

Heteroallelism at the *het-c* Locus Contributes to Sexual Dysfunction in Outcrossed Strains of *Neurospora tetrasperma*

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Saenz, G. S., Stam, J. G., Jacobson, D. J., and Natvig, D. O. 2001. Heteroallelism at the *het-c* locus contributes to sexual dysfunction in outcrossed strains of *Neurospora tetrasperma*. *Fungal Genetics and Biology* 34, 123–129. *Neurospora tetrasperma* is naturally heterokaryotic, with cells possessing haploid nuclei of both *a* and *A* mating types. As a result, isolates are self-fertile (pseudohomothallic). Occasional homokaryotic ascospores and conidia arise, however, and they produce strains that are self-sterile and must outcross to complete sexual reproduction. Invariably, laboratory crosses employing sibling *a* and *A* strains from the same parental heterokaryon restore the pseudohomothallic, heterokaryotic state. In contrast, outcrosses employing *a* and *A* strains from different wild isolates typically result in sexual dysfunction. Diverse sexual dysfunction types have been observed, ranging from complete sterility to reduced viability. We report that one type of dysfunction, characterized by spontaneous loss of the heterokaryotic state upon ascospore germination, can result from the interaction of incompatible alleles at heterokaryon incompatibility loci. Specifically, we demonstrate that homoallelism at the *het-c* locus in *N. tetrasperma* is required for heterokaryon stability. Heterokaryon incompatibility there-

fore provides an obstacle to outcrossing in this species, an observation with important implications for fungal life-cycle evolution. © 2001 Academic Press

Index Descriptors: *het-c*; pseudohomothallism; *Neurospora*; self/nonself recognition; compatibility; heterokaryon.

Self fertility in *Neurospora tetrasperma* results from programmed ascus development that produces four heterokaryotic ascospores per ascus. Each ascospore possesses both *A* and *a* mating-type nuclei. This life cycle, termed pseudohomothallism, contrasts with that of the closely related species *N. crassa*, which possesses a life cycle typical of truly heterothallic (self-sterile) pyrenomycetes, with four *A* and four *a* homokaryotic ascospores per ascus. Despite its self fertility, the genetic control of mating compatibility in *N. tetrasperma* is that of heterothallic species such as *N. crassa*. Homokaryotic strains derived from single-mating-type ascospores and conidia are self-sterile and exhibit heterothallic mating behavior (Raju, 1992).

In principle, the ability to form ascospores and conidia carrying only one mating type gives *N. tetrasperma* the potential to outcross in nature. The question of whether outcrossing potential is realized in nature has been addressed previously with both molecular genetic and crossing experiments, with the revelation of discordant features in *N. tetrasperma* reproductive biology. Recent studies of *het-c* allele diversity among closely related isolates dem-

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onstrated that *N. tetrasperma* does engage in some outcrossing in nature (Powell *et al.*, 2001). This result contrasts with the fact that laboratory outcrossing experiments employing strains from diverse locations revealed a high frequency of sexual dysfunction, with dysfunctional phenotypes ranging from complete sterility to loss of self fertility (Jacobson, 1995). Although the severity of sexual dysfunction generally increases with genetic distance, it is clear that sexual dysfunction is not simply the result of genetic divergence, because it is also observed among closely related strains (Jacobson, 1995; Merino *et al.*, 1996; Skupski *et al.*, 1997).

Studies of the *het-c* heterokaryon incompatibility locus in *N. tetrasperma* provide evidence of outcrossing in nature and an hypothesis to explain at least one type of sexual dysfunction observed in laboratory outcrosses (Powell *et al.*, 2001). Outcrossing in nature is indicated by the fact that different ancient *het-c* alleles are present in otherwise closely related wild-type strains. Crosses between such strains result in f_1 progeny that break down into single-mating-type components upon ascospore germination and subsequent vegetative growth, thereby disrupting pseudohomothallism. In *N. crassa*, two strains must possess functionally equivalent alleles at the *het-c* locus before a stable heterokaryon can be formed (Glass and Kulda, 1992; Saupe and Glass, 1997). We hypothesized a role for *het-c* incompatibility in the heterokaryon instability observed in certain outcrosses with *N. tetrasperma* (Powell *et al.*, 2001). Here, we report experimental evidence that this is the case. We show that in a cross between isolates with different *het-c* alleles only progeny homoallelic at the *het-c* locus form stable heterokaryons.

