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An incompatibility system determined by three factors in a species of *Psathyrella* (Basidiomycetes)

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SUMMARY

Evidence is given for a trifactorial system of incompatibility in an isolate identified as *Psathyrella coprobia*. The three factors are designated A, B and C. They are thought to be inherited independently. The function of factor A is most probably the same as that of factor A of bifactorial species. Factor B is concerned with the initiation of fruit body primordia, while all three factors must be heterozygous for the occurrence of nuclear migration and the formation of mature fruit bodies.

1. INTRODUCTION

Incompatibility in fungi was first described by Blakeslee (1904) in *Rhizopus nigricans*. He used the term 'heterothallism' to describe the situation where sexual reproduction can only take place between hyphae of genetically different mycelia. In this species as in many other phycomycetes the incompatibility system is determined at a single incompatibility locus with two alleles. The opposite of 'heterothallism' is 'homothallism' and this indicates that the sexual cycle can be completed by a mycelium derived from a single spore.

Heterothallism in the basidiomycetes was first described by Bensaude (1918). She found that the incompatibility system in the basidiomycete *Coprinus cinereus* (= *C. lagopus sensu* Buller) was determined at two incompatibility loci both with multiple alleles. Incompatibility of this type is generally referred to as 'tetrapolar' following the initial use of the term 'tetrapolar sexuality' by Bauch (1930) in *Ustilago longissima*. Some basidiomycetes such as *Ustilago violacea* (Burgeff, 1920) were found to have a single factor with multiple alleles. This system is known as 'bipolar incompatibility', though Burgeff had initially used the term 'bipolar sexuality'.

The terms 'incompatibility factor' and 'incompatibility locus' are used synonymously in most of the literature. The term 'factor' is now preferred as it is known that the mating-type determinants consist of at least two subunits which may not always be closely linked (Papazian, 1950; Takemaru, 1957). Since Quintanilha (1939) specifically referred to the incompatibility factors of *Coprinus radiatus* (= *C. fimetarius*) the use of the terms 'unifactorial' and 'bifactorial' have been

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replacing the terms 'bipolar' and 'tetrapolar'. In an incompatibility system determined by three factors the term 'trifactorial' is probably preferable to 'octopolar'.

In a unifactorial system the single factor is designated A, and in a bifactorial system the two factors are designated A and B. In no species are the A and B factors linked. In a bifactorial system heterozygosity for the A factor is responsible for most stages of clamp formation. Heterozygosity for the B factor alone is sufficient to permit nuclear migration (Fulton, 1950). As heterozygosity for both factors is necessary for the formation of complete clamp connexions and for the development of mature fruit bodies the incompatibility mechanism involves some form of complementation.

Basidiospores obtained from the same fruit body are of two mating types in a unifactorial species and of four mating types in a bifactorial species. Each monokaryon can mate with half of the sister monokaryons in a unifactorial species but with only a quarter of the sister monokaryons in a bifactorial species. This restriction in the level of inbreeding is presumably of evolutionary advantage to the species. The presence of a third factor would reduce sister mating even further to one in eight. In theory there is no reason why a trifactorial system should not exist. The results described below show that such a system of incompatibility does exist in an isolate of the genus *Psathyrella*.

2. THE METHOD OF DETERMINING MATING TYPES

The number of mating types present among the basidiospores obtained from a single fruit body is relatively easy to determine by mating a randomly selected sample of monokaryotic mycelia in all combinations, and scoring the matings for the extent of dikaryotization. Reciprocal dikaryotization of the mated mycelia is detected by the presence of clamp connexions throughout both mycelia. It indicates that the two mycelia are fully compatible. Absence of clamp connexions throughout indicates that the two mycelia have incompatibility alleles in common. In bifactorial isolates there is a third class of mating in which clamps can be isolated from the junction line only. Careful examination of the clamps may reveal that they are incomplete or false clamps, but in determining mating types it is necessary to test for nuclear migration as well as for clamps (Aschan, 1954). This can be done by placing the initial inocula some distance apart and by taking sample plugs from the edges of the mycelia as well as from the junction line. If sample plugs are taken from the junction line only it is possible to score a heterokaryotic mycelium with false clamps as a compatible dikaryotic mating because no information on nuclear migration is available. Examples from the literature where incompatibility systems have been wrongly determined because false clamps have been scored as normal clamps are mentioned in the discussion.

