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Evidence for a multi-allelic heterokaryon incompatibility (*het*) locus detected by hybridization among three heterokaryon-compatibility (h–c) groups of *Aspergillus nidulans*

R. B. G. DALES,* J. MOORHOUSE & J. H. CROFT
School of Biological Sciences, The University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

A strain of heterokaryon-compatibility (h–c) group A was crossed sexually to strains of h–cB and h–cGL of *Aspergillus nidulans*. A back-crossing programme established that there were seven hetero-allelic heterokaryon compatibility (*het*) genes controlling somatic incompatibility between strains of h–cA and h–cB. A similar back-crossing programme between strains of h-cA and h-cGL confirmed that these two groups differ at six *het* loci. Previous work has shown that h-cB differs from h-cGL at two *het* loci, *hetA* on linkage group V and *hetB* on linkage group VI. As an allelic difference at a single *het* locus is enough to cause two strains to be heterokaryon incompatible, 15 alleles spread over seven *het* loci are necessary to explain the h-cA, h-cB, h-cGL triangular compatibility relationship. One *het* locus is multi-allelic and this locus must be either *hetA* or *hetB*.

Keywords: *Aspergillus nidulans*, h–c groups, *het* genes, heterokaryon incompatibility, multi-allelic *het* locus, vegetative incompatibility.

Introduction

Systems of intraspecific vegetative incompatibility have been detected in a number of filamentous fungi. In *Aspergillus nidulans* 100 wild isolates, collected from various sites in Britain and maintained in the Birmingham strain collection, have been classified into 19 heterokaryon compatibility (h–c) groups. The commonly used lineage of strains derived from NRRL 194 (Thom & Raper, 1945) are all of the same compatibility and effectively constitute a twentieth h–c group, known as h–cGLASGOW or h–cGL. Members of each h–c group are fully compatible with each other and vegetatively incompatible with members of other groups. The easiest methods to screen for these compatibility differences is to look at paired mixed cultures in which the component strains differ for conidial colour. Heterokaryon compatible pairings produce a proportion (~1–10 per cent) of heterokaryotic sporeheads within mixed cultures. In these sporeheads adjacent chains of conidia can be of different spore colour giving the sporehead a vertically striped appearance. Mixed cultures of heterokaryon incompatible pairings do not normally generate heterokaryotic sporeheads, though Grindle (1963) showed that a small number (~0.1 per cent) can be observed occasionally.

Heterokaryon incompatibility in *A. nidulans* is controlled by nuclear *het* genes (Jinks & Grindle, 1963). The system is a heterogenic one in that strains which carry identical genes at all *het* loci are compatible, while allelic differences at one or more *het* loci will render the two strains heterokaryon incompatible. Allelic differences at *het* loci do not seem to prevent or even significantly reduce the capacity for sexual crossing between strains (Jinks *et al.*, 1966; Butcher, 1968). Parasexual and sexual methods have been used to investigate heterokaryon incompatibility (Dales *et al.*, 1983; Dales & Croft, 1983, 1990). Protoplast fusion was used to produce somatic hybrids between heterokaryon incompatible parents (Dales & Croft, 1977, 1983, 1990; Dales *et al.*, 1983). Some hybrids were shown to be diploid, and haploid segregants, obtained

*Correspondence: Dr. R. B. G. Dales. Present address: Department of Biology, The University of the West Indies, Cave Hill Campus, P.O. Box 64, Bridgetown, Barbados.