MATERIALS AND METHODS

Strains and crossing procedure. The two *N. tetrasperma* wild-type isolates employed, P556 and P581, were originally collected from Hanalei and Lihue, Hawaii, respectively (Perkins *et al.*, 1976) and obtained from the Perkins collection (Stanford University). These strains are part of a geographically diverse sample of *N. tetrasperma* isolates previously characterized for phylogenetic relationships (Merino *et al.*, 1996) and reproductive dysfunction (Jacobson, 1995).

Analyses reported here employed f_1 progeny from an initial outcross between the single-mating-type components P556A and P581a (Fungal Genetic Stock Center Nos. 2510 and 2509, respectively), which was reported in

TABLE 1
Viability and Self Fertility of Progeny from Crosses Examined

Cross	Viable progeny (%)	Self-fertile progeny (%)
P556A (<i>het-c^{OR}</i>) × P581a (<i>het-c^{PA}</i>)	121/200 (60.5%)	23/121 (19%)
f_1 141A (<i>het-c^{PA}</i>) × f_1 141a (<i>het-c^{OR}</i>)	185/200 (92.5%)	101/185 (54.6%)
f_1 128A × f_1 145a	148/200 (74%)	34/148 (23%)

Gallegos *et al.* (2000). In addition, f_1 progeny were crossed to obtain f_2 progeny (see Table 1), some of which were scored for *het-c* genotype and heterokaryotic stability to assess the relationship between *het-c* function and sexual dysfunction phenotype. Methods used for culturing, crossing, and isolation of unordered tetrads of *N. tetrasperma* were as described in Jacobson (1995) and Gallegos *et al.* (2000). When required, self-fertile progeny were separated into their single-mating-type components by isolation and testing of single-conidium cultures for self fertility and mating type (Jacobson, 1995). Isolation of single-conidium cultures was also used to judge stability of heterokaryotic progeny subsequent to ascospore germination (see below).

Determination of *het-c* allele type. The *het-c* functional allele classes for P556 and P581 were previously determined to be *het-c* Oak Ridge (*het-c^{OR}*) and *het-c* Panama (*het-c^{PA}*), respectively (Powell *et al.*, 2001). Progeny (f_1 and f_2) were typed for *het-c* by PCR amplification and sequence analysis. The primer pair *hetc-1* (5'-GGGTCTCAGGATAGGCTGGGG-3') and *hetc-2* (5'-CCCAACTTTGACCCCGTCAA-3') amplified a 1300-bp fragment that included the region of allele functional specificity and flanking sequences (Saupe and Glass, 1997). The functional region was then sequenced with the internal sequencing primer *hetc-3* (5'-CCCTGGAG-GACTTTCCTGCCC-3'), which is located approximately 100 nucleotides 5' of the functional region. Specific *het-c* functional types are readily distinguished by sequence variability that includes a 45-nucleotide insertion in *het-c^{PA}* relative to *het-c^{OR}*.

RESULTS

The specific type of sexual dysfunction exhibited when strains P556A and P581a are outcrossed has been de-

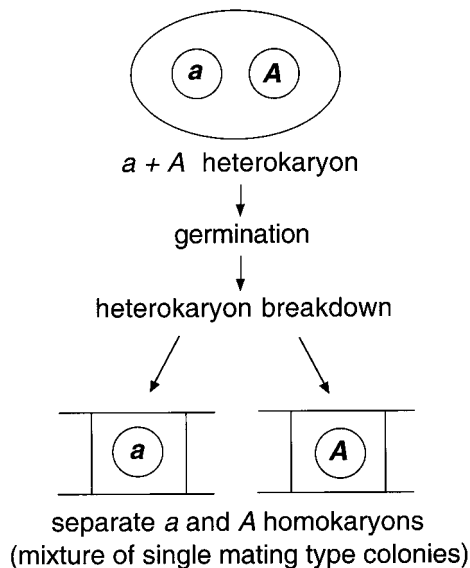


FIG. 1. Resolution of the *N. tetrasperma* mating-type heterokaryon (self-fertile) into single-mating-type homokaryons (self-sterile). Occasional single-mating-type homokaryons arise naturally from stable wild-type heterokaryons (Raju, 1992). Crosses between such sibling *a* and *A* homokaryons derived from the same parental heterokaryon reconstruct a stable heterokaryon. In contrast, in certain outcrosses, most notably between Hawaiian isolates P581 and P556, progeny exhibit heterokaryon instability and break down rapidly into separate homokaryotic components. The result is that conidial isolates are invariably self-sterile.