3. MATERIAL AND METHODS

All cultures were grown on horse-dung extract medium (LH) following the recipe of Lange (1952) and incubated at 25 °C. In the mating-type determination experiment matings were made on LH agar, four per plate, placing the inocula about 1 cm apart. The inocula were incubated for 5 days before the matings were tested for clamps and nuclear migration. Inocula were initially isolated from the junction line and at 1 cm intervals on both sides, making a total of five samples. In later tests it was found adequate to sample from the junction line and at a distance of 1 cm on each side.

In the growth-rate experiment small plugs of mycelia were taken from the junction line of each mating, were inoculated four per plate on LH, and were incubated for 4 days. The diameter of each colony was measured in millimetres.

In the experiment involving fruit-body formation, plates were inoculated with nine monokaryons in the form of a 3×3 square. The four corner inocula and the central one were of one mating type and the middle inoculum on each of the four sides was of the other type. The plates were incubated in the dark until the mycelia had met and were then transferred into the light at a room temperature of 20 °C. They were examined daily for the formation of primordia and mature fruit bodies over a period of 4 weeks.

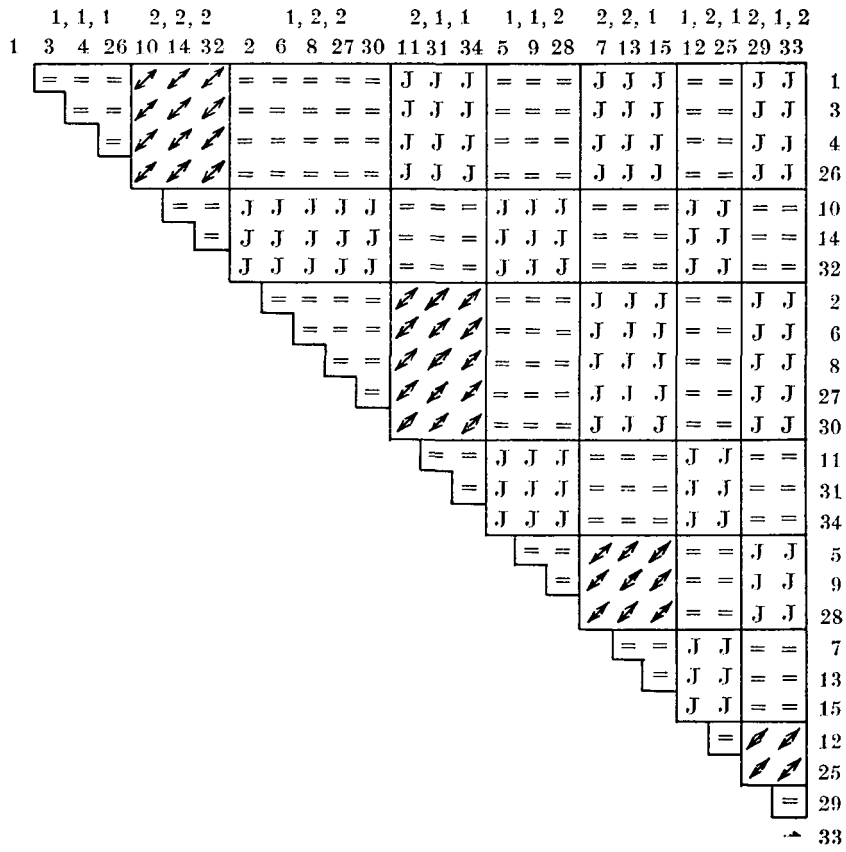
The original isolate was collected by R. F. O. Kemp from horse dung found at Penicuik, Midlothian, in April 1967. The specimen was initially identified as *Psathyrella coprobia* by R. Watling and was placed in the herbarium of the Royal Botanic Garden, Edinburgh, under accession number R.W. 5243. No other isolates belonging to the same breeding group were found. In December 1969 a dikaryotic mycelium was obtained from a mass plating taken from the herbarium. The monokaryolic components of this mycelium were isolated by maceration in May 1970, and shortly afterwards fruit bodies were formed in culture. These were harvested, dried and stored in silica gel. In November 1970 spores from these dried fruit bodies were germinated to initiate the monokaryotic stocks which were used in this study. A total of 73 monokaryons were isolated and these were mated together in various combinations as described below.

4. RESULTS

After mating a random sample of 15 monokaryons in all combinations, seven different mating types were found and not four as expected for a bifactorial species. An eighth mating type was identified when representatives of the seven known mating types were mated with a further sample of monokaryons. A total of 25 monokaryons, including representatives of the eight mating types were then mated again in all combinations and the results are shown in Table 1. The table has been arranged so that strains having the same mating type are next to each other. From the table it can be seen that:

(1) There are four classes of mating in which clamp connexions were isolated

Table 1. *Results of mating 25 monokaryotic mycelia in all combinations*



Dikaryotic mycelia isolated from both sides of the junction line.

