

Maternal inheritance of extranuclear mitochondrial markers in *Aspergillus nidulans*

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SUMMARY

Maternal inheritance of extranuclear mitochondrial genes has been demonstrated in *Aspergillus nidulans* using the 'blue' ascospore colour mutants in combination with heterokaryon incompatible strains. It appears that heterokaryosis is not a prerequisite of sexual outcrossing, and that recombination of extranuclear mitochondrial markers does not occur in the sexual stage of the cell cycle.

1. INTRODUCTION

We have previously reported (Rowlands & Turner, 1973), using the extranuclear mitochondrial mutant (*oliA1*), that the cleistothecia of *Aspergillus nidulans* were always unitype with respect to extranuclear mitochondrial characters, whether selfed or hybrid with respect to nuclear genes. Subsequent reports (Waldron & Roberts, 1973; Gunatilleke, Scazzocchio & Arst, Jr., 1975; Mason & Turner, 1975) confirmed this finding for this and other extranuclear mitochondrial markers. These extranuclear mitochondrial characters were found to sector in heterokaryons, and we suggested at the time (Rowlands & Turner, 1973) that the all-or-none transmission was due to the areas of mycelium producing the cleistothecia having already sectoried by the onset of cleistothecium formation. Handley (1975) suggested the alternative explanation that the all-or-none transmission was due to maternal inheritance, which she had demonstrated for the extranuclearly inherited character 'ragged' in *Aspergillus amstelodami*.

The details of the initial stages of the sexual reproductive cycle of the essentially homothallic ascomycete *Aspergillus nidulans* (Pontecorvo *et al.* 1953) are not known. One school of thought suggests that the sexual cycle is distinct from the asexual, with fertilization occurring via as yet unidentified sexual organs. A second school suggests that the dikaryotic ascogenous hyphae arise directly from the mycelium, so that the heterokaryotic state is a prerequisite for sexual outcrossing. The use of heterokaryon-incompatible strains provides one line of investigation of this problem. Heterokaryon incompatibility is defined by the failure of the two prototrophic strains concerned to produce mixed conidial heads (Grindle, 1963*a*). Using such strains, Jinks *et al.* (1966) and Butcher (1968) have demonstrated the formation of hybrid cleistothecia between pairs of heterokaryon-in-

compatible isolates, which would appear to discredit the second of the above hypotheses. However, in heterokaryon pairs defined as incompatible on the basis of the above test, the possibility of hyphal anastomosis is not excluded. Failure to produce mixed heads could be due to 'heterokaryotic disadvantage' (Caten & Jinks, 1966) rather than failure to anastomose; Jinks *et al.* (1966) and Butcher (1968) themselves favoured this interpretation. It is therefore possible that hyphal anastomosis lead to sufficient heterokaryon formation to initiate the sexual cycle in crosses between such strains, and so the problem remains unresolved. In *A. amstelodami*, failure of the infectious cytoplasmic condition 'ragged' to transfer has been used as a more rigorous test for heterokaryon incompatibility. This has shown that many, but not all, pairings defined as heterokaryon incompatible on the basis of failure to produce mixed conidial heads do in fact show transfer of ragged, demonstrating that hyphal anastomosis does occur in these cases (Handley, 1975; Handley & Caten, 1975). This criterion has not previously been used in *A. nidulans*.

Although the existence of morphologically distinct sexual organs has never been conclusively established in *Aspergillus nidulans*, the existence of physiological sexual differentiation was demonstrated by Apirion (1963) working with ascospore colour mutants. It appears that ascospore colour in this organism is not determined by the ascospore nucleus itself. Instead, all the ascospores in a given cleistothecium regardless of their genotype, as well as the cleistothecial wall, are of the same colour. This colour appears to be determined by the parental nuclei of the area of mycelium which gives rise to the cleistothecial material. By analogy with other ascomycetes (e.g. Alexopoulos, 1962) this implies a female role for these nuclei, the male role being filled by the single nucleus which fuses with one of these nuclei to form the zygote.

The present set of experiments was designed to determine whether maternal inheritance of the extranuclear mitochondrial markers could be demonstrated in *Aspergillus nidulans*, to determine whether the all-or-none segregation could be caused by a fertilization mechanism.

2. MATERIALS AND METHODS

(i) *General*

General materials and methods were as described previously (Rowlands & Turner, 1973; 1974*a, b*).