scribed previously in detail (Jacobson, 1995; Gallegos *et al.*, 2000). Asci and ascospores appear to develop normally with ascospores possessing nuclei of both mating types, typical of *N. tetrasperma*. However, progeny viability is reduced compared to selfing crosses. Moreover, the heterokaryotic state invariably breaks down upon ascospore germination, resulting in either self-sterile cultures of single-mating type or cultures that are mixtures of separate *A* and *a* mycelia (Fig. 1). These separate mycelia are easily recognized because only self-sterile single-mating-type cultures are recovered from single-conidium isolations. In contrast, heterokaryotic *N. tetrasperma* mycelia isolated from nature or selfing crosses produce a majority of conidia that contain both mating-type nuclei and therefore give rise to self-fertile cultures (Raju, 1992). Recovery of single-mating-type isolates of *N. tetrasperma* from nature is rare (Turner *et al.*, 2001).

Two crosses were made with *A* and *a* f_1 progeny, selected for alternative *het-c* alleles (*het-c^{PA}* and *het-c^{OR}*), from a cross of P556A and P581a (Table 1). The parents of one cross were sister *A* and *a* strains that resulted from the heterokaryon breakdown of a single ascospore-derived

progeny (No. 141). The second set of parents originated from ascospores (Nos. 128 and 145) from two different asci and represented two separate meioses. Both crosses showed less severe sexual dysfunction than that observed for the parental cross between P556 and P581, as indicated by increased proportions of both viable f_2 ascospores and self-fertile f_2 progeny (Table 1). A similar trend toward improved sexual reproduction was noted for crosses of f_1 progeny from other *N. tetrasperma* outcrosses (Jacobson, 1995).

Progeny from the 141A \times 141a cross exhibited higher levels of viability and self fertility than that observed for the progeny from the 128A \times 145a cross. We assumed that milder dysfunction could reflect fewer genetic factors involved in causing dysfunction and therefore chose f_2 progeny from the 141A \times 141a cross to assess the role of *het-c* in heterokaryon stability. Thirty-five progeny were selected arbitrarily as one each from different unordered tetrads and therefore were derived from separate meioses. All f_2 progeny gave rise to self-fertile cultures, producing perithecia at least near the point of ascospore germination and thereby demonstrating the heterokaryotic status of ascospores. Heterokaryon stability was assessed by evaluation of 16–60 single-conidium subcultures from each f_2 individual for self fertility. The percentage of self-fertile conidial isolates for each progeny ranged from 0 to 72%. Twenty-two of the 35 f_2 progeny produced few to no (0–3%) self-fertile conidial isolates, whereas 13 f_2 progeny produced at least 14% self-fertile conidial isolates (partial results shown in Table 2). We defined these two groups as heterokaryon unstable and stable, respectively.

We obtained separate *A* and *a* components for nine unstable and eight stable f_2 progeny and typed each component for *het-c* allele (Table 2). All eight stable heterokaryons were homoallelic, possessing identical *het-c* alleles in *A* and *a* component pairs. In contrast, 8 of 9 unstable heterokaryons were heteroallelic, possessing different *het-c* alleles in *A* and *a* components. The statistical significance of this outcome was verified with a χ^2 test of independence that employed a 2×2 table comparing stability categories (stable, unstable) and genotypes (heteroallelic, homoallelic). This test resulted in rejection of the hypothesis that heterokaryon stability is independent of genotype ($P < 0.001$). The strong correlation between *het-c* functional allele status and heterokaryon stability, and particularly the absence of *het-c* heteroallelism among stable progeny, indicates a substantial role for *het-c* in the heterokaryon instability observed among progeny from the P556 \times P581 outcross. It further appears, however, that

