HETEROKARYON INCOMPATIBILITY IN IMPERFECT SPECIES OF *ASPERGILLUS*

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1. INTRODUCTION

HETEROKARYOSIS has generally been considered an important component of the genetic systems of fungi (Pontecorvo, 1946; Raper, 1955; Buxton, 1960). This conclusion should be questioned, however, in view of the demonstration in recent years of systems of heterokaryon or vegetative incompatibility which restrict heterokaryon formation to strains of like genotype, and thus greatly reduce the genetic significance of heterokaryosis (*see* Caten and Jinks, 1966, for review). The evolutionary consequences of restrictions on heterokaryon formation will be most marked in imperfect species, where heterokaryosis coupled with parasexuality (Pontecorvo, 1956) provides the only known method of recombination. However, the detailed investigations of heterokaryon formation between unrelated strains which led to the discovery of heterokaryon incompatibility all involved perfect species (Garnjobst, 1953, 1955; Beisson-Schecroun, 1962; Grindle, 1963*a, b*; Jones, 1965; Esser, 1966; Kwon and Raper, 1967), and no comparable studies with imperfect species have been reported. In view of the added significance of heterokaryosis in the absence of sexual reproduction, it seemed likely that heterokaryon incompatibility might be either absent or less extensive in imperfect forms.

Gossop, Yuill and Yuill (1940) reported the absence of heterokaryotic conidial heads in mixtures of unrelated strains (inter-strain combinations) of *Aspergillus niger*, but gave no information concerning the number of mixtures examined. A similar result was obtained by Raper and Fennell (1953) in two out of three inter-strain combinations of *A. fonsecaeus*, but the interpretation was complicated by the failure of certain combinations of mutants from the same parent (intra-strain combinations) to produce heterokaryotic heads. Jinks (personal communication) failed to synthesise heterokaryons by pairing unrelated, but morphologically similar, English strains of the *Asymmetrica* group of the genus *Penicillium*, despite the fact that similar attempts to reconstitute naturally occurring heterokaryons from their component homokaryons were successful. These brief reports suggest that heterokaryon incompatibility does occur in at least two groups of imperfect fungi, but allow no quantitative statements. The present work was undertaken to extend the investigations of heterokaryon formation between unrelated strains to further imperfect species, and to provide estimates of the extent of heterokaryon incompatibility which could be compared with those previously reported for related perfect species.

The production of mixed conidial heads (Gossop *et al.*, 1940) in mixed cultures of wild-type and conidial colour mutants has many advantages as a criterion of heterokaryon formation for a study of this type (Grindle, 1963*a*). It has one major disadvantage, however, in that it demands a species which produces well-defined conidial heads and in which the conidia are coloured

and uninucleate. Although these requirements placed a severe restriction on the choice of organism, it was considered that a number of imperfect species of *Aspergillus* would be suitable. Soil platings revealed that two of these, *A. versicolor* and *A. terreus*, occurred at a sufficient frequency to provide a collection of unrelated isolates of known origin. New isolations were preferred to strains from culture collections because of (i) the uncertain origin of many such strains, (ii) the possibility of change due to the accumulation of mutations in culture, and (iii) the wish to use isolates from the same and from different localities.

2. MATERIALS AND METHODS

Platings were made from soil samples collected throughout England and Wales in the manner described by Grindle (1963*b*). The soil plates were incubated for 5 days, after which they were examined under the low power of a dissecting microscope for colonies of *A. versicolor* and *A. terreus*. Any cultures of these two species, recognised on the basis of the descriptions given by Thom and Raper (1945), were transferred to plates of Czapek minimal medium (MM). If a number of morphologically identical cultures were obtained from the same soil sample, one only was retained for testing. No difficulty was encountered in identifying cultures of *A. terreus*. The species *A. versicolor* is less clearly defined, and to confirm the initial identifications all isolates were compared on MM with ten type cultures supplied by the Commonwealth Mycological Institute, and morphologically exceptional cultures were discarded. In addition, five isolates representative of the range of variation encountered were identified as *A. versicolor* by Mr J. J. Elphick of the C.M.I. staff. Stock cultures of all isolates were maintained on potato dextrose agar (PDA) in small, screw cap bottles and stored in a refrigerator.

