Analysis of heterokaryon incompatibility between heterokaryon-compatibility (h-c) groups R and GL provides evidence that at least eight *het* loci control somatic incompatibility in *Aspergillus nidulans*

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Protoplast fusion was used to hybridize strains 99-6 (h-cR) and 7-141 (h-cGL) in the Birmingham collection of *Aspergillus nidulans*. A sparsely conidiating, brown-pigmented diploid strain (MA7) was isolated. Inocula from MA7 were haploidized on medium containing Benlate and a haploid progeny sample collected. Heterokaryon compatibility testing of selected progeny strains assayed each linkage group in turn and established that h-cR and h-cGL were hetero-allelic for heterokaryon incompatibility (*het*) loci located on linkage groups III and V. Two selected MA7 progeny strains were sexually crossed to an h-cGL strain. Compatibility classification of the progenies of these crosses indicated that three *het* genes were located on linkage group III and two on linkage group V. Combination of these results with previous work using h-c groups A, B and Q in comparison with h-cGL indicates that a minimum number of eight and a maximum number of 18 *het* loci, spread over five linkage groups, are necessary to explain the compatibility relationships among these five h-c groups.

Introduction

Somatic incompatibility was first demonstrated in *Aspergillus nidulans* by Grindle (1963*a, b*). Pairs of isolates that could not form detectable heterokaryons in mixed culture were identified. Subsequently, 100 wild isolates in the Birmingham strain collection were classified into 19 heterokaryon-compatibility (h-c) groups (Croft & Jinks, 1977), labelled h-cA, B, C, D, E, F, G, H, I, J, K, L, M, N, P, Q, R, U and V. The strains of *A. nidulans* derived from NRRL 194, used in many laboratories, are all compatible with each other and incompatible with members of these 19 h-c groups. The NRRL 194 derivatives are known as Glasgow strains

and form a 20th group, labelled h-cGL. Parasexual and sexual approaches involving strains from h-c groups A, B, Q and GL have been used to map heterokaryon incompatibility (*het*) loci, that control somatic compatibility, to linkage groups II, III, V, VI and VII (Dales *et al.*, 1983; Dales & Croft, 1983, 1990). The parasexual approach was facilitated by protoplast fusion (Dales & Croft, 1977). The regeneration products of inter-h-c group fusions were severely abnormal morphologically but both heterokaryons and diploids could be recovered. Diploid strains were haploidized and the progenies analysed for the segregation of compatibility determinants (Dales *et al.*, 1983; Dales & Croft, 1983, 1990). The sexual approach is not impeded in any significant way by the heterokaryon incompatibility system (Jinks *et al.*, 1966; Butcher, 1968; Dales *et al.*, 1993) as hybrid cleistothecia can be isolated readily from heterokaryon incompatible parents and ascospore samples analysed for compatibility segregation.

In comparison with strains of h-cGL, strains of h-cA were found to differ at six *het* loci (Dales & Croft, 1990; Dales *et al.*, 1993), h-cB strains at two loci (Dales *et al.*, 1983), and h-cQ strains at five loci (Dales & Croft, 1983). A sexual crossing programme between strains of h-cA and h-cB has shown that these two groups differ at seven *het* loci (Dales *et al.*, 1993). Seven *het* loci is the minimum

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Abbreviations: Cz agar, Czapek agar; h-c, heterokaryon-compatibility.

number needed to explain the compatibility relationships among these four h-c groups if none of the five *het* genes that segregate in h-cQ × h-cGL crosses are different loci from those in the h-cA, B, GL triangular relationship. Twelve is the maximum number of *het* loci that could possibly be segregating among these four h-c groups if all of the h-cA × h-cGL hetero-allelic *het* genes are new loci. This paper reports the parasexual and sexual investigation of the number and linkage group location of *het* genes that are hetero-allelic between h-cR and h-cGL strains. This analysis shows that the minimum number of *het* loci required to explain the compatibility relationships among the five h-c groups A, B, Q, R and GL is eight.

Methods

Strains. The strains of *A. nidulans* used are described in Table 1.

Media. The minimal medium was Czapek (Cz) agar as described previously (Dales & Croft, 1990). Auxotrophic strains were cultured on Cz agar supplemented with the specific nutrient(s) required. D-Methionine at a final concentration of 0.03 g l⁻¹ was used to supplement the *sa* mutation. Alternatively, auxotrophic strains were cultured on Cz agar supplemented with a vitamins solution, casein hydrolysate and adenine HCl (CzVCA) at the following final concentrations within the medium (g l⁻¹): biotin, 0.001; nicotinic acid, 0.02; *p*-aminobenzoic acid, 0.002; pantothenic acid, 0.002; pyridoxine HCl, 0.002; riboflavin, 0.01; thiamine HCl, 0.004; casein hydrolysate, 3.0; adenine HCl, 0.1. Acriflavine resistance was tested using CzVCA agar containing acriflavine at 0.0035 g l⁻¹. Lactose utilization was tested using fully-supplemented Cz agar in which sucrose had been replaced by lactose at 10.0 g l⁻¹.

