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Evolution

Sexual origins of British *Aspergillus nidulans* isolates

(fungi/recombination/heterokaryon compatibility/linkage disequilibrium)

DAVID M. GEISER*, MICHAEL L. ARNOLD, AND WILLIAM E. TIMBERLAKE†

Department of Genetics, University of Georgia, Athens, GA 30602

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ABSTRACT **_Aspergillus nidulans_ is a holomorphic fungus, capable of producing both meiotically and mitotically derived spores. Meiosis may be an evolutionary relic in this species because it is potentially capable of mitotic recombination and because most _Aspergilli_ lack the ability to produce meiotic spores. We tested the null hypothesis that meiosis has been a major factor in the origin of strains of _A. nidulans_ from Great Britain by estimating linkage disequilibrium among restriction fragment length polymorphisms. These strains belong to different heterokaryon compatibility groups and are thus incapable of undergoing mitotic recombination with one another, so any recombination evidenced by linkage equilibrium is assumed to be the result of meiosis. Eleven cosmid clones of known chromosomal origin were used to generate multilocus genotypes based on restriction-pattern differences for each heterokaryon compatibility group. Low levels of genetic variation and little linkage disequilibrium were found, indicating that the heterokaryon compatibility groups represent recently diverged lineages that arose via meiotic recombination. The null hypothesis that loci are independent could not be rejected. Additionally, low levels of electrophoretic karyotype variation were indicative of meiosis. We conclude that although _A. nidulans_ probably propagates in a primarily clonal fashion, recombination events are frequent enough to disrupt the stable maintenance of clonal genotypes. We further conclude that the British heterokaryon compatibility groups arose via recombination and not through novel mutation.**

Aspergillus nidulans is a filamentous fungus capable of four modes of propagation (Fig. 1). Both its meiotic spores (ascospores) and mitotic spores (conidia) can be produced in clonal and recombinant fashion in the laboratory (1). The relative roles of the four modes of propagation in nature are unknown. Most *Aspergillus* species are not known to produce ascospores (2). In the laboratory, British strains of *A. nidulans* produce conidia profusely relative to ascospores, suggesting that its primary mode of propagation is mitotic. These observations have led to the suggestion that meiosis may not contribute significant amounts of recombination in *A. nidulans* populations (3) and that its propagation may be primarily clonal.

To determine if meiosis plays a role in the population dynamics of *A. nidulans*, we estimated linkage disequilibrium in nuclear restriction fragment length polymorphism (RFLP) loci among 20 British isolates. This sample represents the 20 heterokaryon compatibility groups (hc groups) identified among >100 British isolates; isolates belonging to the same hc group readily form heterokaryons, whereas those from different hc groups do not (4). Genetic differences between members of the same hc group are very rare (ref. 3; also unpublished data). Therefore, we assume that this population is for the most part represented by just these 20 individuals and that multiple occurrences of the same hc group, even if found in different geographical areas, represent multiple samplings of the same individual. Various tests of linkage disequilibrium have been applied to obtain evidence for clonality in pathogenic bacterial (5, 6) and parasitic protozoan (6) populations. If genetically distinct individuals outcross, either meiotically or mitotically, the consequences are segregation and recombination. In the case of clonal reproduction, different alleles at independent loci are expected to be nonrandomly associated with one another—that is, to be in linkage disequilibrium although not physically linked (6). We assumed any recombination indicated by linkage equilibrium to be the result of meiosis because mitotic recombination is blocked between hc groups and because virtually no variation is found within them.

MATERIALS AND METHODS

DNA Extraction and Manipulation. Fungal mycelium was grown, and DNA was extracted using standard methods (7). One-microgram samples of fungal genomic DNA were digested in microtiter dishes. Digested DNA was separated overnight in 1.1% agarose gels in 1× TAE [0.5 M Tris·HCl/50 mM EDTA/0.7% (vol/vol) glacial acetic acid] and transferred to positively charged nylon membranes (Amersham) in the presence of 20× SSC (1× = 0.3 M sodium citrate/3 M sodium chloride). Cosmid DNAs were isolated from bacterial strains by using a standard alkaline lysis protocol (8). Cosmid DNA was labeled with [α-^{32}P]dCTP by the random primer method (9) according to manufacturer instructions (Promega). Radiolabeled cosmid DNA was incubated with membrane-bound DNA at 68°C in hybridization buffer [5× SSC/5× Denhardt's solution (0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin)/0.25 M sodium phosphate buffer/0.2% SDS], washed in 2× SSC/0.2% SDS at 68°C for 45 min, and exposed to x-ray film at −70°C. Alleles were scored as unique restriction patterns across all four enzymes used; the most common allele was designated "1," the second most common allele was designated "2," etc.