from these diploids, were analysed for heterokaryon compatibility. The parasexual cycle normally leads to the reassortment of whole linkage groups and the demonstration of parasexual linkage of heterokaryon incompatibility with a standard genetical marker indicates the location of a *het* locus or loci on the same linkage group as the marker. A chromosome assay method (Dales & Croft, 1983, 1990) has been used to test each linkage group and in this way the number of linkage groups carrying *het* loci can be determined. The analysis of a sexual back-cross to h-cGL for each linkage group shown to carry *het* genes is all that is further required to define fully the compatibility genotypes of the initial parents used in the protoplast fusion. Using this approach it has been shown that h-cB strains differ from h-cGL at two *het* loci, *hetA* and *hetB*, on linkage groups V and VI, respectively (Dales *et al.*, 1983); that h-cQ and h-cGL strains differ at five *het* loci, two on linkage group III and one on each of linkage groups V, VI and VII (Dales & Croft, 1983); and that h-cA strains differ from h-cGL at six *het* loci, two on linkage group III and one on each of linkage groups II, V, VI and VII (Dales & Croft, 1990). This approach relies upon the availability of a range of genetical markers of known location that cover the eight linkage groups. Such a comprehensive range of markers is currently only available within the GLASGOW strains and an h-cGL master strain has been used as one parent in each of these between h-c group analyses. Unfortunately, this prevents the use of the chromosome assay technique to analyse parasexually derived progeny from pairings of non-GLASGOW h-c groups.

Het loci segregate in Mendelian fashion among the sexual progenies of heterokaryon incompatible parents. In sexual crosses between strains of h-cA and h-cGL, 64 distinct heterokaryon compatibility classes would be expected among the progeny. The classification of such a large number of compatibility classes within a single progeny would be an onerous undertaking and at least 62 further crosses would be necessary to fully define the compatibility genotypes of the recombinant compatibility classes. If sexual back-crosses using progeny strains that are heterokaryon incompatible with both parental classes were used this would reduce the number of compatibility classes to be analysed within a single progeny. Serial back-crossing could be performed until the number of compatibility classes within a progeny are manageable. The number of *het* genes segregating in these back-crosses could be determined and the total number of *het* gene differences between the two initial parents could be deduced by summation. This approach can be used to determine the number of *het* gene differences between pairings of non-GLASGOW h-c groups and has been used here to establish the number of *het* gene differences between strains of h-cA and h-cB. The completion of the triangular h-cA, h-cB, h-cGL compatibility relationship has allowed the determination of the total number of *het* genes involved and indicated the presence of a multiple allelic series at a *het* locus. This approach has also been used with strains of h-cA and h-cGL to confirm that these groups differ at six *het* loci.

Materials and methods

Strains

The parental strains of *A. nidulans* used in the sexual crossing programmes are described in Table 1.

Media

The minimal medium was Czapek (Cz) agar and was derived from that described by Thom & Church (1926). The composition was $NaNo_3$ 2.0 gl^{-1}; KCl 0.5 gl^{-1}; $MgSO_4.7H_2O$ 0.5 gl^{-1}; $ZnSO_4.7H_2O$ 0.01 gl^{-1}; $FeSO_4.7H_2O$ 0.01 gl^{-1}; sucrose 30.0 gl^{-1}; agar (Oxoid No. 3) 15.0 gl^{-1}. K_2HPO_4 was prepared as a sterile stock solution at 50.0 gl^{-1} and added to Cz agar to give a final concentration of 1.0 gl^{-1} prior to pouring to minimize phosphate precipitation. Auxotrophic strains were cultured on Cz agar supplemented with the specific nutrient(s) required. Proline was added to a final concentration of 0.1 gl^{-1}, whereas both *p*-aminobenzoic acid and pyridoxine HCl were added to final concentrations of 0.002 gl^{-1}. Alternatively, auxotrophic strains were cultured on Cz agar supplemented with a solution of vitamins, casein hydrolysate and adenine HCl (CzVCA) at the following final concentrations within the medium: biotin 0.001 gl^{-1}; nicotinic acid 0.02 gl^{-1}; *p*-aminobenzoic acid 0.002 gl^{-1}; pantothenic acid 0.002 gl^{-1}; pyridoxine HCl 0.002 gl^{-1}; riboflavin 0.01 gl^{-1}; thiamine HCl 0.004 gl^{-1}; casein hydrolysate 3.0 gl^{-1}; and adenine HCl 0.1 gl^{-1}.