(ii) *Strains*

The strains of *Aspergillus nidulans* (Eidam) Winter used in this investigation are listed in Table 1. The origins of strains O^R6, B3, B4 and B6 have been described previously (Rowlands & Turner, 1973; 1975). Strains G227 and G043 were obtained from the University of Glasgow stock collection by courtesy of Dr A. J. Clutterbuck, and strains JC9-18 and JC9-190 were obtained from Dr J. H. Croft of the University of Birmingham. The remainder were constructed as follows: strain B10 from the heterokaryon B3/G227; strain B16 from the heterokaryon B4/G227; strain B17 from the heterokaryon B105 (Mason & Turner, 1975)/G043.

(iii) *Sexual crosses*

Sexual crosses were carried out as described in Mason & Turner (1975), with the exception that 2% glucose was incorporated into the growth medium and the cleaned cleistothecia were crushed on 4% plain agar in order to determine ascospore colour, after which the other characters were determined by stabbing the ascospores on to test plates using glass needles. All crosses were carried out in duplicate, and the largest cleistothecia always chosen.

Table 1. *Strains used in this investigation*

Strain	Markers
O ^R 6	<i>pabaA1, yA2; hetA1; hetB1 (oliA1)</i>
B3	<i>wA3; pyroA4; hetA1; hetB1 (oliA1)</i>
B4	<i>wA3; pyroA4; hetA1; hetB1 (camA112)</i>
B6	<i>pabaA1, yA2; hetA1; hetB1 (camA112)</i>
B10	<i>pabaA1, yA2; blaA4; hetA1; hetB1 (oliA1)</i>
B16	<i>pabaA1, yA2; blaA4; hetA1; hetB1 (camA112)</i>
B17	<i>yA2; wA2, blaA1; sC12; hetA1; hetB1 (csA67, camA112)</i>
G043	<i>yA2; wA2, blaA1; sC12; hetA1; hetB1</i>
G227	<i>pabaA1, yA2; blaA4; hetA1; hetB1</i>
JC9-18	<i>pyroA4; hetA2; hetB2</i>
JC9-190	<i>yA2; hetA2; hetB2; ribB2</i>

The designation of mutant alleles is as follows: *pabaA1*, *pyroA4*, *ribB2*, *sC12*, nutritional requirements respectively for *p*-amino-benzoic acid, pyridoxine, riboflavin, sulphite (thio-sulphate); *blaA1*, *blaA4*, blue ascospores; *yA2*, yellow conidia; *wA2*, *wA3*, white conidia; *hetA*, *hetB*, heterokaryon incompatibility loci; (*camA112*), extranuclear chloramphenicol resistance; (*csA67*), extranuclear cold sensitivity; (*oliA1*), extranuclear oligomycin resistance. () denotes extranuclear genes.

The *het* loci are designated according to the following convention: as each new heterokaryon incompatibility locus is discovered it is named A, B etc., and the allele number 1 is given by definition to the allele carried in the Glasgow strains (J. H. Croft, personal communication).

(iv) *Theory of experiment*

It is not possible to demonstrate maternal inheritance in the strains of *Aspergillus nidulans* currently in general use (ultimately derived from the original Glasgow isolate of Yuill (1939)) because reassortment of extranuclear mitochondrial markers as a result of the unavoidable heterokaryon formation which occurs during the induction of the sexual cycle in the laboratory destroys the maternal pattern of inheritance which may be present. Results already published (Rowlands & Turner, 1973) showing the transfer of the extranuclear mitochondrial marker (*oliA1*) into nucleary selfed cleistothecia of the partner strain in a sexual cross clearly demonstrate this.

However, the species *Aspergillus nidulans* is genetically heterogeneous, and independent isolates in the collection of the University of Birmingham differ from each other and from the Glasgow strains in several characters, one of which is their ability to form heterokaryons with each other (Grindle, 1963*a, b*). Therefore by using strains from the Birmingham collection which differ from the Glasgow

strains at several heterokaryon incompatibility loci (Caten, Butcher & Croft, 1972), it should be possible to reduce or prevent the asexual transmission of extranuclear mitochondrial genes when carrying out sexual crosses.

