Sex Slows Down the Accumulation of Deleterious Mutations in the Homothallic Fungus *Aspergillus nidulans*

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ABSTRACT

Coexistence of sexual and asexual reproduction within the same individual is an intriguing problem, especially when it concerns homothallic haplonts, like the fungus *Aspergillus nidulans*. In this fungus asexual and sexual offspring have largely identical genotypes. This genetic model organism is an ideal tool to measure possible fitness effects of sex (compared to asex) resulting from causes other than recombination. In this article we show that slightly deleterious mutations accumulate at a lower rate in the sexual pathway than in the asexual pathway. This secondary sex advantage may contribute to the persistence of sexual spores in this fungus. We propose that this advantage results from intra-organismal selection of the fittest gametes or zygotes, which is more stringent in the costly sexual pathway.

MANY organisms are able to produce offspring both sexually and asexually (Bell 1982). The apparent stability of this dual reproductive system raises questions about its functional significance. The coexistence of a sexual and an asexual reproductive pathway within the same individual is especially intriguing in homothallic organisms with predominantly haploid life cycles like many algae and fungi, because their sexual and asexual offspring have identical genotypes. A good example is the fungus *Aspergillus nidulans* (Figure 1). In its vegetative state, it consists of a mycelial colony that typically originates from a single haploid spore. All nuclei in this colony are therefore genetically identical, except for novel mutations that have arisen spontaneously during the growth of the mycelium. A mature colony produces offspring in the form of spores using both an asexual and a sexual pathway. The conidiospores are produced by mitotic division in 3 days after germination, while the ascospores are produced by zygote formation and subsequent meiosis after at least 7 days (Pontecorvo 1953). The sexual ascospores are produced in fruiting bodies, or cleistothecia, each of which is the result of a single fertilization event and may contain up to 100,000 spores. Because *A. nidulans* is homothallic, a single colony can produce ascospores by self-fertilization. This implies that two identical haploid nuclei fuse to a diploid meiocyte only to produce meiotic products (the haploid ascospores) that are genetically identical to the parental nuclei and to the asexually produced conidiospores. Whatever fitness difference, if any, exists between sexually and asexually produced offspring in such a situation, it cannot be a consequence of genetic recombination, which is thought to be a key factor in the evolutionary success of sex (Otto and Lenormand 2002). Thus, in *A. nidulans*, recombination in the sexual cycle has no genetic consequences in the case of selfing. Therefore, this genetic model organism is an ideal tool to measure possible fitness effects of sex (compared to asex) resulting from causes other than recombination. One such cause may be the early intra-organismal selection of potential offspring.

The existence of intra-organismal prezygotic (Otto and Hastings 1998; Walter *et al.* 1998; Extavour and Garcia-Bellido 2001) and postzygotic mechanisms (Gosling 1986; Stephenson and Winsor 1986; Stearns 1987; Forbes and Mock 1998) to increase offspring fitness has been suggested for several organisms with obligate sexuality. The key idea is that parents create an enlarged array of gametes and/or zygotes from which they choose a genetically superior subset. In this way, energy for reproduction is invested in the most promising zygotes. In principle, intra-organismal selection for offspring quality may occur in the sexual and the asexual cycle of *A. nidulans*.

Interestingly, some auxotrophic mutations of *A. nidulans* confer sexual self-sterility as pleiotropic effect while asexual sporulation is normal. This self-sterility manifests itself in very tiny empty cleistothecia and has been described for the *hisB* locus (Millington Ward *et al.* 1984; Busch *et al.* 2001), the *argB* locus (Serlupi Crescenzi *et al.* 1983), the *yB* locus (Kurtz and Champe 1981), and the *trpC* locus (Yelton *et al.* 1983; Eckert *et al.* 1999). Restoration of sexual self-fertility needs higher supplementation of the deficient nutrient than restoration of asexual spore production needs. We tested other loci for this differential restoration of asexual and sexual