Identifying Pairwise Associations Between Loci. Two allelic classes were defined for each locus: one for the most common allele (represented by class A for locus *X* and class B for locus *Y*) and the second for all others (represented by class a for locus *X* and b for locus *Y*). Linkage disequilibrium between loci *X* and *Y* was estimated as follows:

$$D_{XY} = n_{AB}/N - [(n_{AB} + n_{Ab})/N \times (n_{AB} + n_{aB})/N] \quad [1]$$

where n_{AB}, n_{Ab}, and n_{aB} represent the number of individuals in each allelic class for loci *X* and *Y*, and *N* is the total number of individuals ($N = 20$). The variance in this estimate was calculated as follows:

$$\mathrm{Var}(D_{XY}) = p(1-p)q(1-q)/N, \quad [2]$$

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Abbreviations: hc group, heterokaryon compatibility group; RFLP, restriction fragment length polymorphism; CHEF, contour-clamped homogeneous electric field.

*To whom reprint requests should be addressed.
†Present address: Myco Pharmaceuticals, Inc., Building 300, One Kendall Square, Cambridge, MA 02139.

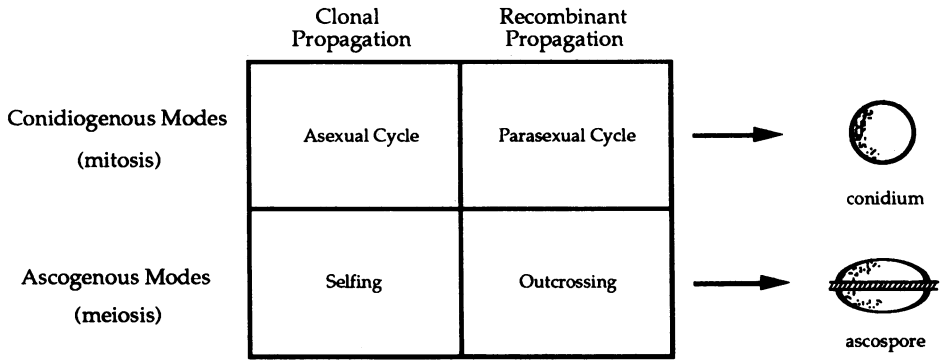

FIG. 1. Four potential modes of propagation in *A. nidulans*. Mitotic spores (conidia) are produced on specialized structures called conidiophores; meiotic spores (ascospores) are produced in structures called cleistothecia. Both spore types can be produced in clonal and recombinant fashion.

where p, the estimated frequency of allele A, $= (n_{AB} + n_{Ab})/N$ and q, the estimated frequency of allele B, $= (n_{AB} + n_{aB})/N$ (10). χ^2 values (df = 1) were calculated by dividing the square of the estimated linkage disequilibrium for each pair of loci, D_{XY}, by the variance of the estimate.

Identifying Overall Allelic Associations. The index of association (I_A; refs. 5 and 11), which estimates overall nonrandom associations of alleles between loci, was calculated as described by Maynard Smith *et al.* (5). We did not lump alleles into two classes in this analysis. First, h_j, a measure of genic diversity, was calculated for each locus j as $1 - \Sigma p_{ij}^2$, where p_{ij} is the frequency of the ith allele at the jth locus. K is the number of differences between two individuals; the average number of differences between any two individuals, $\bar{K}$, is Σh_j. The expected value for the variance of K is $V_E = \Sigma h_j(1 - h_j)$. V_o is the observed variance. The index of association, I_A, is

$$I_A = V_o/V_E - 1. \quad [3]$$

Electrophoretic Karyotype Analysis. Protoplasts were isolated with Novozyme 234 (Novo/Nordisk) and $MgSO_4$ (7), suspended in agarose plugs, and lysed. Chromosomal separations were performed with a Bio-Rad CHEF-DR II contour-clamped homogeneous electric field (CHEF; ref. 12) pulsed-field gel electrophoresis apparatus in 0.5× TAE at 10°C. The following pulsing parameters were used: 50-min pulses for 73 hr, 45-min pulses for 18 hr, and 37-min pulses for 73 hr (modified from ref. 13). The gels were stained with ethidium bromide and photographed on an ultraviolet light source.