Since in *Aspergillus nidulans* either parental strain can act as maternal or paternal parent (in the sense established by Apirion (1963)), a means of labelling the cleistothecia with regard to their parental origin is necessary. This is provided by the 'blue' ascospore mutants. Ascospore colour in this organism is determined not by the ascospore nucleus itself but by the nuclei of the area of mycelium which gives rise to the cleistothecial material (the 'maternal' nuclei). Thus by scoring the *phenotypic* ascospore colour, one obtains the 'maternal' ascospore genotype. If the extranuclear markers in question are found to remain associated with their parental ascospore genotype, this is interpreted as maternal inheritance. In crosses between heterokaryon-compatible strains the female primordium could be heterokaryotic, in which case the term 'maternal inheritance' becomes less clearly acceptable. However, it should be possible from the results of the cross to estimate whether such heterokaryotic primordia occurred to any great extent. In crosses between heterokaryon-incompatible strains this situation is presumably rare, if it occurs at all.

The heterokaryon-incompatible strains together with the blue ascospore mutant thus provide a system for the investigation of the pattern of transmission of extranuclear mitochondrial markers during the purely sexual cycle of this organism, similar to that used by Handley (1975) to demonstrate maternal inheritance of ragged in *Aspergillus amstelodami*.

3. RESULTS

(i) *Attempted transfer of (oliA1) across the heterokaryon incompatibility barrier*

In order to determine the extent of cytoplasmic transmission allowed by differences at the *hetA* plus *hetB* loci, the combinations B10/JC9-18 and B3/JC9-190 were set up as heterokaryons in the usual way (Rowlands & Turner, 1973), using complementary nutritional markers to force growth on minimal medium.

It proved possible to establish poorly growing heterokaryon-like organisms, and to maintain these indefinitely by mass hyphal transfer subculture to fresh minimal medium. However, conidial samples from these failed to show any reassortment of the (+) allele into the Glasgow strains, or of the (*oliA1*) marker into the Birmingham strains, even after growing the B10/JC9-18 combination on minimal medium containing 3 µg/ml oligomycin (Rowlands & Turner, 1973) (the B3/JC9-190 combination failed to establish itself on oligomycin medium). Similar treatment of compatible Glasgow strains always shows reassortment of (*oliA1*) under these conditions.

It was therefore concluded that the barrier to asexual transmission of extranuclear mitochondrial markers presented by heterozygosity at the *hetA* plus *hetB* loci was absolute under our experimental conditions.

(ii) *Sexual crosses between heterokaryon-compatible strains*

Previous results (Rowlands & Turner, 1973) had led us to believe that asexual transmission of extranuclear mitochondrial markers during the induction of the

Table 2. Sexual crosses between heterokaryon-compatible strains

Cross	Phenotypes and numbers of cleistothecia obtained (ascospore colour and extranuclear markers only shown)				% Hybrid cleistothecia showing maternal inheritance
	Parental selfed	Reassorted selfed	Maternally inherited hybrid	Non-maternally inherited hybrid	
B3 <i>wA3; pyroA4; hetA1; hetB1 (oliA1) × G227 pabaA1, yA2; blaA4; hetA1; hetB1</i>	+ (oli) 19 bl (+) 5	+ (+) 3 bl (oli) 0	+ (oli) 2 bl (+) 11	+ (+) 3 bl (oli) 0	81
O ^h 6 <i>pabaA1, yA2; hetA1; hetB1 (oliA1) × G043 yA2; wA2, bla1, sCI2; hetA1; hetB1</i>	+ (oli) 2 bl (+) 39	+ (+) 0 bl (oli) 0	+ (oli) 0 bl (+) 1	+ (+) 0 bl (oli) 0	—
B4 <i>wA3; pyroA4; yA2; hetA1; hetB1 (camA112) × G227 pabaA1, yA2; blaA4; hetA1; hetB1</i>	+ (cam) 0 bl (+) 2	+ (+) 1 bl (cam) 0	+ (cam) 3 bl (+) 11	+ (+) 12 bl (cam) 0	54
B6 <i>pabaA1, yA2; hetA1; hetB1 (camA112) × G043 yA2, wA2, bla1, sCI2; hetA1; hetB1</i>	+ (cam) 0 bl (+) 25	+ (+) 0 bl (cam) 2	+ (cam) 2 bl (+) 6	+ (+) 2 bl (cam) 0	

No classes other than those shown were recovered.

The term 'reassorted' is used to designate segregation of extranuclear markers with respect to nuclear, regardless of whether recombination of extra-nuclear genes with respect to themselves or nuclear genes with respect to themselves has occurred.