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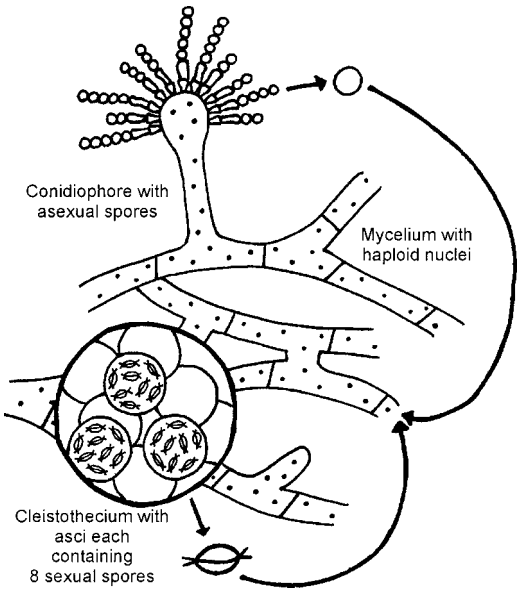

FIGURE 1.—Life cycle of the haploid fungus *A. nidulans*. A mature colony can produce both asexual conidiospores by mitotic division and sexual ascospores by zygote formation and subsequent meiotic division. Inside the cleistothecium in each ascus eight sexual spores are formed, the result of meiosis followed by two mitotic divisions. Both an asexual and a sexual spore can develop into a new mycelial network.

fertility. Literature references and our own data point to the possibility of a more stringent selection in the sexual cycle than in the asexual cycle (Table 1).

This hypothesis predicts that in *A. nidulans* sexual spores produced by selfing will have a higher average fitness than that of the asexual spores with the same genotype. To test our hypothesis, we started a mutation accumulation experiment with 40 asexual and 40 sexual selfing lines, all derived from a common ancestral strain of *A. nidulans*. In these lines, mutations were allowed to accumulate during 40 generations by single-spore transfer. One generation refers to a full cycle of spore germination, mycelial colony growth, and (sexual or asexual) spore development. If intra-organismal selection operates more effectively in the sexual reproduction route, then the sexual lines are expected to carry a smaller load of deleterious mutations than the asexual lines carry. After 40 generations of mutation accumulation, the fitness of each line was estimated relative to that of the founder. Fitness was estimated by measuring the colony diameter grown in a fixed time period. For these measurements, we used stored conidiospores from the founder, the asexual, and the sexual lines, respectively. We observed that mutations had accumulated in both groups, but with a significantly lower "fitness impact" in the sexual lines. We discuss this finding and argue that genetic recombination can be excluded as an explanation for our result. Instead, we propose that the advantage of sex results from intra-organismal selection of the fittest gametes or zygotes, which is more stringent in the costly sexual pathway.

MATERIALS AND METHODS

Strain, media, and culture conditions: The wild-type *A. nidulans* strain JC256 *veA*$^{+}$ was used in this study. This strain was isolated from cereal field soil in Hungary in 1981 (Birmingham collection of J. H. Croft). Minimal medium (MM) and complete medium (CM) were essentially as described by PONTECORVO (1953). All incubations were done at 37°. Asexual spore suspensions were prepared in saline (0.8% NaCl) + 0.005% Tween, and sexual spore suspensions were prepared in saline.

Mutation accumulation protocol: The mutation accumulation procedure is depicted in Figure 2. The asexual and sexual mutation accumulation (MA) lines all started from one single founder colony of strain JC256. An *A. nidulans* colony develops asexual spores 3 days after germination and sexual spores after at least 7 days. Therefore, in the asexual lines a new generation was started every 3 days while in the sexual lines a new generation was started every 12 days. For asexual MA, 40 CM plates were inoculated with an asexual spore suspension from the founder colony, so that individual colonies could originate from single spores. The colonies grown on these plates represented the first generation of the asexual MA lines. We refer to a generation as a full cycle of spore germination, mycelial colony growth, and spore development. After 3 days, from each of the 40 plates, a single colony was randomly chosen by taking the most central colony on the plate. Asexual spore suspensions were made from each of these 40 colonies, after which they were spread for the second MA generation. This procedure was repeated every 3 days until generation 40. This procedure creates for each line a history of 40 successive bottlenecks of a single conidiospore.