Conidial colour mutations were induced by ultraviolet irradiation of wild-type conidia suspended in a 0.001% (v/v) solution of Tween 80 (a wetting agent). Irradiated samples were plated on to PDA at a density of about 10^5 survivors per petri dish and, after 7 days' incubation, examined under a dissecting microscope for white or yellow conidial heads. Conidia were picked from mutant heads with a fine glass needle and the mutants purified by further transfers on PDA. Several mutants were obtained from each wild isolate in this way, and compared on MM and PDA with their parent. For each wild isolate, a single mutant which apart from spore colour closely resembled the wild type was retained for the heterokaryon compatibility tests.

The heterokaryon compatibility tests were carried out following the procedure described by Grindle (1963*a*). Preliminary trials were run with several media and incubation temperatures to determine suitable conditions for the formation and detection of mixed heads. As a result PDA with 25° C. incubation and 2 per cent. malt agar (MT) with 25° C. incubation were selected for the tests with *A. versicolor* and *A. terreus* respectively. Because of differences in rate of growth between the isolates, the two strains in each test were inoculated 1-2 mm. apart to avoid the faster growing swamping the slower. Under these conditions the strains met and intermingled along an interface between the points of inoculation. After 12-14 days' incubation, this interface was examined under a dissecting microscope for mixed conidial heads.

Any particular pair of isolates is referred to throughout as a combination. Each combination consisted of two reciprocal tests, differing in which of the isolates carried the spore colour mutation, *e.g.* the combination T1 + T2 was examined by the reciprocal tests T1w + T2 and T1 + T2w. These reciprocal tests provided the only means of replication of the *A. terreus* combinations. With *A. versicolor*, however, each individual test was repeated on at least two occasions.

With *A. versicolor* an attempt was made to quantify the extent of heterokaryon formation. This was achieved by counting the number of mixed heads in four lengths of interface. These were selected at random and defined by the width of the microscope field at $\times 100$ magnification (2 mm.).

TABLE 1

Morphological characteristics of group A and B isolates of A. versicolor

Morphological group	Increase in colony dia. mm./day MM*	Colony colour-surface†		Colony colour-reverse		Conidiation	
		MM	PDA*	MM	PDA	MM	PDA
A	mean = 2.95	green	green	colourless — reddish brown	colourless	high	high
	range = 2.8-3.2						
B	mean = 2.24	yellow	dark green	yellow	yellow	low	high
	range = 2.1-2.6						

* MM = Czapek minimal medium; PDA = Potato dextrose agar.

† Surface colour determined mainly by density of conidiation and amount of aerial mycelium.

Although this method of scoring allows no estimation of the proportion of heterokaryotic heads as was possible for *A. nidulans* (Grindle, 1963*a, b*), it does indicate the relative extent of heterokaryon formation in different combinations. No attempt was made to measure the amount of heterokaryotisation with *A. terreus*. Individual combinations were scored as compatible or incompatible on the basis of the presence or absence of mixed heads.

For growth rate comparisons isolates were grown on 20 ml. of MM in 9 cm. diameter petri dishes. Four isolates were inoculated on to each plate at random, and six (*A. versicolor*) or five (*A. terreus*) replicates of each isolate were grown. The increase in diameter of each colony over the period from the fourth to the ninth day (*A. versicolor*) or the third to the sixth day (*A. terreus*) after inoculation was recorded, and the increase in diameter per day used for analysis.

All incubation was at 25° C.

3. RESULTS

(i) *Description of isolates*

(a) *A. versicolor*

A. versicolor occurred frequently in soil samples collected throughout England and Wales, and a large collection of isolates was readily obtained. Sixty-two of the new isolates and the 10 CMI type cultures were compared

on MM and PDA. There were large differences in rate of growth, surface colour, sporulation, production of sterile aerial hyphae and degree of pigment production in the substrate. The ten type cultures were themselves very variable. On the basis of colony morphology on these media, the majority of the 62 new isolates could be classified into two distinct groups,