J Heterothallic mycelia bearing false clamp connections isolated only from the junction line.

= Matings indistinguishable from the monokaryons.

from both sides of the junction line. This indicated that nuclear migration took place.

(2) There are 12 classes of mating from which heterokaryotic hyphae bearing clamps were isolated only from the junction line. Detailed microscopic observations showed that the clamps were false.

(3) There were 12 classes of mating which were indistinguishable from the monokaryons.

For a single fruit body to produce monokaryons having eight different mating types three factors must be involved in their determination. As the two factors of a bifactorial species have been given the symbols A and B the additional factor present in the trifactorial system has been designated C. Further studies may,

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however, reveal that the A and B factors of the two systems are not homologous. Mating types have been assigned to the monokaryons on this basis and the eight combinations of mating type alleles are represented by their numbers only along the top of Table 1. For example 1, 1, 1 represents A1 B1 C1.

In order to make clearer a more detailed analysis of the results shown in Table 1 it is first necessary to consider some of the characteristics of bifactorial incompatibility, which have been revealed by the studies on *Schizophyllum commune* by Raper and his co-workers and of *Coprinus cinereus* (= *C. lagopus sensu* Buller) by Day. In these two species it has been found that heterozygosity for the A factor ($A \neq B =$) results in the formation, at the junction line, of heterokaryotic mycelia bearing false clamp connexions. No nuclear migration takes place and the hyphae which bear false clamps often from a ridge at the junction line which is known as a 'barrage'. When mated strains are heterozygous for the B factor ($A = B \neq$) nuclear migration can occur into both of the established monokaryotic mycelia but the resulting heterokaryotic mycelium has neither true nor false clamps. In *Schizophyllum* these mycelia can be distinguished by their flat morphology (Papazian, 1950).

Table 2. *The genotypes and mating characteristics of bifactorial and trifactorial systems of incompatibility (the symbols used are those of Raper (1966))*

Bifactorial		$A \neq B \neq$ Compatible	$A \neq B =$ False clamps 'Barrage'	$A = B \neq$ Migration 'Flat'	$A = B =$
Trifactorial	$C \neq$	$A \neq B \neq C \neq$ Compatible	$A \neq B = C \neq$ False clamps	$A = B \neq C \neq$ As monokaryon	$A = B = C \neq$ As monokaryon
	$C =$	$A \neq B \neq C =$ False clamps	$A \neq B = C =$ False clamps	$A = B \neq C =$ As monokaryon	$A = B = C =$

On the basis of this evidence Fulton (1950) distinguished between the functions of the A and B factors in a bifactorial system. He concluded that the A factor controls clamp formation and the B factor controls nuclear migration. Although this difference in function seems to be common to the few species which have been studied in detail it is unsafe to generalize too widely as *Coprinus lagopides* has been found to form $A \neq B =$ heterokaryons by migration (Kemp, unpublished).

A comparison between the mating characteristics in bifactorial and trifactorial systems is summarized in Table 2. From the table it can be seen that in a bifactorial system there are four possible combinations of factors and that there are at least three different mating reactions. The functions of the A and B factors can therefore be distinguished. In the trifactorial system there are eight combinations of factors but only three different mating reactions. The two systems are similar in that mycelia bearing false clamps can be isolated from the junction line in the incompatible matings which involve different A factors. However whereas in a bifactorial system only two factors have to be heterozygous for the formation of dikaryotic mycelia throughout both the initial mycelia, all three have to be heterozygous in a trifactorial system. In the bifactorial system heterozygosity for the B factor alone

is sufficient to permit nuclear migration and in *Schizophyllum commune* heterokaryons of the genotype A = B ≠ can be distinguished by their flat mycelia. In common A matings of trifactorial systems where either or both of the B and C matings were heterozygous, mycelia with a characteristic growth form were not isolated.

From the above information it can be concluded that in the trifactorial system, as in the bifactorial system, the A factor is largely responsible for the formation of clamp connexions. More information is, however, needed to distinguish further between the functions of the three factors. Further experiments were carried out.

(i) *Nuclear migration experiment*

In order to determine whether nuclear migration takes place in the common A matings of the trifactorial system, eight different common A matings were set up and after the matings had been in contact for several days sample plugs from the peripheries of the colonies were isolated. The sample inocula were each paired with two mating types of genotype opposite to that of the original mating. For example, if the original mating was A1B1C1 × A1B2C2, then the plugs from the peripheries were each mated with A2B2C2 and A2B1C1. Compatible matings resulted only if the peripheral plug was mated with a mating type opposite to that of the original colony. For example, a sample plug from the periphery of the original A1B1C1 colony gave a compatible result only when mated with A2B2C2. This indicated that nuclear migration did not take place between the original mycelia of the common A mating. All three factors must be different for nuclear migration to occur.

