−	+	−	+	−	+	−	
bw	+	10	?	5	5	8	2	4	6	10*
	−	37	?	23	14	20	17	12	25	37
w	+	—	—	28	19	28	19	16	31	47
	−	—	—	33	29	39	23	17	45	62
lys	+	—	—	—	—	39	22	18	43	—
	−	—	—	—	—	28	20	15	33	—
nic	+	—	—	—	—	—	—	13	54	—
	−	—	—	—	—	—	—	20	22	—
Unselected markers				61	48	67	42†	33	76†	—

* Only 10 wA^+ bwA^+ (green) haploid segregants selected.
? *bwA* genotype could not be scored in *wA* segregants.
† Significantly different from 1:1 segregation.

Diploids were then synthesized to examine the segregation of the *adA*, *proA*, *methA*, *niaD*, *argA*, *adB* and *dilA* markers with the five tester markers defining linkage groups I–V. Generally each new marker was introduced separately in two independent combinations of haploid strains and around 50 segregants isolated from each diploid (Table 3). The results from replicate diploids were entirely consistent and in all cases the markers for linkage groups I–V segregated independently as expected. The recombinant to parental ratios for each new mutation with the tester markers showed a marked discontinuity between values in the range 2·75–0·41 (i.e. approximately one) and values less than 0·02 (Table 3). This clear dichotomy suggests that the values in the former range represent independent assortment while the very low values indicate that the two markers are linked. On this basis *adA* was located in linkage group V, *proA* in group III, *methA* in group V, *niaD*

Table 3. *Allocation of* adA, proA, methA, niaD, argA, adB *and* dilA *genes to linkage groups I–VI*

		Segregation		Number of parental (P) and recombinant (R) segregants									
				*bwA**		*wA*		*lysA*		*nicA*		*oclA*	
Mutation	Diploid	+	−	P	R	P	R	P	R	P	R	P	R
adA1	A1/9+A24	27	30	15	13	26	31	23	34	33	24	56	1
	A1/9+A55	26	30	11	12	29	27	25	31	26	30	56	0
proA1	A1/14+A24	39	16†	13	12	31	24	55	0	33	22	39	16
	A49+A68	38	18†	18	38	—	—	56	0	27	29	29	27
methA1	A1/15+A24	17	37†	8	22	21	33	19	35	24	30	53	1
	A1/15+A53	39	24†	14	14	24	39	28	35	36	27	63	0
niaD1	A1/15+A53	23	40†	13	15	23	40	29	34	35	28	62	1
argA1	A1/9+A55	16	40†	23	0	39	17	33	23	30	26	26	30
	A1/38+A55	22	37	29	0	32	27	39	20	31	28	36	23
adB2	A1/38+A79	72	76	38	38	148	0	80	68	75	73	69	79
dilA1	A49+A68	28	28	28	28	—	—	20	36	25	31	23	33
	A1/38+A78	15	26†	14	27	26	15	17	24	21	20	23	18

* Could only be scored in wA^+ segregants.
— Marker not included in diploid.
† Significantly different from 1:1 segregation.

in group V, *argA* in group I and *adB* in group II (Table 4). The *dilA* gene reassorted with all five markers and was considered to define linkage group VI.

Considering the 6 pairs of linked genes examined only 3 mitotic recombinants were recovered from a total of 604 haploid segregants (Table 3). This represents a frequency of crossing over prior to haploidization (0·50 %) similar to that reported by Lewis (1969) for the same organism and by Pontecorvo & Käfer (1958) in *A. nidulans*.

Table 4. *Linkage groups I–VI in* Aspergillus amstelodami

Linkage groups	Markers*			
I	*bwA*	*argA*	—	—
II	*wA*	*adB*	—	—
III	*lysA*	*proA*	—	—
IV	*nicA*	—	—	—
V	*oclA*	*adA*	*methA*	*niaD*
VI	*dilA*	—	—	—

* See text for gene symbols.