Culture methods. General strain culture methods and sexual crossing procedures were derived from those of Pontecorvo *et al.* (1953) and Clutterbuck (1974).

Table 1. *Strains of A. nidulans*

Strain 7-141 is the same as FGSC A375. 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 that refer to the strain in which they were isolated, e.g. *y-99.1* represents the first yellow conidial mutant to be isolated from wild isolate 99 in the Birmingham strain collection.

Birmingham collection strain no.	Genotype	Compatibility
7-37	<i>pabaA6, yA2</i>	h-cGL
7-141	<i>suA1adE20, adE20, biA1; acrA1; sA4; pyroA4; pA2; lacA1; nicB8; riboB2</i>	h-cGL
99-6	<i>paba-99.1, y-99.1</i>	h-cR
MA7-1	<i>suA1adE20, adE20, biA1; acrA1; pa2; lacA1; nicB8; riboB2</i>	h-cR × h-cGL recombinant
MA7-34	<i>suA1adE20, adE20, biA1; acrA1; sA4; pyroA4; lacA1; nicB8; riboB2</i>	h-cR × h-cGL recombinant

Compatibility. Methods for the investigation of heterokaryon incompatibility by a parasexual approach, including the isolation, fusion and regeneration of protoplasts, haploidization of diploids, heterokaryon compatibility testing, and *het* gene location to linkage group by chromosome assay, have been described previously (Dales & Croft, 1977, 1983, 1990; Dales *et al.*, 1983).

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

Results and Discussion

Protoplasts suspensions were prepared from strains 7-141 (h-cGL) and 99-6 (h-cR) and mixed in approximately equal concentrations. Protoplasts within this mixture were fused using 30% (w/v) polyethylene glycol (PEG) 4000 in 0.1 M-CaCl₂. The mixed suspension was diluted in 0.6 M-KCl and plated out in osmotically stabilized (0.6 M-KCl) Cz agar. The colonies that grew on the regeneration plates after incubation at 35 °C for 7 d were of four general types. The majority were non-conidiating, thin mycelial colonies of a dark brown colour. The brown pigment was mostly retained within the sparsely branched hyphae, though some diffused into the surrounding agar. About 20% of the regeneration products appeared to be white, thin mycelial colonies. Half of these colonies were totally aconidial and unpigmented, while the remainder produced some poorly organized, yellow conidial heads. The fourth type of regeneration product was rare (approx. 1%) and appeared to be mixed culture colonies of normal morphology with both yellow and green conidial heads.

The removal of nutritional selection from the conidiating and non-conidiating white types as well as the normal morphology colonies, by transferring block inocula to CzVCA agar, produced both green and yellow sporing sectors that were of parental genotypes for standard markers. These three protoplast fusion product types were thought to be heterokaryotic. Inocula from the brown-pigmented colonies were relatively stable on CzVCA agar. Transfers to Cz agar gave colonies that were a lighter brown in colour with rare light green, loose conidial heads. The conidia were on average 3.6 µm in diameter. The brown colonies were presumed to be diploid and a single culture (MA7) was retained.

Four hundred and fifty small agar block subcultures from the MA7 strain were made onto CzVCA agar containing 1.3 µg Benlate ml⁻¹ and incubated for 7 d to induce haploidization. Sectors of normal morphology and of the four different conidial colours, yellow, pale yellow, green and pale green, were produced, indicating that MA7 was probably diploid. A progeny sample of 95 randomly isolated and purified haploid strains was collected onto 4 × 4 master plates. The master plates were replicated onto a range of differential media and the

Table 2. *Heterokaryon compatibility tests between selected pairs of MA7 progeny strains to test for het gene interaction*