TABLE 1

Differential restoration of asexual and sexual spore production in auxotrophic mutants

Mutation	Nutrient required	Supplementation for asexual spore formation (mM)	Supplementation for sexual spore formation (mM)	Reference
pyrD23	Uridine	1	5	Personal observation
adD3	Adenine	0.1	2	Personal observation
hisB	Histidine	0.3	30	BUSCH *et al.* (2001)
trypC	Tryptophan	4	20/30	ECKERT *et al.* (1999)
pyroA4	Pyridoxine	0.001	0.01	Personal observation
riboB2	Riboflavin	0.01	0.1	Personal observation
yB	Copper	0.0016	0.005	KURTZ and CHAMPE (1981)

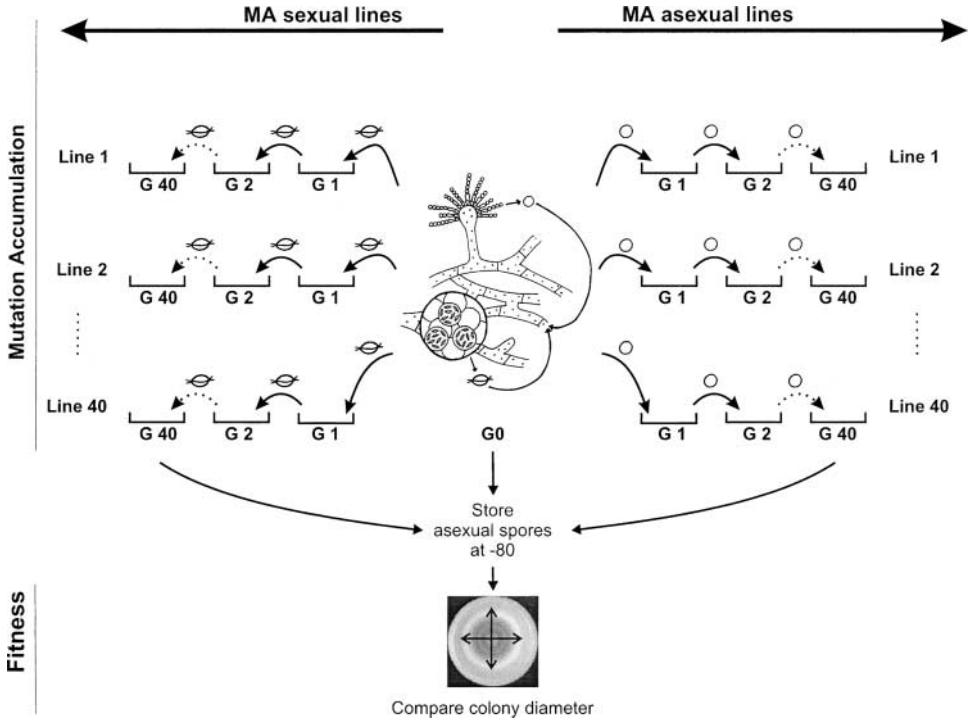

FIGURE 2.—Representation of the mutation accumulation protocol. Forty sexual and asexual lines were established from a single founder colony of *A. nidulans* and maintained during 40 generations. The successive generations were established by the inoculation of one single sexual or asexual spore from the preceding generation. As a fitness assay we measured the relative colony diameter. G, generation.

We started the sexual MA lines with 40 randomly chosen cleistothecia from the founder colony. The 40 cleistothecia were crushed in 1 ml saline each. We inoculated 40 plates with these suspensions so that individual colonies could originate from single sexual spores. The colonies grown on these plates represented the first generation of the sexual MA lines. After 12 days, a sexually sporulating colony was randomly chosen on each of the 40 plates by taking the most central colony. From each of these 40 colonies, a cleistothecium was randomly chosen to start the next round of propagation. This procedure was repeated every 12 days until generation 40. This procedure creates for each line a history of 40 successive bottlenecks of a single ascospore. At regular intervals, asexual spore suspensions were stored at −80° in glycerol/peptone (29%/0.67%) for both the sexual and the asexual lines.