(iv) *Meiotic linkage*

The haploidization analysis revealed that linkage groups I, II, III and V each contained two or more genes (Table 4). The following pairs of genes located in the same group were tested for meiotic linkage in two factor crosses: *bwA* and *argA*, *lysA* and *proA*, *oclA* and *adA*, *oclA* and *methA*, *oclA* and *niaD*. The first four pairs segregated independently. However, the cross (A1/38 × A53) which involved *oclA* and *niaD* in coupling gave the following results for a sample of 200 progeny: *oclA niaD* 60, *oclA niaD*$^+$ 38, *oclA*$^+$ *niaD* 43, *oclA*$^+$ *niaD*$^+$ 59. This indicates loose meiotic linkage ($\chi^2_1 = 7{\cdot}22$, $P < 0{\cdot}01$) between these two genes (recombination frequency = 40·5 % with 5 % limits of 33·7 % and 47·3 %).

4. DISCUSSION

Heterozygous diploids of *A. amstelodami* were isolated from all 10 combinations tested at a frequency among conidia plated of from 1×10^{-6} to 5×10^{-6}. They arose spontaneously and exposure of heterokaryons to D-camphor was not necessary, in contrast to the experience of Lewis (1969). Although the clonal origin of heterozygous diploid conidia makes comparisons difficult, their frequency in *A. amstelodami* appears higher than in *A. nidulans* (1×10^{-7} to 1×10^{-6}, Pontecorvo *et al.* 1953; Clutterbuck, 1974). This enhanced frequency may be attributed to an increased probability of fusion between unlike nuclei consequent upon the better mixing of nuclei of the component genomes in heterokaryons of *A. amstelodami*.

The selection of heterozygous diploid cultures from heterokaryons is most readily accomplished in species with uninucleate spores since this prevents direct propagation of the heterokaryons which would obscure the rare diploid colonies. However, it is not the nuclear number *per se* that is important but the frequency

of heterokaryotic spores. Because of non-random distribution of nuclei heterokaryotic spores may rarely be produced even in species with multinucleate spores, thereby permitting efficient selection of heterozygous diploids. This is the case in *A. amstelodami* where heterokaryons occurred on the selective plates at a frequency only ten to a hundred times that of heterozygous diploids despite the fact that 77 % of conidia contained two or more nuclei. This emphasizes the importance of developmental information, in addition to knowledge of nuclear number, when predicting the genetical composition of spores produced by heterokaryons (Buxton, 1960).

By haploidization analysis two natural and ten induced mutations in *A. amstelodami* were assigned to six linkage groups (Table 4). Successful allocation of genes to linkage groups by this method requires efficient haploidization, a low frequency of mitotic crossing over, no strong selection for or against particular segregant genotypes and the absence of chromosomal rearrangements in the mutant strains. The clear distinction between linked and unlinked genes and the consistency of the data (Table 3) suggest that these conditions were realized in the present study. Bearing in mind that only 12 markers were mapped, six is unlikely to be the final number of linkage groups in this species and it is anticipated that the mapping of further mutations will reveal additional groups. No cytological information on the chromosome number of *A. amstelodami* is available. Meiotic linkage was detected between only one of the five pairs of linked genes tested. This suggests that the linkage groups in this species, in common with those of *A. nidulans* (Clutterbuck, 1974) and *Saccharomyces cerevisiae* (Sherman & Lawrence, 1974) consist of many meiotic units and clearly demonstrates the value of haploidization analysis for assigning markers to linkage groups.