RESULTS

We randomly chose 42 clones (averaging ≈35 kb) from a chromosome-specific cosmid library (14). These 42 clones represented ≈5% of the *A. nidulans* genome, and the chromosomal origin of each clone is known. An initial screen for polymorphism between seven strains from different hc groups (A, B, E, F, Q, R, and GL) with eight six-base-recognizing restriction enzymes identified only 14 polymorphic cosmids out of the 42 screened. This result contrasts with findings with other fungi, where similar levels of polymorphism have been found by surveying much smaller (<4 kb) genomic regions (15–17). Six of the sampled loci contained polymorphic restriction sites, and 12 showed complex rearrangements (caused by insertion, deletion, duplication, inversion, or a combination of these) between the hc groups. We conclude that many of the nonsite polymorphisms mapped just outside the regions covered by the cosmid

Table 1. Eleven locus genotypes for strains from 20 hc groups

strain ref. no.	isolation location	hc group	cosmid W03E01	W13E04	W04H04	W09H07	W27H05	W02H09	W21E10	L03H06	L30H09	L03B10	L13C04
65	Birmingham	A	1	3	3	1	2	1	2	2	2	1	1
1	Birmingham	B	1	1	1	1	1	1	2	1	1	2	1
31	Birmingham	C	1	3	2	3	2	1	1	6	4	1	1
34	Birmingham	D	1	1	1	1	1	1	2	4	2	3	1
43	Durham	E	1	1	2	4	1	2	1	3	1	4	1
108	Kent	F	1	2	3	1	1	1	2	1	1	1	2
143	Cornwall	G	2	2	1	1	1	2	2	6	5	5	1
109	Kent	H	1	4	3	1	1	1	2	2	3	1	1
66	Birmingham	I	1	2	4	1	3	1	2	8	1	6	1
67	Birmingham	J	3	2	5	5	1	1	1	5	2	1	3
68	Birmingham	K	2	1	2	1	1	1	1	1	1	1	1
80	Pembrokeshire	L	1	2	1	1	1	1	2	3	2	1	1
85	Pembrokeshire	M	2	1	1	2	1	1	1	1	1	1	1
89	Cambridgeshire	N	2	1	1	1	1	1	1	1	1	1	1
94	Hampshire	P	1	2	2	1	4	1	1	4	1	1	1
106	Warwickshire	Q	1	1	3	1	1	1	2	2	3	1	1
99	Hampshire	R	1	1	1	1	1	2	1	3	6	7	2
114	Pembrokeshire	U	2	1	2	1	1	1	1	5	1	1	1
154	Devon	V	2	1	2	1	1	1	1	1	1	1	1
FGSC4	Glasgow (?)	GL	2	5	1	2	1	1	1	9	2	1	1

The strain reference number, isolation location, and hc group identity are given for each isolate, along with the allele found at each cosmid locus. FGSC4, the "Glasgow wild-type" strain used widely in research, is of unknown geographical origin and is heterokaryon compatible only with itself (22). For each locus, allele 1 is the most common allele, 2 is the second most common, etc.