Colony diameter measurements: After 40 generations of MA we measured, after 88 hr, colony diameter in millimeters of all MA lines relative to that of the founder (resulting in a relative colony diameter, or RCD). For all lines (sexual as well as asexual, including the ancestor) we used stored asexual spores at −80° for this fitness measurement. Measurements were performed on five replicate MM plates with point inoculation in the center of a plate using 10 μl asexual spore suspension obtained from 3-day-old cultures grown on CM. After 88 hr, we transferred all plates to 4°, where, after 1 hr rest, we measured diameters of each colony in two perpendicular directions. Mean RCD was calculated for each plate and used as the basic replicate fitness estimate in the analysis.

Data analysis: Differences between founder and mean sexual or asexual MA lines were tested one-tailed using a one-sample *t*-test. Among-group differences were analyzed using a general linear model in which the effect of groups (asexual or sexual) was treated as fixed and the effect of lines (nested within groups) as random effect. We used a Dunnett's test to compare all individual-line means with the ancestral value. Mutational parameters were estimated with the classical Bateman-Mukai method, in which U_{min} and s_{max} are derived from the decrease of average fitness and the increase of the genetic variance in fitness under the assumption of equal mutational effects (MUKAI *et al.* 1972). Bootstrap 95% confidence intervals for U_{min} and s_{max} were obtained from 1000 bootstrapped pseudovalues. Mutational parameters were also estimated using the maximum-likelihood method (KEIGHTLEY 1994), which produces estimates of U and s themselves (instead of their limits) by assuming s to vary according to a gamma distribution. Confidence intervals of 95% for individual parameters were obtained by a drop of 2 log likelihood from the maximum. The kurtosis of this distribution is described by γ_2; with $\gamma_2 \rightarrow$ infinity the distribution becomes increasingly leptokurtic. Differences between two parameter estimates are significant when their point estimates lie outside each other's 95% confidence interval.

RESULTS

After 40 generations of mutation accumulation, we found that fitness was significantly lower in both the asexual lines and the sexual lines than in the founder (asex: mean (SE) = 0.961 (0.0018); $t_{38} = -12.78$; $P < 0.001$; sex: mean (SE) = 0.973 (0.0020); $t_{37} = -6.67$; $P < 0.001$; Figure 3). The sexual lines had a significantly higher fitness than the asexual lines ($F_{1,75} = 5.60$; $P = 0.021$). Thus, mutations had accumulated in both groups, but with a significantly lower "fitness impact" in the sexual lines. Moreover, 11 of the 38 sexual lines (29%) had a colony diameter significantly smaller than that of the founder, while this was true for 21 of the 39 asexual lines (54%). This difference between sexual and asexual lines was significant ($\chi^2_1 = 4.913$; $P = 0.027$). These findings are consistent with our prediction that more effective intra-organismal selection in the sexual lines results in slowing down the accumulation of mutations. In addition, we found that the error variance of