TABLE 2
List of isolates used in the heterokaryon compatibility studies

Isolate No.	Morphological group	Increase in colony dia., mm./day	Site of isolation	Date of isolation
(a) <i>A. versicolor</i>				
V1	A	3.0	Dudley A*	1962
V5†	B	2.4	Suffolk	1947
V13	A	2.9	Tottenham	1962
V16	B	2.3	Cardiff	1962
V20	A	2.8	Pembroke M	1962
V22	B	2.2	Oxford	1962
V26	B	2.1	Cheddar	1962
V29	B	2.6	Stratford B	1962
V30	A	2.9	Stratford B	1962
V32	B	2.2	Pembroke A	1962
V34	A	3.1	Pembroke J	1962
V39	B	2.1	Ely	1962
V43	A	3.2	Sutton Coldfield	1962
V44	B	2.3	Sutton Coldfield	1962
V50	B	2.1	Pembroke E	1962
V51	B	2.2	Pembroke E	1962
V52	A	2.9	Birmingham	1962
V55	A	3.0	Dudley B	1962
V56	A	2.8	Dudley B	1962
V74	B	2.1	St Ives	1963
V91	B	2.1	Warkworth	1963
(b) <i>A. terreus</i>				
T1	X	6.6	Pembroke A	1962
T2	X	6.8	Pembroke P	1962
T4	Y	8.0	Pembroke Q	1962
T5	Y	8.0	Pembroke E	1962
T6	Y	8.0	Bewdley	1963
T7	Z	4.9	Bodmin Moor	1963
T8	Z	5.1	Hawkey, Hants.	1963

* The letters denote different soil samples from the same area.

† = I.M.I. 28645.

A and B. Group A was comprised of 27 isolates which fitted the taxonomic description of *A. versicolor* but did not correspond to any of the type cultures. Group B consisted of 15 isolates which closely resembled the type culture IMI 28645. The remaining new isolates formed several additional morphological groups, each represented by a small number of isolates. The main morphological features of the group A and B isolates are listed in table 1.

(b) *A. terreus*

A. terreus was rarely recovered from soil samples and a total of only seven isolates was collected. The isolation sites of these cultures (table 2b)

suggest that this species is largely restricted to the south-west of the British Isles. The seven isolates differed in a number of morphological characters including, rate of growth (table 2), amount of aerial mycelium, and colony colour in reverse. On the basis of colony morphology they could be arranged in three morphological groups as follows: group X = T1, T2; group Y = T4, T5, T6; group Z = T7, T8. Despite these differences, the *A. terreus* isolates were far more uniform than a comparable sample of *A. versicolor*.

TABLE 3

Mean number of mixed conidial heads in 8 mm. of interface for the set I tests with *A. versicolor*. Each intra-strain frequency is the mean of 13 observations. The majority of the inter-strain values are based on two independent tests and each combination is further replicated by the reciprocals

		Green spored wild types													
		V1	V5	V13	V20	V29	V30	V32	V34	V43	V44	V50	V52	V55	V56
Conidial colour mutants*	V1w	3.9	0†	0	0	0	0	0	0	0	0	0	0	0	0
	V5y	0	5.5	0	0	0	0	0	0	1.0	0	0	0	0	0
	V13w	0	0	3.3	0	0	0	0	0	0	0	0	0	0	0
	V20w	0	0	0	4.2	0	0	0	0	0	0	0	0	0	0
	V29y	0.5	0	0	1.0	12.4	0	0	0	0	0	0	0	0	0
	V30w	0	0	0	0	0	3.6	0	0	0	0	0	0	0	0
	V32w	0	0	0	0	0	0	2.0	0	0	0	0	0	0	0
	V34w	0	0	0	0	0	0	0	2.7	0	0	0	0	0	0
	V43w	0	0	0	0	0	0	0	0	2.5	0	0	0	0	0
	V44y	0	0	0	0	0	0.5	0	0	0	14.2	0	0	0	0
	V50y	0	0	0	0	0	0	0	0	0	0	16.8	0	0	0
	V52w	0	0	0	0	0	—‡	0	0	0	0	0	3.3	0	0
	V55w	0	0	0	2.0	0	0	0	0	0	0	0	0	4.2	0
	V56w	0	0	0	0	0	0	0	0	0	0	0	0	0	2.6

* w and y = White and yellow conidial colour mutants respectively.

† A zero score indicates that each of the duplicate tests failed to produce a single mixed head.

‡ Combinations denoted by a minus sign have not been tested.

(ii) Induction of conidial colour mutants

Twenty-one isolates of *A. versicolor* were used for heterokaryon compatibility studies. Details of these are given in table 2a. White mutants were readily induced in all 21 cultures. All group B isolates, except V16, V22 and V32, also produced yellow mutants, although these occurred at a lower frequency than white. A yellow mutant was never observed in any of the group A isolates.