The terms 'hybrid' and 'selfed' refer to the segregation or otherwise of nuclear markers. The cleistothecia were classified as hybrid or selfed with respect to nuclear markers by the segregation of conidial colour and/or nutrient requirements; this data was omitted from the table for the sake of clarity. The ascospore colours given above refer to the phenotypes only; the genotypes with respect to ascospore colour were not determined.

sexual stage in crosses between heterokaryon-compatible strains would obscure the maternal pattern of inheritance that may be present. In order to test this, sexual crosses between compatible Glasgow strains involving the 'blue' markers *blA1* and *blA4* and the extranuclear mitochondrial loci (*oliA1*) and (*camA112*) were carried out and analysed. The results are shown in Table 2.

The occurrence of a large proportion of heterokaryotic female primordia among the total would result in a preponderance of red cleistothecia among the nuclear hybrids. It can be seen from the table that this is not the case, so we can assume that the proportion of such primordia was small. The maximum possible frequency of cleistothecia showing a maternal pattern of inheritance if random reassortment of the extranuclear and ascospore colour markers occurs (i.e. no maternal inheritance) is 50%. As can be seen from Table 2, the percentage of hybrid cleistothecia showing a maternal pattern of inheritance ranged from 54 to 81%, suggesting that at least in some cases maternal inheritance is occurring, but the pattern is overshadowed by a second phenomenon. That this second phenomenon is probably asexual reassortment of the extranuclear markers is shown by the fact that even in the nuclear selfed cleistothecia, reassortment of the extranuclear markers has occurred.

(iii) *Sexual crosses between heterokaryon-incompatible strains*

The results of sexual crosses between heterokaryon-incompatible strains involving both 'blue' markers and the extranuclear mitochondrial markers (*oliA1*), (*csA67*) and (*camA112*) are shown in Table 3.

It is clear from this table that all hybrid cleistothecia from all crosses show strict maternal inheritance, and none of the nuclear selfed cleistothecia show reassortment of the extranuclear markers. Also, it will be noted that in any given cross all the hybrid cleistothecia are of the same type, indicating that the same strain has always acted as the maternal parent. This appears to be governed by the Birmingham strains, since a particular Birmingham strain has consistently acted as maternal or paternal parent whatever the cross, while the Glasgow strains vary. Thus strain JC9-18 consistently acted as the maternal parent towards either the G227-derived strain or the G043-derived strain, while strain JC9-190 consistently acted as the paternal parent towards both strains.

Finally, no recombination between the extranuclear markers has been detected in any crosses between heterokaryon-incompatible strains.

4. DISCUSSION

Uniparental inheritance of extranuclear organelle genes, both mitochondrial and chloroplast, appears to be a general phenomenon, occurring in a wide range of organisms including *Neurospora crassa* (Mitchell & Mitchell, 1952; Bertrand & Pittenger, 1972), *Podospira anserina* (Belcour, 1975), *Xenopus* (Dawid & Blackler, 1972), *Equus* (Hutchison III, *et al.* 1974), higher plants (see Sager, 1972 for review) and *Chlamydomonas reinhardtii* (Sager, 1972). *Saccharomyces cerevisiae* is atypical in this respect, though in a recent report Birky (1975) suggests that uniparental

Table 3. Sexual crosses between heterokaryon-incompatible strains

Cross	Phenotypes and numbers of cleistothecia obtained (ascospore colour and extranuclear markers only shown)					% Hybrid cleistothecia showing maternal inheritance
	Parental selfed	Reassorted selfed	Maternally inherited hybrid	Non-maternally inherited hybrid		
B16 <i>pabaA1, yA2; blaA4; hetA1; hetB1 (camA112) ×</i>	+ (+)	+ (cam)	+ (+)	+ (cam)	0	100
JC9-190 <i>yA2; hetA2; hetB2; ribB2</i>	bl (cam)	bl (+)	bl (cam)	bl (+)	0	
B17 <i>yA2; wA2, bla1; sC12; hetA1; hetB1 (csA67, camA112) ×</i>	+ (+, +)	+ (cs, cam)	+ (+, +)	+ (cs, cam)	0	100
JC9-190 <i>yA2; hetA2; hetB2; ribB2</i>	bl (cs, cam)	80bl (+, +)	bl (cs, cam)	bl (+, +)	0	
B10 <i>pabaA1, yA2; blaA4; hetA1; hetB1 (oliA1) ×</i>	+ (+)	+ (oli)	+ (+)	+ (oli)	0	100
JC9-18 <i>pyroA4; hetA2; hetB2</i>	bl (oli)	bl (+)	bl (oli)	bl (+)	0	
B17 <i>yA2; wA2, bla1; sC12; hetA1; hetB1 (csA67, camA112) ×</i>	+ (+, +)	+ (cs, cam)	+ (+, +)	+ (cs, cam)	0	100
JC9-18 <i>pyroA4; hetA2; hetB2</i>	bl (cs, cam)	bl (+, +)	bl (cs, cam)	bl (+, +)	0	

No classes other than those shown were recovered.