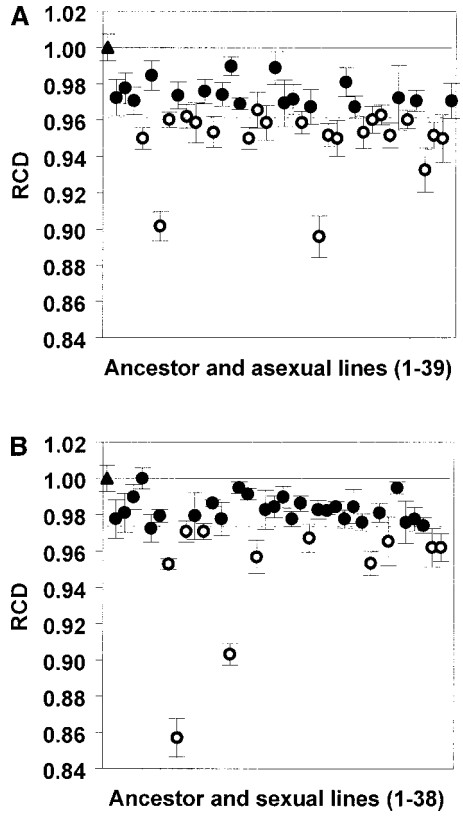

FIGURE 3.—Colony diameter of the mutation accumulation lines relative to the ancestor (RCD). ▲, the ancestor. Circles represent the MA lines: ●, lines not significantly different from ancestor; ○, lines significantly different from ancestor. Error bars show standard errors. The solid line indicates the mean of the ancestor and the dashed line the mean of the asexual and sexual lines, respectively. (A) Asexual lines. (B) Sexual lines.

sexual lines is (slightly) lower than that of asexual lines ($F_{149, 147} = 1.3603$, $P = 0.031$).

The higher fitness of sexual relative to asexual lines can be the result of a lower number of mutations that accumulated or a lower fitness effect of the mutations, or both. However, without *a priori* knowledge of the mechanism responsible for intra-organismal selection, we expect, first of all, a lower number of mutations to have accumulated. This is because *any* mechanism would reduce the number of mutations, while only a subset of mechanisms (namely those with selection bias toward mutations with large effect) would also reduce the average fitness effect. As a first approach to estimating the parameters underlying the mutation accumulation process, we used the classical Bateman-Mukai method (MUKAI *et al.* 1972). This method estimates the lower limit of the mutation rate (U_{min} per generation per whole genome) and the upper limit of the mean fitness effect of a mutation (s_{max}). U_{min} and s_{max} are derived from the decrease of average fitness and the increase of the genetic variance in fitness under the assumption of equal mutational effects. This method, however, did not reveal significant differences between the asexual and sexual lines, as the point estimates did not lie outside each other's 95% confidence interval (Table 2A).

As a second approach, we used maximum-likelihood estimation, which estimates U and s, rather than U_{min} and s_{max}. This procedure assumes that mutational effects follow a gamma distribution, thus avoiding the unrealistic assumption of equal mutational effects. In maximum-likelihood estimation, log likelihood is maximized for three parameters: U, s, and γ_2, *i.e.*, the kurtosis of the gamma distribution. Maximization of log likelihood for all three parameters simultaneously resulted in an infinitely high U, an infinitely small s, and an infinitely high γ_2 for both sexuals and asexuals. Fixing one of the parameters resulted in biologically more realistic parameter estimates. As we expected in any case effect on U, we fixed s by filling in the Bateman-Mukai estimates of s for sexuals and asexuals. In this way, we obtained maximum-likelihood estimates of U that are significantly lower for sexuals than for asexuals, as their point estimates lie outside each other's 95% confidence interval (Table 2B). However, since U and s are confounded in the estimation procedure, the lower U for sexuals could depend on the (nonsignificantly) higher estimate of s for this group. To exclude this possibility, we then fixed the same Bateman-Mukai estimate of s for both sexuals and asexuals. We used the low s estimated for asexuals for this test, since this value resulted in a higher likelihood than did the higher value estimated for the sexuals (see asexual lines in Table 2B and sexual lines in Table 2C). In doing so, the estimated U was still significantly lower for sexuals. Hence, the mechanism causing the smaller load of deleterious mutations in the sexual lines reduces the number of mutations. Whether it also reduces the average fitness effect remains unclear.

The values we obtained for the mutation rate in *A. nidulans* agree with estimates in other organisms (DRAKE *et al.* 1998). On the basis of a genome size of 3.10×10^7 bp, the Bateman-Mukai estimates of U_{min}, and 20 nuclear divisions between generations, we estimated the number of mutations per base pair per nuclear division to be 2.26×10^{-10} for the asexual and 5.81×10^{-11} for the sexual lines.