White mutants were readily isolated from all seven *A. terreus* cultures. In addition yellow mutants were obtained in T4, T5, T6 and T7. These latter mutants were further distinguished from wild type by a marked reduction in the size of their conidial apparatus.

(iii) Appearance of heterokaryotic conidial heads

Heterokaryotic conidiophores will only produce mixed conidial heads, i.e. heads showing a segregation for conidial colour, if the determination of the colour of the conidia is autonomous (Pontecorvo, 1946). Mixed conidial heads were produced in both species when white mutants were mixed with their own wild type. To confirm the autonomous control indicated by these

observations, intra-strain mixtures of the white and yellow mutants of V5, V44, T4, T5 and T6 were examined for wild type conidial heads. In both species, while wild type conidial heads were only rarely seen, mixed wild type/white heads occurred at a significant frequency along with the expected mixed white/yellow heads. Progeny testing confirmed that the wild type and mixed wild type/white heads were heterokaryotic. These observations indicate a degree of non-autonomy in the yellow mutants and resemble the situation described by Kwon and Raper (1967) in *A. heterothallicus* and termed autonomous-non-autonomous control. It appears that the type of nuclear

TABLE 4

Mean number of mixed conidial heads in 8 mm. of interface for the set II tests with *A. versicolor*. Each intra-strain frequency is the mean of at least five observations. The majority of the inter-strain values are based on two independent tests and each combination is further replicated by the reciprocals

		Green spored wild types								
		V1	V5	V16	V22	V26	V39	V51	V74	V91
Conidial colour mutants*	V1w	3.8	0†	0	0	0	—‡	—	0	0
	V5y	0	4.3	0	0	0	0	0	0	0
	V16w	0	0	6.2	0	0	0	0	0	0
	V22w	0	0	0	6.3	0	0	0	0	0
	V26y	0	1.0	0	0	9.7	0	3.5	0	0
	V39y	0	0	0	0	1.0	5.2	2.0	0	0
	V51y	0	0	0	0	1.5	—	9.0	0	0
	V74y	0.5	0	0	0	0	0	0	7.0	0
	V91y	0	0	0.5	0	0	0	0	0	6.9

* w and y = White and yellow conidial colour mutants respectively.

† A zero score indicates that each of the duplicate tests failed to produce a single mixed head.

‡ Combinations denoted by a minus sign have not been tested.

action in heterokaryotic heads is not solely a property of the species, but is also dependent on the particular mutants used. In general white mutants lie more towards the autonomous end of the spectrum of nuclear action than other colour mutants.

In mixtures of a wild type with a single spore colour mutant, partial non-autonomy will have the effect of reducing the number of mixed heads seen relative to the number of heterokaryotic heads present. This complication should not influence the relative compatibilities of different combinations considered in the present study, unless the degree of autonomy is markedly affected by the type of combination.

(iv) *Heterokaryon compatibility*

(a) *A. versicolor*

The heterokaryon compatibility of the 21 isolates (table 2a) was investigated in two sets. Set I consisted of all combinations between 14 isolates chosen to give all permutations of morphological similarity or dissimilarity with common or different geographic origins. The results of the set I tests are summarised in table 3. Although each of the isolates was compatible

with its own conidial mutant, no instances of comparable inter-strain compatibility were found. Only six mixed heads were observed in the inter-strain combinations as opposed to 1032 in the same number of intra-strain. Of the six inter-strain mixed heads, two occurred in the combination 20g + 55w. However, mixed heads were not found in subsequent repeats of this combination or its reciprocal and these two strains cannot be considered fully compatible. No pattern is apparent in the occurrence of the remaining inter-strain mixed heads.

In *A. nidulans* compatible isolates are invariably very similar in colony

TABLE 5

Formation of mixed conidial heads in all pairwise combinations of seven wild isolates of A. terreus with their white conidial colour mutants. Six replicate tests of each intra-strain combination were examined. The inter-strain combinations are replicated by the appropriate reciprocal tests

		Brown spored wild types						
		T1	T2	T4	T5	T6	T7	T8
White conidial mutants	T1w	+	+	0	0	0	0	0
	T2w	+	+	0	0	0	0	0
	T4w	0	0	+	0	0	0	0
	T5w	0	0	0	+	0	0	0
	T6w	0	0	0	0	+	0	0
	T7w	0	0	0	0	0	+	0
	T8w	0	0	0	0	0	0	+

+ = Abundant mixed conidial heads.