During the analysis of mating types it was noticed that sample plugs taken from the various matings had a number of characteristic growth rates. Mycelial growth rate and fruiting experiments were therefore carried out to test this observation in an attempt to obtain more information about the functions of the B and C factors.

(ii) *Mycelial growth rate, and heterokaryon stability*

Two monokaryons were selected and mated in all combinations. Small plugs of mycelia were taken from the junction line of each mating and were inoculated, incubated and measured as described in the Materials and Methods. The colony measurements after 4 days' growth are shown in Table 3. The table shows that the samples derived from the matings which resulted in the formation of clamp connexions were of two sizes. The dikaryons (A ≠ B ≠ C ≠) and the heterokaryons of genotype A ≠ B ≠ C = had a mean diameter of 32·56 and 32·00 mm respectively. The heterokaryons of genotypes A ≠ B = C ≠ and A ≠ B = C = had a mean diameter of 23·00 and 21·25 mm respectively. The difference between the diameters of the 'fast' and 'slow' clamp bearing mycelia was shown to be very highly significant by an analysis of variance.

Mycelia were isolated from the junction line of all the 120 matings made in the growth-rate experiment and were stored in bijoux bottles. When the mycelia were subcultured after 2 or 3 weeks of storage it was found that in some of the matings

Table 3. *The relative growth rates (mm) of mycelia isolated from the junction-line of a mating between two members of each of the eight mating types*

	A1B1C1		A2B2C2		A1B2C2		A2B1C1		A1B1C2		A2B2C1		A1B2C1		A2B1C2		
	3	4	10	14	6	8	11	34	5	9	7	13	12	25	29	33	
A1B1C1		26	34	32	26	29	23	26	30	30	30	31	27	28	23	23	3
			30	31	28	30	19	22	31	33	30	30	25	25	21	19	4
A2B2C2				28	19	21	25	26	34	35	34	35	25	24	29	30	10
					19	22	30	30	31	32	30	27	21	25	30	30	14
A1B2C2						25	30	33	26	27	24	20	25	26	33	33	6
							31	34	33	33	26	24	31	32	31	32	8
A2B1C1								28	23	22	31	30	31	32	30	33	11
									25	23	31	29	33	34	29	29	34
A1B1C2										30	33	33	28	28	23	21	5
											34	34	28	28	22	22	9
A2B2C1												30	19	21	27	28	7
													18	23	27	28	13
A1B2C1														30	32	33	12
															31	33	25
A2B1C2																29	29
																	33

Fast growing matings in which nuclear formation, true clamp formation and mature fruit body production takes place.

Fast growing matings in which false clamps are formed at the junction line.

Slow growing matings in which false clamps are formed at the junction line.

false clamps were not recovered. False clamps were recovered in all $A \neq B \neq C =$ matings but not in matings of genotype $A \neq B = C =$ and $A \neq B = C \neq$. On the basis of these results it is possible to conclude that whilst false clamps are formed when the A factor is different, stable false clamps are formed when in addition the B factor is different.

(iii) *Fruit-body production*

In order to test whether heterozygosity for all three factors is necessary for the formation of fruit-bodies, plates were inoculated with monokaryotic inocula as

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described in Materials and Methods. The inocula were arranged according to this pattern because it has been found in certain species of *Psathyrella* and *Coprinus* that fruiting may occur where two monokaryotic inocula come in contact but fails if a plate is inoculated with one or several dikaryotic inocula.

Mature fruit-bodies were formed in five pairs of matings all of which were heterozygous for the three mating type factors. The matings of the type $A \neq B \neq C =$ all developed small primordia but no mature fruit-bodies were formed. All other matings failed to develop any primordia. By referring to Table 3 it can be seen that the fast-growing heterokaryons with false clamps which were isolated from the junction line were the strains which produced primordia, and these all had the genotype $A \neq B \neq C =$. The slow-growing heterokaryons with false clamps which were $A \neq B = C =$ or $A \neq B = C \neq$ developed no primordia. It is therefore possible to conclude that primordium initiation only takes place in matings in which the B factor is different. The heterokaryons with the genotype $A \neq B \neq C =$ although growing at the same rate as the fully compatible matings are distinguishable morphologically by their side branches which often grow in contact with the main axis instead of at an angle of about 45°. This gives the periphery of the colony a rather spiky appearance.