DISCUSSION

The stable coexistence of asexual and sexual reproduction in the homothallic fungus *A. nidulans* is an intriguing problem, since offspring from both pathways have the same genotype. We hypothesize that more stringent intra-organismal selection against deleterious mutations in the sexual than in the asexual reproduction route of *A. nidulans* contributes to the stability of the dual reproductive system. This selection would immediately give a higher fitness to the sexually pro-

TABLE 2

Parameters underlying the mutation accumulation process in the sexual and asexual lines

	A[a]		B[b]				C[c]			
Line	U_{min}	s_{max}	U	s	γ_2	LL	U	s	γ_2	LL
Sex	0.036 (0.0080, 0.19)	0.019 (0.0034, 0.049)	0.014 (0.0053, 0.029)	0.019[d]	11 (3.1, 59)	484.9	0.041 (0.016, 0.081)	0.0069[d]	33 (10, 159)	485.4
Asexl	0.14 (0.042, 0.35)	0.0069 (0.0028, 0.017)	0.10 (0.057, 0.16)	0.0069[d]	2.8 (1, 10)	466.0	0.018 (0.0090, 0.037)	0.019[d]	1.1 (1, 28)	463.6

[a] U_{min} and s_{max} according to the Bateman-Mukai method. Bootstrap 95% confidence intervals for U_{min} and s_{max} were obtained from 1000 bootstrapped pseudovalues and are shown in parentheses.

[b] Maximum-likelihood estimation of U and γ_2, with s fixed at the Bateman-Mukai estimates for the corresponding lines. The value of the maximum log likelihood (LL) is indicated. Confidence intervals of 95% were obtained by a drop of 2 log likelihood from the maximum and are shown in parentheses.

[c] Maximum-likelihood estimation with s fixed at the Bateman-Mukai estimates from the other lines.

[d] s is fixed.

duced spores than to the asexually produced spores. We tested this hypothesis with a mutation accumulation experiment in 40 asexual and 40 sexual lines during 40 generations. As a fitness estimate we measured colony diameter relative to the ancestor. We (deVisser *et al.* 1997) and others (Pringle and Taylor 2002) have shown that colony diameter is a good and reliable measure of fitness, since it is highly correlated with spore production (*i.e.*, fertility). In the sexual lines, mutations accumulated with a significantly lower "fitness impact" than those in the asexual lines, supporting our hypothesis of more efficient intra-organismal pre- and/or postzygotic selection in the sexual reproduction route. As a surprising result the error variance of sexual lines is (slightly) lower than that of asexual lines ($F_{149, 147} = 1.3603$, $P = 0.031$). This observation is consistent with the accumulation of more deleterious mutations in the asexuals if some mutations cause these genotypes to be less robust against environmental variation (largely reflected by the error variance of the fitness assay). However, we do not have any direct evidence for this explanation.

Our experiment aimed to reveal a possible fitness difference between asexual lines and sexual lines, but not to reveal the actual mechanistic cause. However, from the fitness data we could estimate parameter values underlying the mutation accumulation process in the asexual and sexual lines. We found that the mechanism causing the smaller load of deleterious mutations in the sexual lines at least reduced the *number* of mutations. This is in agreement with the fact that all possible mechanisms should cause a lower U, while only a subset of mechanisms (*i.e.*, those with a bias against large mutations) should also cause a lower s.

Although we could not discriminate between intra-organismal prezygotic and postzygotic mechanisms, some observations favor postzygotic selection. Development of a zygote consists of a huge proliferation resulting in 100,000 or more ascospores. If this development is highly autonomous—for example, resources from the parental mycelium are not readily available—efficient selection is possible since only mutation-free fruiting bodies will be able to complete development. As mentioned in the Introduction, the phenotypic effect of auxotrophic mutations is very suggestive in this respect. A low level of supplementation restores asexual spore formation, but does not allow the formation of cleistothecia: externally normal cleistothecia are formed, but these are tiny and do not contain ripe ascospores. Only higher supplementation allows full sexual development (Table 1).