0 = No mixed conidial heads.

morphology (Grindle, 1963*b*). Therefore in order to increase the probability of finding compatible combinations, eight group B isolates were selected for set II purely on the basis of their morphological similarity. To these was added isolate V1 as a representative of group A. The mean number of mixed heads in all pairwise combinations of these nine isolates is given in table 4. Mixed heads were again frequent in all intra-strain combinations and, in addition, were regularly formed in the inter-strain combinations between isolates V26, V39 and V51. The mixture V39 + V26y appeared to be an exception to this inter-strain compatibility. There was no indication of regular heterokaryon formation in any other inter-strain combination and the majority of such combinations produced no mixed heads. The frequency of mixed heads in the three compatible inter-strain combinations (mean = 1.6) was less than in the intra-strain (mean = 6.5), suggesting that the proportion of heterokaryotic mycelium was less in the former type of combination.

(b) *A. terreus*

The results of heterokaryon compatibility tests involving all pairwise combinations of the seven *A. terreus* isolates are shown in table 5. Mixed heads were abundant in all intra-strain combinations, and in the reciprocal combinations between isolates T1 and T2. No mixed heads were seen in any other inter-strain combination.

(v) *Growth rate comparisons*(a) *A. versicolor*

Growth rate comparisons were carried out separately for set I and set II. There are significant differences in this character among the set I isolates (table 6a). A major part of this variation is accounted for by the comparison

TABLE 6

Analysis of variance of the increase in diameter (mm./day) of colonies of isolates of A. versicolor and A. terreus

Source of variation	N	MS	VR	P
(a) <i>A. versicolor</i> , set I				
Isolates (I)	13	0.721	43.95	<0.001
I { Between morphological groups A and B	1	7.583	50.89*	<0.001
Within group A	8	0.102	6.24	<0.001
Within group B	4	0.242	14.77	<0.001
Replicates	70	0.016	—	—
(b) <i>A. versicolor</i> , set II				
Isolates (I)	8	0.335	17.65	<0.001
I { Between h-c groups	6	0.443	23.29	<0.001
Within h-c groups	2	0.014	<1.0	NS
Replicates	45	0.019	—	—
(c) <i>A. terreus</i>				
Isolates (I)	6	9.043	62.66	<0.001
I { Between h-c groups	5	10.832	75.06	<0.001
Within h-c groups	1	0.098	<1.0	NS
Replicates	28	0.144	—	—

* Tested against within A and B pooled mean square.

between the two morphological groups, although there are also differences between the isolates within each group. Set II also shows significant variation in rate of growth (table 6b), although as these isolates were selected for their morphological similarity the differences are not as great as among set I. The variation among the set II isolates has been partitioned into that between heterokaryon incompatible isolates (between h-c groups) and that between compatible isolates (within h-c groups). All the variation is accounted for by the former comparison and the three compatible isolates V26, V39 and V51 do not differ in rate of growth. The similarity of these three isolates is further emphasised by their morphologies on MM and PDA. V39 and V51 cannot be distinguished on these media, while V26 differs only in a reduced amount of aerial mycelium and lighter colouration in reverse.

(b) *A. terreus*

There are significant differences in rate of growth among the *A. terreus* isolates (table 6c). As with set II *A. versicolor* all this variation is accounted for by comparisons between heterokaryon incompatible isolates and the two compatible isolates T1 and T2 do not differ significantly in this respect.

(vi) *Macroscopic reactions between isolates*

In the course of the heterokaryon compatibility tests with *A. terreus*, a regular morphological reaction visible to the naked eye was observed in certain of the inter-strain combinations. This reaction occurred at the interface between the two isolates and was comprised of two components: (1) the production of a mound of aerial mycelium, and (2) the production of a clear zone of reduced sporulation. The mound of mycelium was formed as a result of the profuse branching of one of the interacting isolates (the reacting isolate) when adjacent to the other (the inducing isolate). Where present the zone of reduced sporulation was also formed by the reacting