Table 2. χ^2 values for each pair of loci

chr:	VIII	II	V	III	IV	I	II	III	VII	VII	III
Clone:	W03E01	W13E04	W04H04	W09H07	W27H05	W02H09	W21E10	L03H06	L30H09	L03B10	L13C04
W03E01	-	0.83	0.55	2.54	3.33	0.07	**5.69**	2.54	0.83	1.94	0.07
W13E04		-	0.83	0.00	**5.00**	0.39	1.82	3.81	3.20	0.95	0.39
W04H04			-	0.16	3.33	1.05	0.14	0.36	0.83	2.54	0.07
W09H07				-	0.06	0.02	**7.01**	0.73	0.00	0.73	0.02
W27H05					-	0.88	0.05	2.14	0.00	0.06	0.88
W02H09						-	0.19	1.51	0.39	**8.24**	0.93
W21E10							-	0.47	1.82	1.63	0.19
L03H06								-	**8.57**	0.73	0.02
L30H09									-	0.00	0.39
L03B10										-	0.02
L13C04											-

Values > 3.84 (in boldface type) are significant at the 5% level. The chromosomal origin of each cosmid clone is given (chr).

inserts rather than within them because some polymorphisms were not apparent in all enzyme digests. These polymorphisms may be underrepresented in the cosmid library used if the polymorphisms are associated with repeated elements because repeated DNA was selected against in the construction of the libraries (14). The complex nature of the polymorphisms made the inference of relationships between restriction patterns (and thus between hc groups) difficult, underscoring the need for genetic markers useful for inferring relationships at the population level in fungi.

We expanded the analysis to all 20 known British hc groups by using four six-base-recognizing restriction enzymes and 11 cosmids as probes. Unique patterns across all four restriction enzymes were used as the basis for defining alleles (Table 1). Linkage disequilibrium between each pair of loci was estimated, and a χ^2 with 1 df was calculated by dividing the square of the disequilibrium estimate by its variance (10). The number of samples was small (N = 20). Thus, two allelic classes were defined at each locus: that of the most common allele and that of all other alleles combined. Five of 55 (9.1%) pairwise comparisons showed nonrandom associations significant at the 5% level (Table 2). Although 9.1% is only slightly higher than the 5% expected by chance alone, we do not rule out the possibility that some of these associations may be real, maintained by natural selection. The observation that all of the significantly associated loci were from different chromosomes rules out physical linkage as a factor. Because combining alleles into classes can cause linkage disequilibrium to be under-estimated (18, 19), we also analyzed this data without combining alleles into two classes, finding more (17 of 55 = 25.8%) loci with significant associations. However, in 14 of these 17 pairwise comparisons, a single rare genotype (expected frequency, 0.0025) occurring once (observed frequency, 0.05) was alone responsible for the significant χ^2 value.

We calculated the index of association (I_A; ref. 5) to test further the hypothesis that loci are independently associated. Here we did not pool alleles into two classes. When I_A is significantly different from zero, the null hypothesis that loci are independent is rejected. $I_A = -0.050$ for this data set (V_o = 2.279, V_E = 2.399). By assuming that the sampling distribution of the error variance of I_A, which is calculated (11) as

$$\mathrm{Var}(V_E) = [\Sigma h_i - 7\Sigma h_i^2 + 12\Sigma h_i^3 - 6\Sigma h_i^4 - 2(\Sigma h_i - h_i^2)^2]/N, \quad [4]$$

approximates normality, the upper 95% confidence limit for $\mathrm{Var}(V_E)$ is (11)

$$L \approx \Sigma h_j - \Sigma h_j^2 + 2[\mathrm{Var}(V_E)]^{1/2}. \quad [5]$$

For these data, $L \approx 3.78$. V_o does not exceed this value, so the null hypothesis of independence is not rejected. However, our analysis was limited to single members of each hc group; this treatment is similar to that of taking clusters of