Strain culture

General culture methods were derived from those of Pontecorvo *et al.* (1953) and Clutterbuck (1974).

Sexual crosses

Parental strains were used that differed in conidial colour so that hybrid cleistothecia could be readily identified. Dense conidial suspensions of both parents, made up in 0.001 per cent (v:v) Tween 80, were spread

Table 1 Strains of *A. nidulans*

Birmingham collection strain no.	Genotype	Compatibility
65-3	*pyro-65.2*	h-cA
JC1-8	*paba-1.1, yA2*	h-cB
JC1-39	*proA1; pyroA5*	h-cGL
JC6-45	*paba-1.1, yA2*	h-cGL
JM1-2	*pyro-65.2*	h-cA×h-cB recombinant
JM1-3	*paba-1.1, yA2*	h-cA×h-cB recombinant
JM2-10	*pyro-65.2*	h-cA×h-cB recombinant
JM3-21	*paba-1.1, yA2*	h-cA×h-cB recombinant
JM4-11	*paba-1.1, yA2*	h-cA×h-cGL recombinant
JM4-33	*pyro-65.2*	h-cA×h-cGL recombinant
JM6-27	*paba-1.1, yA2*	h-cA×h-cGL recombinant
JM6-35	*pyro-65.2*	h-cA×h-cGL recombinant

Gene symbols are as defined by Clutterbuck (1974). Loci are defined by upper-case letters where known. Mutations that have not been characterized to a locus are defined by a hyphen. Alleles are numbered sequentially. Uncharacterized mutant alleles are given numbers which refer to the strain in which they were isolated, e.g. *pyro-65.2* represents the second pyridoxine requiring mutant to be isolated from wild isolate 65 in the Birmingham strain collection.

together on Cz agar supplemented individually for all auxotrophic requirements or on CzVCA agar to give between 10^5 and 10^6 spores of each strain per plate. The plates were incubated at 35°C for 1–3 weeks until mature cleistothecia were visible. Cleistothecia were selected from areas of mixed parental growth and transferred to plates of Cz agar or 3 per cent (w:v) water agar. The cleistothecia were rolled over the surface of the agar to remove adhering conidia, Hülle cells and hyphal fragments. Cleaned cleistothecia were individually crushed to liberate ascospores. A small sample of ascospores from each crushed cleistothecium was inoculated onto supplemented Cz agar and incubated at 35°C for 2–3 days while the remaining ascospores were maintained at 4°C. Inoculations that gave approximately equal proportions of both conidial colours indicated that the ascospore inoculum was probably derived from a hybrid cleistothecium. Further ascospores, from a single putative hybrid cleistothecium per cross, were spread on plates of supplemented Cz agar to give 20–80 colonies per plate following 3 days' incubation at 35°C. These progeny colonies were scored for conidial colour. Progenies that gave approximately equal conidial colour ratios were taken to be of hybrid cleistothecial origin. Progeny samples were collected, purified and analysed for genotype.

Compatibility testing

Compatibility tests were performed on Cz agar supplemented for all auxotrophic requirements, or CzVCA. Strain pairs differing for conidial colour were point or spread inoculated together and incubated for 3 days at 35°C. The mixed colonies produced were screened for the presence of striped, heterokaryotic sporeheads using × 100 magnification and incident illumination.

Terminology

The terms compatible and incompatible will be used to mean heterokaryon compatible and heterokaryon incompatible, respectively.

Results

Sexual crosses to establish the number of het *gene differences operating between strains of h-cA and h-cB*

A sexual cross, numbered JM1, was established between strain 65-3, which was of h-cA, and strain JC1-8 of h-cB. A sample of 150 JM1 progeny strains was collected. Initially, 28 of the green sporing JM1 strains were compatibility tested, in all possible pairwise combinations, with 21 of the yellow sporing progeny. Only 11 pairings, out of these 588 tests, seemed to be compatible and none was compatible with either parental compatibility class. Further testing within the JM1 progeny lead to 63 out of the 150 progeny being classified into 19 compatibility classes. Therefore, at least five *het* genes were segregating between the two parental strains.