TABLE 7
Occurrence of the barrage reaction between seven isolates of A. terreus

		Brown spored wild types						
		T1	T2	T4	T5	T6	T7	T8
White conidial mutants	T1w	0	0	0	0	0	0	0
	T2w	0	0	0	0	0	0	0
	T4w	0	0	0	+ ^{4w} _{S-}	+ ^{4w} _{S-}	0	0
	T5w	0	0	+ ⁴ _{S-}	0	+ ^{5w} _{S+}	0	0
	T6w	0	0	+ ⁴ _{S-}	+ ⁵ _{S+}	0	0	0
	T7w	0	0	0	0	0	0	+ ^{7w} _{S+}
	T8w	0	0	0	0	0	+ ⁷ _{S+}	0

+ = Mound present. The superscript indicates the reacting strain.

S⁺ = Zone of reduced sporulation present.

S⁻ = Zone of reduced sporulation absent.

0 = No reaction.

isolate and appeared on the side of the mound away from the inducing isolate. Thus a profile of the reacting isolate from the interface passes through three regions in the sequence: (1) the mound, (2) the zone of reduced sporulation, and (3) normal growth and sporulation. The inducing isolate is not entirely unaffected, a tendency for a reduction in the size of the conidial heads at the interface was apparent. Rizet (1952) observed a phenotypically similar reaction between strains of *Podospora anserina*, which he termed the barrage phenomenon. Following Rizet, the reaction in *A. terreus* will also be referred to as barrage.

Table 7 shows that the barrage reaction occurred in a regular manner and was consistent over reciprocals. Not all interacting combinations showed both components however, T4 × T5 and T5 × T6 produced only the mound. In any combination the reacting and inducing isolates were always the same irrespective of which carried the spore colour marker (table 7), although a single isolate may act in both roles in different combinations, *e.g.* T5. The reaction in the three combinations involving T4, T5 and T6 were not all equal in intensity. That in T4 × T6 was more marked than in T4 × T5, which in turn was more marked than T5 × T6.

All intra-strain and the majority of the inter-strain combinations produced no such morphological reaction. In these cases there was complete mixing of the two mycelia along the interface, and apart from the spore

colour difference the mixed colonies were indistinguishable from colonies developed from point inoculation of a single culture.

Three points concerning the occurrence of the barrage reaction emerge from table 7: (1) isolates do not react with themselves, (2) heterokaryon compatible isolates do not react, and (3) the interacting isolates fall into two groups (group 1 = T4, T5, T6; group 2 = T7, T8) such that isolates within the same group react with each other, but not with those from the other group. The two groups recognised on this basis correspond to the morphological groups Y and Z respectively (section 3 (1*b*)). The remaining isolates T1 and T2 form a third distinct group both on the basis of morphological similarity and heterokaryon compatibility.

4. DISCUSSION

(i) *The occurrence of heterokaryon incompatibility*

The results of the heterokaryon compatibility tests in the two species were very similar and have been pooled for ease of discussion. All the isolates examined were able to form heterokaryons with their own spore colour mutants under the experimental conditions used. Similar heterokaryon formation occurred in only a small proportion of the combinations between unrelated isolates, however. In all 147 different inter-strain combinations were examined. Heterokaryotic conidial heads were regularly formed in only four of these (2.7 per cent.). The occasional mixed head was seen in a further eight combinations (5.4 per cent., all *A. versicolor*), but in these cases the positive result was not repeatable. In most (91.9 per cent.) of the inter-strain combinations, mixed heads were never observed. The present experiments have shown, therefore, that under the conditions used, which of the various methods of detecting heterokaryosis approximate closest to those in nature (Caten and Jinks, 1966), the vast majority of pairs of unrelated strains in these two species fail to form heterokaryons.

In both species a clear-cut distinction between heterokaryon compatible combinations with mixed heads regularly observed in each length of interface examined, and incompatible combinations with mixed heads absent or very rare and only sporadically produced could be made. With the exception of the eight *A. versicolor* combinations in which one or two mixed heads were observed, the incompatibility appeared absolute. Although for each species the complete set of heterokaryon compatibility tests was carried out in only a single environment, a number were repeated with different media or incubation temperatures. In no case did the environmental conditions of the test alter the classification of a particular combination as compatible or incompatible, although the frequency of mixed heads in the compatible combinations was influenced.