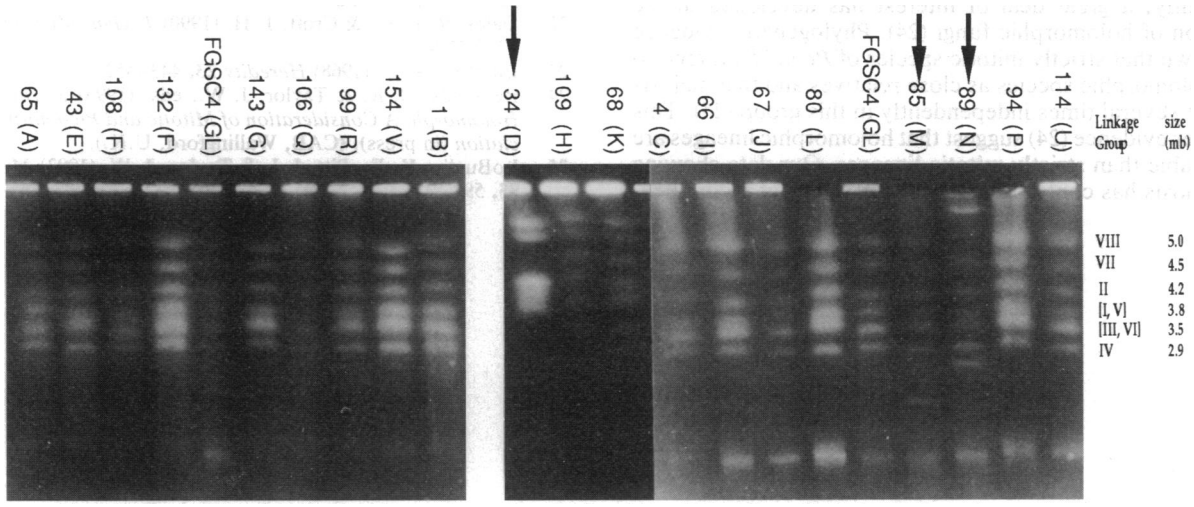

FIG. 2. Electrophoretic karyotypes of *A. nidulans* isolates from 20 hc groups. The isolate number is given for each lane, with its hc group designation in parentheses. The linkage group assignments for each chromosomal band and their estimated sizes in megabase pairs are given on the right, as they were determined for strain FGSC4 (13). Strains 34 (hc D), 85 (hc M), and 89 (hc N) (arrows) have atypical karyotype patterns.

electrophoretic types (ETs) as units of analysis (5), which has the effect of hiding clonal structure. So, while we conclude that the 20 hc groups are of recombinant origin, we do not rule out that propagation may be primarily clonal. The observation that these strains produce far more conidia than ascospores in the laboratory, along with the fact that so few genotypes are observed in this population, suggest that following their recombinant origin, the hc groups may reproduce by predominantly clonal means (6).

We determined the electrophoretic karyotypes of strains from each hc group by using CHEF gel electrophoresis (refs. 12 and 13; Fig. 2). Three of 20 hc groups (D, M, and N) had karyotypes differing from the majority. These findings contrast with those from other, primarily plant pathogenic fungi, where most strains have numerous differences in chromosome size and number at the population level (20, 21). Low levels of karyotype variation support the hypothesis that hc groups arise via meiotic recombination. Progeny of crosses between individuals with different karyotypes are often expected to be inviable due to deletion or duplication. An alternative (but not mutually exclusive) hypothesis to explain the low levels of RFLP and karyotype variation may be very recent common ancestry.

DISCUSSION

A. nidulans from Great Britain appears to consist of primarily clonally propagating individuals or hc groups. However, our data strongly suggest that the various hc groups arose from meiotic recombination and do not exist as distinct, diverging lineages. This finding is consistent with what is known about the genetics of heterokaryon compatibility. The eight loci known to control the phenomenon (*het* genes) must be isoallelic between individuals to form heterokaryons (22). Crosses between individuals from different hc groups produce unusual, recombinant *het* genotypes specifying previously unknown hc group phenotypes. Strains from different hc groups are fully capable of meiotic outcrossing in the laboratory, although the progeny of such crosses are often less vigorous than their parents (23). These findings together with ours suggest that meiotic recombination occurs with significant frequency in British strains of *A. nidulans*. Most of the progeny of these crosses possess novel hc group phenotypes that are either lost or become stable in the population through processes of genetic drift, natural selection, or both.

Recently, a great deal of interest has developed in the evolution of holomorphic fungi (24). Phylogenetic evidence has shown that strictly mitotic species of *Penicillium* tend to have holomorphic species as close relatives and that meiosis was lost several times independently in this group (25). This and other evidence (24) suggest that holomorphic lineages are more stable than strictly mitotic lineages. Our data showing that meiosis has contributed significant levels of recombination in a fungal population corroborates the notion that meiosis plays an important role in fungal evolution.

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