The following conclusions can thus be made. The A factor is concerned with most aspects of clamp formation and is similar in function to the A factor of a bifactorial species. The B factor is concerned with the initiation of primordia while all three factors must be heterozygous for nuclear migration and for the formation of mature fruit-bodies. However, it is still not possible to detect an independent function for C. It is possible that the function of C is epistatic to both of the A and B functions, so that the effect of the C factor would only be detected in compatible matings.

(iv) *The relative position of the three factors on the chromosomes*

In a bifactorial species sister mating occurs at the level of one in four if the factors are not linked and infra-factor recombination is low. All bifactorial species have been found to show independent segregation of the A and B factors. The reduction in the level of sister mating is thought to be the evolutionary advantage of the bifactorial system over a unifactorial one. The halving of sister mating with each additional locus will only occur if the factors segregate independently.

As 73 monokaryotic mycelia were analysed for mating type (Table 4) an indication of the linkage relationships of the three factors is possible. If the three factors are not linked the number of monokaryons in each of the mating type classes should be equal. A chi-square test was therefore carried out to test whether the observed totals in each class differed significantly from the mean of 9·125. The result showed that there was no significant difference from the mean ($\chi^2 = 6{\cdot}67$ with 7 D.F.; $P = 0{\cdot}5 - 0{\cdot}3$). The result for this small sample suggests that the three factors are inherited independently.

Table 4. *The total numbers of monokaryons in the eight mating type classes*

Mating type			Total
1	1	1	8
2	2	2	8
1	2	2	15
2	1	1	8
1	1	2	9
2	2	1	12
1	2	1	6
2	1	2	7
			73

5. DISCUSSION

These results indicate that a system of incompatibility determined by three factors exists in an isolate of *Psathyrella*. Such a system has never before been found in any group of fungi.

Most of the studies of incompatibility in the basidiomycetes have been done on the classical species of *Schizophyllum commune* and *Coprinus lagopus*. It is possible that different systems may be found in other basidiomycetes. Studies in a number of *Psathyrella* and *Coprinus* species show that exceptions from the recognized types of mating behaviour are common. Some species, for example, have no clamp connexions (e.g. *Coprinus congregatus*) although dikaryotic mycelia are formed. But the presence of clamp connexions is not restricted to heterothallic species. Clamps have also been found on the hyphae of several homothallic species. In most species the dikaryotic mycelia are more vigorous than the monokaryons but in a few species of *Coprinus* the monokaryons grow in culture more rapidly than the dikaryons and it is therefore not always possible to isolate compatible dikaryons after migration. The occurrence of nuclear migration in common-B heterokaryons of *C. lagopides* has already been mentioned. In *C. congregatus*, *Psathyrella gracilis* and many other species of *Coprinus* and *Psathyrella* nuclear migration in compatible matings may not occur into monokaryons which have a particular genotype. In this case the restriction in nuclear migration also appears to be controlled genetically but not by the incompatibility factors. In other strains nuclear migration may only be possible over a limited range of temperatures.

Hyphal fusion, nuclear migration and the development of properly formed clamp connexions and fruit bodies may all be controlled by the incompatibility factors. The use of an incomplete scoring procedure has probably accounted for several wrong determinations of incompatibility systems. If no migration information is available and if false clamps are scored as true clamps, then it is possible to misinterpret a bifactorial system as a unifactorial one. Several examples are given below where the incompatibility system of a species has been wrongly determined because false clamps have been scored as normal clamps. Dickson (1936) found *C. macrorhizus* (= *C. cinereus* = *C. lagopus sensu* Buller) to be bifactorial, which agrees with Bensaude (1918), who called the species *C. fimetarius*. However,

Routien (1940) found *C. macrorhizus* to be unifactorial. Similarly Dickson (1934) recorded *C. sphaerosporus* (= *C. lagopides*) as unifactorial but Quintanilha *et al.* (1950) found *C. funariorum* (= *C. lagopides*) to be bifactorial. Recent studies of species in *Coprinus* section *lanatuli*, to which all the above-mentioned species belong, have shown all the described species and several new ones to be bifactorial. Misidentification is therefore unlikely to account for these differences in mating behaviour. Some more examples where incompatibility systems of a species have been wrongly determined are cited by Aschan (1954).

The results given in this paper also show that it is important to test for all aspects of incompatibility and not just for the appearance of clamps. It also seems likely that the function of mating type factors in the basidiomycetes as a whole may not necessarily be similar to those found in the classical bifactorial species.

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