The ascospores from all extranuclearly bifactorial crosses were tested for heteroplasmons, but none were found.

The term 'reassorted' is used to designate segregation of extranuclear markers with respect to nuclear, regardless of whether recombination of extranuclear genes with respect to themselves or nuclear genes with respect to themselves has occurred.

The terms 'hybrid' and 'selfed' refer to the segregation or otherwise of nuclear markers. The cleistothecia were classified as hybrid or selfed with respect to nuclear markers by the segregation of conidial colour and/or nutrient requirements; this data was omitted from the table for the sake of clarity.

The ascospore colours given above refer to the phenotypes only; the genotypes with respect to ascospore colour were not determined.

inheritance occurs in some crosses. The recent demonstration of maternal inheritance in *Aspergillus amstelodami* (Handley, 1975) prompted us to investigate its possible occurrence in *A. nidulans*.

It is clear from the results presented above that maternal inheritance of extranuclear mitochondrial genes does occur in *Aspergillus nidulans*, confirming the existence of a fertilization mechanism in this organism. In sexual crosses between heterokaryon-compatible strains the maternal inheritance pattern is overshadowed by asexual reassortment of the extranuclear markers. A similar effect has been reported in *Podospira anserina* for crosses carried out by confrontation as opposed to spermatization (Belcour, 1975). Our results also indicate that the heterokaryotic state is not a prerequisite for sexual outcrossing in *A. nidulans*, since the *hetA* plus *hetB* genes present a complete barrier to heterokaryotic reassortment of extranuclear mitochondrial genes, yet these can reassort with respect to nuclear genes during the sexual cycle.

An unusual feature of maternal inheritance in this organism is that, despite its homothallic nature, in sexual crosses between heterokaryon-incompatible strains differing at the *hetA* plus *hetB* loci, a given Birmingham strain would consistently act as only maternal or paternal parent with respect to Glasgow strains. This point is under further investigation.

We may therefore interpret the all-or-none segregation of extranuclear mitochondrial markers in sexual crosses as being the consequence of maternal inheritance, though of course when cleistothecia arise from sectored areas of heterokaryons only one mitochondriotype is available for transfer into the cleistothecia. Maternal inheritance also implies the absence of recombination of extranuclear mitochondrial genes during the purely sexual stage of the cell cycle. This is borne out by the results so far obtained with crosses between heterokaryon-incompatible strains, though it is possible that analysis of greater numbers of cleistothecia may yield 'exceptional zygotes' (Sager, 1972). This means that attempts to map the mitochondrial genes by random cleistothecium analysis (Mason & Turner, 1975) of sexual crosses between heterokaryon compatible strains do little more than analyse the results of recombination of extranuclear mitochondrial markers which has previously occurred during the heterokaryon stage, exactly as is done with random conidial analysis (Rowlands & Turner, 1975). However, the different 'transmission strengths' observed by Mason & Turner (1975) for different mitochondriotypes in such crosses cannot be obtained from the results of random conidial analysis of the heterokaryons reported in Rowlands & Turner (1973, 1974*b*, 1975). These must therefore reflect a selection mechanism operating at the cleistothecial level.

The limitation of extranuclear recombination to the heterokaryon stage of the cell cycle of *Aspergillus nidulans* suggests that its occurrence in nature is severely restricted, since different wild populations are likely to be heterokaryon-incompatible (Grindle, 1963*a, b*). Recombination between the extranuclear genes of these different populations can therefore only occur following transfer of these genes across, the incompatibility barrier when they are present in the maternal parent in a sexual cross between heterokaryon-incompatible strains. It is not

clear whether these mechanisms have been evolved to specifically slow down the evolution of the mitochondrial genome, or whether this is a side-effect of mechanisms developed to inhibit the transfer of fungal viruses and deleterious suppressive cytoplasmic conditions, as suggested by Caten (1972) for the restriction of transmission of ragged or 'vegetative death' by the heterokaryon-incompatibility system of *A. amstelodami*. The fact that uniparental inheritance of extranuclear genes is so widespread a phenomenon, occurring even in higher animals (Dawid & Blackler, 1972; Hutchison III *et al.* 1974), where cytoplasmically transmitted infectious conditions are not known, supports the former hypothesis.

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