In table 8, the proportion of compatible inter-strain combinations in *A. versicolor* and *A. terreus* is compared with that in a number of related perfect species. In so far as the combinations tested represent a random sample, the proportion compatible should indicate the probability of any two strains from the population sampled being able to form a heterokaryon. It is apparent from table 8 that the frequency of compatible combinations in *A. versicolor* and *A. terreus* is no greater, and may in fact be less, than in the other species. Contrary to the evolutionary hypothesis, therefore, the present

studies lead to the conclusion that heterokaryon incompatibility is as extensive in imperfect forms as in perfect.

Grindle (1963*b*) found that wild isolates of *A. nidulans* could be arranged on the basis of their ability to form heterokaryons with each other into a number of distinct compatibility groups. Eighteen isolates collected from two localities fell into five groups. As a result of the low frequency of inter-strain heterokaryon formation, no useful grouping of the *A. versicolor* or *A. terreus* isolates on this basis can be made. Nevertheless, the few compatible combinations detected formed a regular and predictable pattern, which suggested the existence of groups of compatible isolates. In both cases the partitioning of the species by heterokaryon incompatibility appears more extensive than in *A. nidulans*, with more groups each containing fewer isolates.

TABLE 8

The frequency of heterokaryon compatibility between unrelated strains in four perfect and two imperfect species of Aspergillus

Species	Life cycle	No. of isolates	No. inter-strain combinations tested	Percentage inter-strain combinations compatible	Reference
<i>A. amstelodami</i>	Perfect	12	66	1.5	Caten, unpub.
<i>A. glaucus</i> group	Perfect	35	101	11.9*	Jones, 1965
<i>A. heterothallicus</i>	Perfect	11	55	10.9	Kwon and Raper, 1967
<i>A. nidulans</i>	Perfect	18	153	23.5†	Grindle, 1963 <i>b</i>
<i>A. terreus</i>	Imperfect	7	21	4.8	This report
<i>A. versicolor</i>	Imperfect	21	126	2.4	This report

* Total data, counting only combinations giving heterokaryotic heads in both reciprocals as compatible.

† Based on all combinations between the 18 isolates examined, assuming all within h-c group combinations compatible and between h-c group combinations incompatible.

As in *A. nidulans* (Grindle, 1963*b*), heterokaryon compatible isolates were extremely similar both in colony morphology and rate of growth, while incompatible isolates generally differed in these characters. It should be noted, however, that morphological similarity is a necessary but not sufficient criterion for compatibility, since several examples of apparently identical but incompatible isolates were found, *e.g.* T4, T5 and T6. Crosses between compatible isolates of *A. nidulans* revealed that these were nearly isogenic (Jinks *et al.*, 1966). Confirmation in the present species of the genetic homology of compatible isolates, implied by their phenotypic similarity could only be accomplished through the parasexual cycle.

The low frequency of heterokaryon formation also prevents any attempt to relate compatibility to the sites of isolation. The compatible isolates of *A. versicolor* (V26, V39 and V51) came from widely separated localities, while those of *A. terreus* (T1 and T2) were obtained from the same locality (table 2).

(ii) *The significance of heterokaryon incompatibility*

The potential advantages of a genetic system including heterokaryosis are dependent upon the interaction of two different genomes. If as the

evidence from both perfect and imperfect species, suggests, heterokaryon formation is restricted by genetically determined incompatibility systems to strains with more or less identical genotypes, these advantages will not be realised and heterokaryosis will be of only limited significance in the biology of fungi (Caten and Jinks, 1966). Furthermore, the opportunities for gene exchange through the parasexual cycle will also be greatly restricted. This would be of particular significance in imperfect fungi where parasexuality constitutes the sole means of recombination.

It is a natural step from the idea that the parasexual cycle may replace the sexual cycle where the latter has been lost (Pontecorvo, 1959), to extend the concept of the biological species as a group of individuals sharing a common gene pool to imperfect fungi (Buxton, 1960). The present study suggests, however, that as a result of heterokaryon incompatibility the members of imperfect species, as they are presently defined, are not able to exchange genes through the parasexual cycle in a manner comparable to the flow of genes through sexual reproduction. Rather the present species appear comprised of a large number of independent and restricted gene pools, *i.e.* the heterokaryon compatibility groups. To apply the biological species concept to the Fungi Imperfecti would therefore lead to much narrower specific definition than that presently accepted. For example, the seven morphologically very similar isolates of *A. terreus* used in the present work would constitute no less than six "biological species". Apart from the obvious impracticability of such a scheme, it would not necessarily be biologically more meaningful than the present classification. Where detailed genetic analysis has been carried out, heterokaryon incompatibility has been found to be determined by differences at a few identifiable loci (Garnjobst, 1953, 1955; Caten, unpublished) and hence does not necessarily reflect extensive genetic divergence, despite the association of these two characters in wild isolates (Jinks *et al.*, 1966). In view of this consideration, too much reliance should not be placed on the use of the ability to form heterokaryons or heterozygous diploids as taxonomic criteria.