This study confirms and extends the work of Lewis & Barron (1964) and Lewis (1969) on the parasexual cycle in *A. amstelodami*. Lewis (1969) analysed haploid and diploid segregants from four heterozygous diploids involving eleven markers altogether and, on this basis, placed five markers in one linkage group (I) and two in another (II). His data do not permit final allocation of the remaining four markers but it seems likely that a further three or four linkage groups are involved. Both studies are consistent therefore in indicating at least five linkage groups in this organism. The use of different strains and mutations precludes direct comparisons between the linkage groups established in the two studies. However, since only one gene in each case has been found to give rise to the white and brown conidial-colour phenotypes (Caten, 1979), the *wh* and *br* genes of Lewis (1969) may be equated to the *wA* and *bwA* genes. On this basis, linkage group I of Lewis (1969) corresponds to linkage group II in the present study in that both carry the white-conidial gene. Lewis (1969) did not allocate the brown gene to a linkage group although he did show that it was unlinked to the white gene, in agreement with the present results. Several of the linkages established by Lewis (1969) were confirmed by analysis of diploid mitotic segregants. Similarly, the order of the markers in linkage groups I and III has been determined by mitotic recombination analysis (Dhahi & Caten, unpublished). Considered together these studies indicate

that *A. amstelodami* possesses a parasexual cycle similar to that in *A. nidulans* (Pontecorvo *et al.* 1953) and that as in *A. nidulans* (Pontecorvo & Käfer, 1958; Käfer, 1958) this may be readily exploited for genetic analysis. Further application of these techniques should rapidly establish the chromosome number of *A. amstelodami* and provide initial genetic maps of these chromosomes.

Since the initial work with *A. nidulans* (Pontecorvo *et al.* 1953; Pontecorvo & Käfer, 1958; Käfer, 1958) the parasexual cycle has been applied to the genetic analysis of several species of filamentous fungi. In some cases this has provided, as in the present work, a clear definition of linkage groups (e.g. Lhoas, 1967; Ball, 1971; Typas & Heale, 1978). The results in other cases, however, have been ambiguous (e.g. Strømnaes & Garber, 1963; Garber & Beraha, 1965; MacDonald, Hutchinson & Gillett, 1965) and it has been suggested that the pattern of the parasexual cycle may not always conform to that in *A. nidulans* (Lewis, 1969). A number of differences have been reported; for example, the high frequency of mitotic crossing over in *Verticillium albo-atrum* (Hastie, 1967) and the recovery in *Humicola* sp. of heterozygous diploids from mixed cultures of haploids without a detectable, intervening heterokaryon (de Bertoldi & Caten, 1975). It is likely, however, that in many instances the ambiguity has resulted from technical deficiencies such as selection against particular segregants or the presence of chromosomal aberrations, particularly translocations (Garber & Beraha, 1965; MacDonald *et al.* 1965). Selective effects can be reduced by appropriate choice of markers, by modification of the media and by use of a haploidizing agent such as FPA (Lhoas, 1961) or benlate (Hastie, 1970), although with certain markers the agent may itself exert an effect (Van Arkel, 1963). Once linkage groups are defined, haploidization analysis provides an efficient means of detecting translocations in new mutant strains (Käfer, 1962). However, during the initial construction of linkage groups the presence of translocations in some mutants will produce spurious linkages and complicate interpretation of the data. The risk of translocations can be reduced by choice of mutagen and, in sexual fungi, by meiotic screening of new mutants (Upshall & Käfer, 1974).

Considering the great diversity of the fungi, genetical studies have been conducted on very few species (King, 1974; Burnett, 1975; Fincham, Day & Radford, 1979). As a result, generalizations can only be made with caution and few detailed inter-specific comparisons are possible. It is therefore desirable to increase the number of genetically characterized species. *A. amstelodami* appears suitable for this purpose and extension of the present studies will permit comparison of its genetic system with that of *A. nidulans*. Basic similarities in the mitotic and meiotic systems of these two species are already apparent but it is likely that considerable differences exist at a more detailed level. The Aspergilli are a variable group (Raper & Fennell, 1965) and the morphological and physiological divergence of *A. amstelodami* and *A. nidulans* are indicated by their classification into different genera (*Eurotium* and *Emericella* respectively) by some authorities (Benjamin, 1965; Alexopoulous, 1962).

The authors thank Professor J. L. Jinks for the provision of laboratory facilities for one of us (M. de B.) during the course of this work. The excellent technical assistance of Hazel Howell and Heather Bottom is gratefully acknowledged.

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