The meiotic process of recombination itself is very unlikely to have caused our results. First, this process may be mutagenic itself (Watters and Stadler 1995; Russell and Russell 1996). Second, in *A. nidulans*, self-fertilization is a local process and thus the two nuclei that form the basis of a sexual fruiting body must have a very recent common ancestor nucleus because they are located in the mycelium in each other's close vicinity. Recombination can have only genetic consequences in the highly unlikely event that these two nuclei carry at least two different mutations. And even then, all recombinant products will remain present in the tetrads formed since selection is absent at that stage. Consequently, in our protocol all recombinants had an equal chance to be picked for the next generation, so that the net genetic effect of recombination is zero. Last, recombinational repair of double-stranded DNA damage during the process of meiosis could not have caused our results because hyphal expansion can efficiently eliminate damaged nuclei.

Our result potentially could be explained by a lower number of nuclear divisions between generations in the sexual lines than in the asexual lines. However, the opposite is almost certainly the case: the longer sexual reproduction cycle—12 days *vs.* 3 days in the asexual cycle—is very likely to reflect a higher number of divisions in this route.

Ascospores of *A. nidulans* contain two nuclei, resulting

from a final mitosis in the ascospores, whereas asexual spores contain only one nucleus. Could this have any relevance for our findings? The two nuclei in an ascospore derive from a very recent mitotic division within the spore and are therefore identical with very high probability. Nevertheless, the binucleate condition may make a difference, for example, when the nuclei carry a mutation encoding reduced enzymatic function such that sufficient catalytic activity is provided by two copies of the mutant allele but not by a single copy. Thus some mutations may be transmitted via sexual spores but not via asexual spores. Therefore, the presence of two nuclei in the ascospores as opposed to a single nucleus in the conidiospores cannot contribute to an explanation of our results. Moreover, we performed our fitness measurements exclusively on colonies that developed from asexual spores in both the asexual and the sexual MA lines.

An important question concerns the generality of our results. Is *A. nidulans* idiosyncratic in this respect, or is the association of more stringent intra-organismal selection with the sexual cycle a feature of many organisms in which asexual and sexual reproduction coexist? It is difficult to answer this question due to the lack of suitable data. However, many mycelial fungi have basically the same reproductive biology as *A. nidulans*, which is characterized by a quick production of very large numbers of conidiospores, followed by smaller numbers of ascospores. Ascospores are bigger than conidiospores, they require a longer development time, and under most conditions asexual sporulation is much more abundant than sexual sporulation (Adams *et al.* 1998). Moreover, due to their protective spore wall, the sexual spores are more resistant than the asexual spores to adverse environmental conditions. The two types of spores seem to differ in their ecological role: conidiospores enable fast occupation and utilization of locally available substrate, while ascospores allow survival of environmental stresses, often accompanied by severe reduction in numbers and a subsequent building up of the population. It is therefore not unlikely that in many other mycelial fungi intra-organismal selection on offspring quality also will be stricter in the sexual cycle. Clearly, if a population periodically has to go through a bottleneck of relatively few surviving spores, genetic quality control should be highest among those spores, *i.e.*, in the sexual cycle.

We believe our study has revealed a novel functional aspect of sex in an organism with both sexual and asexual reproduction, namely more stringent screening for deleterious mutations in potential offspring in the sexual route than in the asexual route. This secondary sex advantage may form part of the explanation of the persistence of sexual spores in *A. nidulans*. Our findings imply that sexually produced offspring have a higher average fitness than that of asexually produced offspring. This difference may contribute to the somewhat puzzling stable coexistence of both reproductive modes in this homothallic haploid organism, in addition to the above-mentioned ecological specialization of both types of spores. Although we speculate that the more autonomous (that is, less supported by the maternal mycelial resources) production of sexual spores compared with asexual spores is a possible explanation of our findings, the exact mechanism remains to be elucidated.

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