The occurrence of similar systems of heterokaryon incompatibility in association with such diverse breeding systems as heterothallism (*Neurospora crassa*, *A. heterothallicus*), secondary homothallism (*Podospira anserina*), homothallism (*A. nidulans*, *A. glaucus*) and asexual (*A. versicolor*, *A. terreus*) suggests that the primary function of this phenomenon is not the regulation of the flow of genetic variability through heterokaryosis and parasexual recombination. An alternative role should be sought, and it is suggested that incompatibility might serve to restrict the spread of harmful cell inclusions, such as viruses (Hollings, 1962; Banks *et al.*, 1968) or mutant suppressive mitochondria (Wilson, 1969), from one mycelium to another. Without a mechanism of this type any harmful inclusions would be rapidly spread through an entire population by the hyphal anastomoses and cytoplasmic streaming common in fungal mycelia.

(iii) *Macroscopic reactions between isolates*

The regularity of barrage formation in the *A. terreus* combinations suggests that this phenomenon has a genetic basis. In this case the reaction does not result from the interaction of like genes or genotypes, as in many mating type reactions, since the isolates do not react with their own spore colour mutants. Neither is it simply a reaction between unlike genotypes.

Rather barrage formation is limited to morphologically similar but heterokaryon incompatible pairs of isolates. Equating morphological similarity to genetic similarity and heterokaryon compatibility to near genetic identity, both of which assumptions are valid for wild isolates of *A. nidulans* (Jinks *et al.*, 1966), we can state that barrage formation results from the interaction of two similar but not identical genotypes. If the strains are isogenic or too diverse no reaction occurs.

The biological significance of the barrage reaction is obscure. In *Podospira anserina* barrage formation has been shown to be a macroscopic expression of vegetative (heterokaryon) incompatibility (Esser and Kuenen, 1967). Whether a similar causal connection occurs in *A. terreus* is not yet clear. Certainly barrage formation occurs only with heterokaryon incompatible pairs of isolates, but not all such pairs react in this way. Even if not directly related to heterokaryon incompatibility, in so far as such gross morphological reactions reduce or even prohibit the intermingling of two mycelia, they will form an additional restriction on heterokaryon formation.

5. SUMMARY

1. Isolates of the imperfect species *Aspergillus versicolor* and *A. terreus* were obtained from soil samples collected throughout England and Wales. Their morphological characteristics were compared, and the ability of different isolates of the same species to form heterokaryons was examined using the mixed conidial head test.

2. Isolates of both species readily formed heterokaryons with their own conidial colour mutant but generally failed to do so with mutants derived from different wild isolates. In all 126 combinations involving 21 unrelated isolates of *A. versicolor* were examined. Heterokaryotic mycelium was only regularly produced in three of these. Similarly only one of 21 combinations of unrelated *A. terreus* isolates formed a heterokaryon. Within each species the great majority of wild isolates are heterokaryon incompatible.

3. Heterokaryon compatible isolates are very similar both in colony morphology and rate of growth, while incompatible isolates usually differ in these characteristics.

4. The frequency of compatibility between unrelated strains in *A. versicolor* and *A. terreus* is compared with that in related perfect species. It is concluded that heterokaryon incompatibility is as extensive in imperfect forms as in perfect.

5. The effects of incompatibility on the role of heterokaryosis and parasexual recombination in the genetic systems of imperfect fungi and on the use of the ability to form heterokaryons as a taxonomic criterion are discussed.

6. A macroscopic reaction occurring between certain pairs of *A. terreus* isolates and involving the formation of a mound of aerial mycelium is described. This reaction is thought to be genetically determined and to involve isolates of similar but not identical genotype.

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