TABLE 2

Heterokaryon Stability and *het-c* Constitution of f_2 Progeny from Cross 141A (*het-c*^{PA}) × 141a (*het-c*^{OR})

f_2 Progeny number	Single conidial isolates from f_2 progeny			<i>het-c</i> Allele	
	Number self-fertile	Single mating type	Percentage self-fertile	A component	a component
Unstable heterokaryons					
U1	0	19	0%	PA	OR
U2	0	25	0%	OR	PA
U3	0	25	0%	OR	PA
U4	0	29	0%	PA	OR
U5	0	29	0%	OR	PA
U6	0	29	0%	OR	PA
U7	0	30	0%	PA	OR
U8	0	30	0%	PA	OR
U9	0	16	0%	PA	PA
Stable heterokaryons					
S1	8	22	26.7%	PA	PA
S2	8	17	32.0%	OR	OR
S3	10	18	35.7%	PA	PA
S4	12	18	40.0%	PA	PA
S5	22	18	55.0%	PA	PA
S6	14	11	56.0%	PA	PA
S7	18	12	60.0%	OR	OR
S8	18	12	60.0%	OR	OR

het-c homoallelism is a necessary but not sufficient requirement for heterokaryon stability (see Discussion).

DISCUSSION

Filamentous ascomycetes possess heterokaryon incompatibility genes that control the outcome of hyphal fusions between vegetative individuals. Hyphal fusions between incompatible individuals result in the death of cells in the region of contact. Most commonly, compatibility requires functional allelic identity at *het* loci. In *N. crassa*, there are at least 10 different loci involved in allelic heterokaryon incompatibility; the best characterized of these is *het-c* (Glass *et al.*, 2000).

The self recognition system of allelic vegetative (heterokaryon) incompatibility in *N. crassa* and other ascomycete fungi appears to have evolved in life cycles in which a haploid, homokaryotic vegetative phase predominates. It has been recognized that such systems may be in conflict with pseudohomothallic life cycles such as that of *N. tetrasperma*, which naturally possess a vegetative state that is heterokaryotic for mating type and potentially other genes (Powell *et al.*, 2001).

In principle, *N. tetrasperma* could avoid heterokaryon incompatibility in several different ways. One mechanism

of avoidance would require the suppression of the incompatibility function. There is evidence for one example of this type of suppression in *N. tetrasperma*. It is known from studies of *N. crassa* that the mating-type locus (*mat*) has dual functions; in addition to being required for sexual reproduction, it functions as a heterokaryon incompatibility locus preventing stable *matA* + *matA* heterokaryons in the vegetative state (Glass and Kulda, 1992). Mutations at a suppressor locus, *tol*, are required to allow mating-type heterokaryon compatibility (Newmeyer, 1970). In *N. tetrasperma*, however, suppression of the heterokaryon incompatibility function of the mating locus is a normal function of the wild-type *tol* allele (*tol*^T) in this species. This allows formation of the stable mating-type heterokaryons that are critical for pseudohomothallism, but which are prevented in *N. crassa* (Jacobson, 1992).

Other *het* loci are very different from mating type in that there is no requirement for heterozygosity to complete the life cycle. In addition, the *tol* mutant does not suppress incompatibility mediated by other *het* loci in *N. crassa* (Leslie and Yamashiro, 1997). In theory, therefore, individual strains of *N. tetrasperma* could avoid *het* incompatibility by maintaining homoallelism at all *het* loci. Such avoidance could result from either (1) strict selfing or (2) selection against functional *het* allele diversity within populations. We speculated previously that one or both of these avoidance mechanisms might apply to *N. tetra-*

sperma (Merino *et al.*, 1996). To the contrary, recent evidence indicates that *N. tetrasperma* does not completely avoid heteroallelism of *het* genes. Although individual wild isolates are invariably homoallelic for *het-c*, closely related isolates can possess alleles from different functional classes (Powell *et al.*, 2001). This observation led to the conclusion that *N. tetrasperma*, although adapted for selfing, occasionally outcrosses in nature. Moreover, the existence of two different, ancient functional alleles of *het-c* among isolates of *N. tetrasperma* suggested that balancing selection to maintain self recognition exists in this species (Powell *et al.*, 2001), as has been proposed for truly heterothallic species (Wu *et al.*, 1998). This in turn implies that the *het-c* locus retains the heterokaryon incompatibility function in *N. tetrasperma*.