A pair of compatible JM1 progeny strains (JM1-2 and JM1-3), which were incompatible with strains of both h–cA and h–cB, were selected as a starting point for a back-crossing programme (Fig. 1). Cross number JM2 was the back-cross of JM1-2 with JC1-8 and cross JM3 was the back-cross of JM1-3 with 65-3. JM1-2 was also crossed with JM1-3 and all progeny of this cross were compatible.

From cross JM2, 104 progeny strains were collected and 90 of these were compatibility classified into 13 compatibility classes, indicating that there were probably four *het* genes segregating in this cross. Further back-crosses JM6 and JM7 (Fig. 1) were done to test this. Only 39 progeny strains of cross JM3 were compatibility tested but all of these were classified into eight compatibility classes (Table 2). This indicated that there were three *het* genes segregating in this cross. Some compatibility tests among the JM3 progeny, however, showed that these strains could give ambivalent compatibility responses. Strains that were highly compatible in pairings with strains assigned to the same compatibility class could also exhibit a low level of compatibility with strains of certain other compatibility classes.

A single JM2 progeny strain, JM2-10, was selected and backcrossed to JC1-8 (cross JM7) and JM1-3 (cross JM8). Table 2 shows that 40 JM7 and 50 JM8 progeny were grouped into four compatibility classes in both crosses. Therefore, there are two *het* genes segregating in each cross. By summation of the *het* genes segregating in crosses JM3, JM7 and JM8 it can be concluded that strains of h–cA differ from strains of h–cB at seven *het* loci.

Sexual crosses to establish the number of het *gene differences operating between strains of h–cA and h–cGL*

It has been shown using parasexual and subsequent sexual back-cross data that strains belonging to h–c groups A and GL differ by six *het* genes (Dales & Croft, 1990). A sexual crossing programme between strains belonging to h–cA and h–cGl was used to confirm this result. The crossing programme is outlined in Fig. 2. The initial cross (JM4) was between strain 65-3 (h–cA) and strain JC6–45 (h–cGL). As it was assumed that these strains would differ at six *het* loci, very little analysis of the JM4 progeny was performed. Two compatible strains, of different spore colour, were selected (JM4-11 & JM4-33), which were incompatible with both parental classes. Strain JM4-11 was back-crossed to strain 65-3 (cross JM5) and strain JM4-33 was

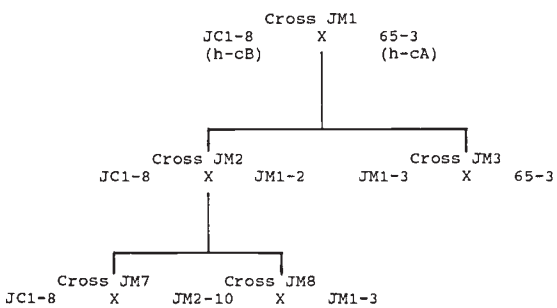

Fig. 1. Sexual crossing scheme to establish the number of *het* gene differences operating between strains of h-cA and h-cB.

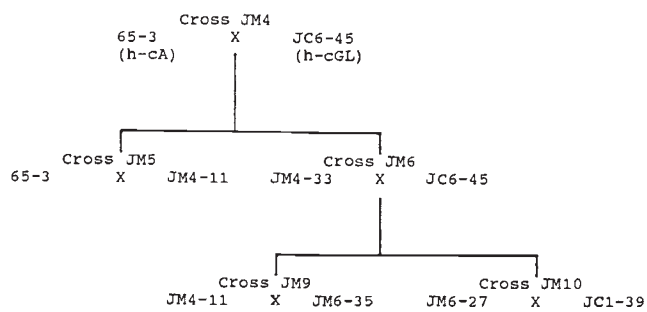

Fig. 2. Sexual crossing scheme to confirm the number of *het* gene differences operating between strains of h-cA and h-cGL.