Linkage group under test	Strain pair	Genotype										Compatibility test*
		<i>paba</i>	<i>y</i>	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	<i>p</i>	<i>lac</i>	<i>nic</i>	<i>ribo</i>	
I	{ MA7-6	+	+	<i>bi</i>	<i>acr</i>	+	<i>pyro</i>	+	+	+	<i>ribo</i>	+
	{ MA7-70	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	+	+	<i>ribo</i>	
	{ MA7-21	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-60	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-24	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	+	+	+	<i>ribo</i>	+
	{ MA7-73	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	+	+	<i>ribo</i>	
	{ MA7-36	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	<i>p</i>	+	<i>nic</i>	<i>ribo</i>	+
	{ MA7-85	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	<i>p</i>	+	<i>nic</i>	<i>ribo</i>	
	{ MA7-37	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-81	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	
II	{ MA7-39	+	+	<i>bi</i>	+	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-86	<i>paba</i>	<i>y</i>	+	+	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-29	+	+	<i>bi</i>	+	+	<i>pyro</i>	+	<i>lac</i>	<i>nic</i>	<i>ribo</i>	+
	{ MA7-91	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	<i>lac</i>	<i>nic</i>	<i>ribo</i>	
	{ MA7-39	+	+	<i>bi</i>	+	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-81	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	
III	{ MA7-40	+	+	<i>bi</i>	+	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-60	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-12	+	+	<i>bi</i>	+	<i>s</i>	<i>pyro</i>	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	—
	{ MA7-72	<i>paba</i>	<i>y</i>	+	+	+	<i>pyro</i>	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-19	+	+	<i>bi</i>	+	<i>s</i>	+	+	+	<i>nic</i>	<i>ribo</i>	—
	{ MA7-55	<i>paba</i>	<i>y</i>	+	+	+	+	+	+	<i>nic</i>	<i>ribo</i>	
IV	{ MA7-31	+	+	<i>bi</i>	+	<i>s</i>	+	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	—
	{ MA7-82	<i>paba</i>	<i>y</i>	+	+	+	+	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-9	+	+	<i>bi</i>	<i>acr</i>	+	+	+	+	+	<i>ribo</i>	+
	{ MA7-70	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	+	+	<i>ribo</i>	
	{ MA7-21	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-81	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	
V	{ MA7-24	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	+	+	+	<i>ribo</i>	+
	{ MA7-63	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	+	+	<i>ribo</i>	
	{ MA7-7	+	+	<i>bi</i>	+	+	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	—
	{ MA7-72	<i>paba</i>	<i>y</i>	+	+	+	<i>pyro</i>	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-11	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	—
	{ MA7-81	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	
VI	{ MA7-23	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	<i>p</i>	<i>lac</i>	+	<i>ribo</i>	—
	{ MA7-60	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	
	{ MA7-5	+	+	<i>bi</i>	<i>acr</i>	+	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-70	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	+	+	<i>ribo</i>	
	{ MA7-21	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-63	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	+	+	<i>ribo</i>	
VII	{ MA7-37	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-73	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	+	+	<i>ribo</i>	
	{ MA7-5	+	+	<i>bi</i>	<i>acr</i>	+	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-91	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	<i>lac</i>	<i>nic</i>	<i>ribo</i>	
	{ MA7-14	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	+	+	+	<i>nic</i>	<i>ribo</i>	+
	{ MA7-73	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	+	+	+	+	<i>ribo</i>	
VIII	{ MA7-32	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	+	<i>nic</i>	<i>ribo</i>	+
	{ MA7-63	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	+	+	<i>ribo</i>	
	{ MA7-7	+	+	<i>bi</i>	+	+	<i>pyro</i>	+	<i>lac</i>	+	<i>ribo</i>	+
	{ MA7-59	<i>paba</i>	<i>y</i>	+	+	+	<i>pyro</i>	+	<i>lac</i>	+	+	
	{ MA7-6	+	+	<i>bi</i>	<i>acr</i>	+	<i>pyro</i>	+	+	+	<i>ribo</i>	+
	{ MA7-57	<i>paba</i>	<i>y</i>	+	<i>acr</i>	+	<i>pyro</i>	+	+	+	+	
	{ MA7-34	+	+	<i>bi</i>	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	<i>nic</i>	<i>ribo</i>	+
	{ MA7-68	<i>paba</i>	<i>y</i>	+	<i>acr</i>	<i>s</i>	<i>pyro</i>	+	<i>lac</i>	<i>nic</i>	+	

* +, Compatibility; —, incompatibility

Table 3. Numbers of sexual cross progeny strains in each compatibility class to establish the number of hetero-allelic *het* genes on linkage groups III and V between strains 99-6 (*h-cR*) and 7-141 (*h-cGL*)

Cross no.	Strains crossed	Linkage group under test	Total progeny sample	No. of progeny compatibility classes identified	No. in each progeny compatibility class					No. of <i>het</i> genes segregating
					Parental		Recombinant			
					R	GL				
MA9	MA7-1 × 7-37	III	176	8	27	29	14 22	16 23	20 25	3
MA8	MA7-34 × 7-37	V	105	4	26	31	25	23		2

Table 4. Number and location of *het* loci detected in comparisons of *h-cA*, *h-cB*, *h-cQ* and *h-cR* with *h-cGL*

The minimum and maximum numbers of *het* gene locations at which *h-cGL* differs from other *h-c* groups, for each linkage group and in total, are presented below the body of the table.