Table 2 Compatibility analysis of sexual cross progenies to establish the number of *het* gene differences operating between strains of h–cA and h–cB

Cross no.	No. of progeny strains classified	No. of compatibility classes identified	No. of progeny strains in each compatibility class	No. of *het* genes segregating
JM3	39	8	2,3,5,5,5,6,6,7	3
JM7	40	4	8,10,11,11	2
JM8	50	4	7,12,15,16	2

Table 3 Compatibility analysis of sexual cross progenies to confirm the number of *het* gene differences operating between strains of h-cA and h-cGL

Cross no.	No. of progeny strains classified	No. of compatibility classes identified	No. of progeny strains in each compatibility class	No. of *het* genes segregating
JM5	50	4	6,9,14,21	2
JM9	24	2	9,15	1
JM10	49	8	3,4,5,6,6,8,8,9	3

back-crossed to strain JC6-45 (cross JM6). The results of cross JM5 are presented in Table 3. Four compatibility classes were detected among 50 progeny strains indicating that strains 65-3 and JM4-11 differed by two *het* genes. Cross JM5 also gave some ambivalent compatibility responses as had been detected in the JM3 cross.

Few compatible strain pairs were detected among the JM6 progeny. One pair, JM6-27 and JM6-35, were chosen for further crosses (Fig. 2). In cross JM9, strain JM6-35 was back-crossed to strain JM4-11 and in cross JM10, strain JM6-27 was crossed to strain JC1-39 of h-cGL compatibility. Results of these crosses are presented in Table 3. Two compatibility classes were detected among 24 progeny of the JM9 cross indicating a single *het* gene difference segregating. Cross JM10 generated eight compatibility classes among 49 progeny strains indicating the segregation of three *het* genes. The results of this crossing programme confirm the conclusion of Dales & Croft (1990), as the total number of *het* genes segregating in crosses JM5, JM9 and JM10 is six.

Crosses to investigate the nature of the ambivalent compatibility responses observed in crosses JM3 and JM5

Cross JM11 was a back-cross between strain JM3-21 and strain 65-3. The analysis of 50 JM11 progeny strains revealed two compatibility classes of 23 and 27 progeny strains, respectively, indicating that strain JM3-21 differs from h-cA at a single *het* locus. Compatibility tests between strains assigned to the same compatibility class gave a high frequency (~ 1-10 per cent) of heterokaryotic sporeheads within the mixed cultures. In compatibility tests between strains assigned to different compatibility classes a high proportion (66 per cent) showed a low level of compatibility, with only one or a few sporeheads in a 3-day-old mixed culture being heterokaryotic. Representative strains from the other five recombinant compatibility classes of the JM3 progeny have also been back-crossed to strain 65-3. In the other two situations where two compatibility classes were detected among the progeny samples, i.e. where single *het* gene differences were segregating between the parent strains, the compatibility scoring has been absolute. This indicates that the ambivalence in some of the compatibility tests within the JM3 progeny can be correlated with one of the three *het* genes segregating in that cross. An allelic difference at this *het* gene can give a low level of heterokaryon compatibility whereas allelic differences at the other two *het* loci were absolute in their incompatibility response.

Back-crosses to the h-cA parent were also made using representatives from the two recombinant compatibility classes of the JM5 progeny. Here again, one of the back-crosses and hence one of the two *het* genes segregating in the JM5 progeny, was found to show a low level of compatibility in hetero-allelic pairings. Strains assigned to this JM5 compatibility class were found to be heterokaryon compatible with strain JM3-21. Thus the cause of the ambivalent compatibility responses in both the JM3 and JM5 progeny could be attributed to the same *het* gene.