h-c group	No. of <i>het</i> gene locations per linkage group:								
	I	II	III	IV	V	VI	VII	VIII	
A	0	1	2	0	1	1	1	0	
B	0	0	0	0	1	1	0	0	
Q	0	0	2	0	1	1	1	0	
R	0	0	3	0	2	0	0	0	
Minimum no. of <i>het</i> loci	0	1	3	0	2	1	1	0	Total 8
Maximum no. of <i>het</i> loci	0	1	7	0	5	3	2	0	Total 18

growth responses used to classify the progeny strains into genotype classes with respect to the standard markers. Allelic ratios and joint marker segregations were determined. All of the MA7 progeny were prototrophic for adenine as the *adE20* marker was masked by the *suAladE20* suppressor. Biased allelic ratios were observed at the *pA* (37 *pA2*:58 +^p), *nicB* (27 *nicB8*:68 +^{nic}) and *riboB* (86 *riboB2*:9 +^{ribo}) loci, with those at *nicB* and *riboB* being highly significant. These values were unexpected and indicate drastically reduced viabilities of the *nicB8* and +^{ribo} genes, or factors linked to them. All other allelic ratios did not deviate significantly from random segregation.

Complete linkage was observed for the *paba-99*, *y-99* and *biA* loci on linkage group I. It is probable that *paba-99.1* and *y-99.1* are alleles of the *pabaA* and *yA* loci, respectively. The joint segregations for the *adE* and *suAadE20* loci with the other standard markers could not be determined absolutely and were disregarded. Non-random assortment was observed between *biA* and *lacA* (39% recombinants) and between *nicB* and *riboB* (64% recombinants). These are probably due to chance variation, compounded, in the latter case, with unequal allele viability. All other marker pairs showed independent assortment.

Six pairs of MA7 progeny strains that were iso-allelic for the markers on linkage groups II–VIII and hetero-allelic for the markers on linkage group I were selected. These pairs of strains were tested for heterokaryon compatibility. All six pairs of strains were compatible (Table 2), indicating that there were no *het* gene differences between strains 99-6 and 7-141 on linkage group I.

Further pairs of strains were selected that were hetero-allelic for the markers on linkage group I, to provide the necessary spore colour difference for compatibility testing, and hetero-allelic for only one other linkage group. Three pairs of strains were compatibility tested for each linkage group assayed. The results presented in Table 2 show that all pairs of strains that were hetero-allelic at the *sA* or *pA* loci were incompatible, whereas all other pairings were compatible. This indicates that *het* gene differences between strains of *h-cR* and *h-cGL* are located on linkage groups III and V.

Two sexual crosses were made to establish the number of hetero-allelic *het* loci segregating between strains of *h-cR* and *h-cGL* on linkage groups III and V. Strain MA7-1 has linkage groups III and IV parasexually derived from strain 99-6 (*h-cR*) and linkage groups I, II, V, VI, VII and VIII derived from the *h-cGL* parent,

7-141. Strain MA7-34 carries linkage group V from the h-cR parent and the remaining seven linkage groups are all of h-cGL origin. These two MA7 progeny strains were crossed to the h-cGL strain 7-37 and the crosses labelled MA8 (MA7-34 $\times$ 7-37) and MA9 (MA7-1 $\times$ 7-37). Progeny samples were collected and analysed for marker segregation (data not shown). Each progeny sample was compatibility tested by doing rounds of pairwise comparisons using randomly chosen progeny strains or parental strains as compatibility testers. The MA9 progeny (Table 3) were classified into eight approximately equal compatibility classes indicating that three unlinked *het* genes were segregating in this cross. The MA8 progeny (Table 3) scored into four approximately equal compatibility classes indicating the presence of two unlinked hetero-allelic *het* loci. Overall, these data show that the original parents, strains 99-6 (h-cR) and 7-141 (h-cGL), carry alternative alleles at five *het* loci. Three genetically unlinked *het* loci located on linkage group III and two genetically unlinked loci on linkage group V. Linkage of these *het* loci with either *sA*, on linkage group III, or *pA*, on linkage group V, was not detected.

Previous work using h-c groups A, B and Q in comparison with h-cGL, combined with the results of the h-cR with h-cGL comparison described here, has detected the number of *het* gene locations shown in Table 4. It is apparent that the minimum number of *het* gene differences from h-cGL, detected to date, within wild isolates of *A. nidulans* is now eight and that there could be a maximum of 18. Further work involving backcrossing to h-cGL strains is required to produce a complete range of 18 strains that carry single *het* gene alleles derived originally from h-c groups A, B, Q or R. Pairwise combinations of these strains could be compatibility tested and, if necessary, inter-crossed. This would establish the exact number of genes and corresponding alleles controlling heterokaryon compatibility among these five h-c groups of *A. nidulans*. It is possible, however, that *het* loci defined by these classical approaches could contain more than one tightly linked

gene but it is unlikely that any such complex locus would be detected until these regions are cloned and sequenced.

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