Data analysis

The marker segregations in all of the crosses described did not deviate significantly from expected values. The sizes of the compatibility classes generated in all crosses described did not deviate significantly from random segregation values with the exception of cross JM5. The $\chi^2_{[3]}$ value, with Yates' correction, for compatibility segregation in this cross is 8.8, which is just significant at the 5 per cent level. This may reflect chance variation, sampling error or difficulties with scoring compatibility in this cross due to the reduced stringency of one of the *het* genes. It is unlikely to represent differential *het* allele viability as these alleles are present in other crosses with no apparent affect on segregation ratios. It is also unlikely to represent linkage between the two *het* genes segregating in the

JM5 cross as the recombinant compatibility classes (21 and 14) are in excess of the parental classes (9 and 6).

Discussion

The results described here indicate that h-c groups A and B differ at seven *het* loci and confirm the results of Dales & Croft (1990), which showed that h-c groups A and GL differ at six *het* loci. It has been shown previously (Croft & Jinks, 1977; Dales *et al.*, 1983) by both sexual and parasexual approaches that h-cB and h-c GL differ at two *het* loci. The total number of *het* alleles necessary to explain the triangular compatibility relationship between h-c groups A, B and GL is given by the sum of the allelic differences between pairs of h-c group types, i.e. $7+6+2=15$ alleles. Consequently, one locus must have three alleles in this triangular relationship.

In the parasexual analysis procedures (Dales *et al.*, 1983; Dales & Croft, 1983, 1990), strains carrying single *het* gene differences from h-cGL compatibility have been isolated. GLASGOW compatibility has, therefore, been the standard against which the other h-c groups have been tested. The *het* alleles of h-cGL strains can be arbitrarily given the allele number one. Different compatibility alleles at a locus can be numbered sequentially. The two *het* genes that segregate in crosses between strains of h-cB and h-cGL (Dales *et al.*, 1983) were given the locus letters A and B. The alleles of these loci derived from h-cB were defined as *hetA2* and *hetB2*. At all other *het* loci, h-cB strains must be iso-allelic with h-cGL strains (Table 4).

There are only two possible ways, shown in Table 4, that the compatibility alleles, derived from h-cA, can be assigned in order to satisfy the triangular compatibility relationship between h-c groups A, B and GL. The *het* gene locus that is multi-allelic is either *hetA* on linkage group V or *hetB* on linkage group VI. Whether *hetA* or *hetB* is the multi-allelic locus will be resolved by further analysis and sexual crossing between the strains that differ at *hetA* or *hetB* by single *het* gene differences from h-cGL.

Table 4 Allele number definitions for the *het* loci that segregate among the heterokaryon compatibility groups A, B and GL

Compatibility group	*het* loci							
	A	B	iii	iv	v	vi	vii	... *n*
h-cGL	1	1	1	1	1	1	1	... 1
h-cB	2	2	1	1	1	1	1	... 1
h-cA	3	1	2	2	2	2	2	... 1
	OR							
	1	3	2	2	2	2	2	... 1

The seven *het* loci that control vegetative compatibility within h-cA, h-cB and h-cGL interactions would all seem to be unlinked, although it is known, from the parasexual investigation of the hetero-allelic *het* loci occurring between strains of h-cA and h-cGl, that two of these *het* genes are located on linkage group III (Dales & Croft, 1990). The four remaining *het* gene differences between h—cA and h-cGL were located on linkage groups II, V, VI and VII. If these locations are superimposed upon the information in Table 4 it indicates that if *hetA* is the multi-allelic locus there must be two *het* gene locations on linkage group VI. Alternatively, if *hetB* is multi-allelic then there must be a second *het* locus on linkage group V. Analysis of the compatibility differences between h-c groups R and GL (Anwar *et al.*, unpublished data) has shown that there are two *het* loci on linkage group V. These two *het* loci may be totally unrelated to the ones described in this paper but it is possible that *hetB*, on linkage group VI, is the multi-allelic locus.

One of the seven *het* genes described is not absolute in preventing the formation of heterokaryotic spore-heads when hetero-allelic in mixed culture. The other six *het* genes all give absolute incompatibility responses. Why similar ambivalent compatibility responses were not detected among the progenies generated between members of h-cA and h-cGL, described previously (Dales & Croft, 1990), is unclear.

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