That *het-c* functions in heterokaryon incompatibility in *N. tetrasperma*, as it does in *N. crassa*, was specifically suggested by crosses between wild strains P556 and P581 from Hawaii, possessing *het-c*^{OR} and *het-c*^{PA}, respectively, which produced unstable heterokaryon progeny. The results presented here indicate that the observed instability is in fact caused in large part by heteroallelism at the *het-c* locus.

Two observations suggest that genes in addition to *het-c* contribute to heterokaryon instability among f_1 and f_2 progeny from the P556 \times P581 cross. First, f_2 progeny that were homoallelic for *het-c* produced an average of only 46% self-fertile isolates from single conidia. This compares with greater than 80% self fertility for conidia from wild-type heterokaryons (Raju, 1992). Second, one f_2 individual was heterokaryon unstable even though homoallelic for *het-c* (Table 2). This non-*het-c*-mediated instability is unlikely to result from an additional *het* gene exhibiting simple allelic incompatibility, given the ratio of homo- to heteroallelism within the unstable group (1:8). It is possible that wild-type levels of heterokaryon stability are in part dependent on multiple unknown genes other than *het* genes. Such genes may control *A:a* nuclear ratios in the mycelium and/or partitioning of nuclei into conidia.

Our *het-c* results strengthen the conclusion that pseudohomothallism in *N. tetrasperma* creates an evolutionary dilemma for a species that at first glance possesses the benefits of both outcrossing and selfing (Powell *et al.*, 2001). The existence of *het-c* diversity among related isolates confirms outcrossing in *N. tetrasperma* and therefore suggests a mechanism for both avoidance of inbreeding depression and preservation of self recognition. However, this also implies that *N. tetrasperma* must in some way balance the benefits of outcrossing against the detrimental effects of heterokaryon incompatibility. The high fre-

quency of sexual dysfunction observed in outcrosses of wild isolates of *N. tetrasperma* highlights this conflict (Jacobson, 1995), especially in comparison with truly heterothallic species, such as *N. crassa*.

In the type of sexual dysfunction observed for the P556 \times P581 outcross, pseudohomothallism is interrupted, with most progeny being effectively self-sterile homokaryons. Because isolates of *N. tetrasperma* from nature are typically recovered as stable heterokaryons (Turner *et al.*, 2001), unstable heterokaryon progeny from such outcrosses must represent a transient condition. This transient unstable state could be resolved either by mating between homokaryotic strains with compatible *het* alleles or by matings between heterokaryon incompatible siblings with a crossover and subsequent second division segregation of *het* alleles (Fig. 2). Either of these mechanisms for restoring homoallelism effectively limits the extent of outcrossing, based on either the frequency of *het*-compatible individuals in a population or the distance of each *het* gene from the centromere.

Extending this effect to multiple *het* genes exponentially lowers the probability of outcrossing between individuals with the same *het* genotype, thus lowering outcrossing potential dramatically. Two *het* genes, *het-c* and *het-6* (both originally identified in *N. crassa*), exhibit polymorphism in a single population of *N. tetrasperma* (Powell *et al.*, 2001, and unpublished). In *N. crassa*, 10 *het* genes have been identified, which assuming a random distribution lowers the probability of two individuals having the same *het* genotype to at least one in 2^{10} . Even if the number of functional *het* genes is lower in *N. tetrasperma*, incompatible progeny and transient homokaryons are the very likely result of outcrossing in *N. tetrasperma*.

Functional vegetative incompatibility has long been known in another pseudohomothallic ascomycete, *Podospora anserina*. *P. anserina* has systems of both allelic and nonallelic incompatibility. The effect of vegetative incompatibility on sexual reproduction has also been noted but not well characterized, especially on a population basis (Esser and Blauch, 1994).

More broadly, the potential conflict between allelic heterokaryon incompatibility and the pseudohomothallic life cycle, which includes a heterokaryotic vegetative state, invites questions in regard to constraints on the evolution of life cycles in filamentous fungi. For example, nearly all ascomycete species are homokaryotic (*n*) in the vegetative state. In contrast, most basidiomycetes exist vegetatively as dikaryons (*n* + *n*). At first glance, *N. tetrasperma* appears to represent a transition toward a basidiomycete type of life cycle and perhaps even toward diploidy. Closer scru-

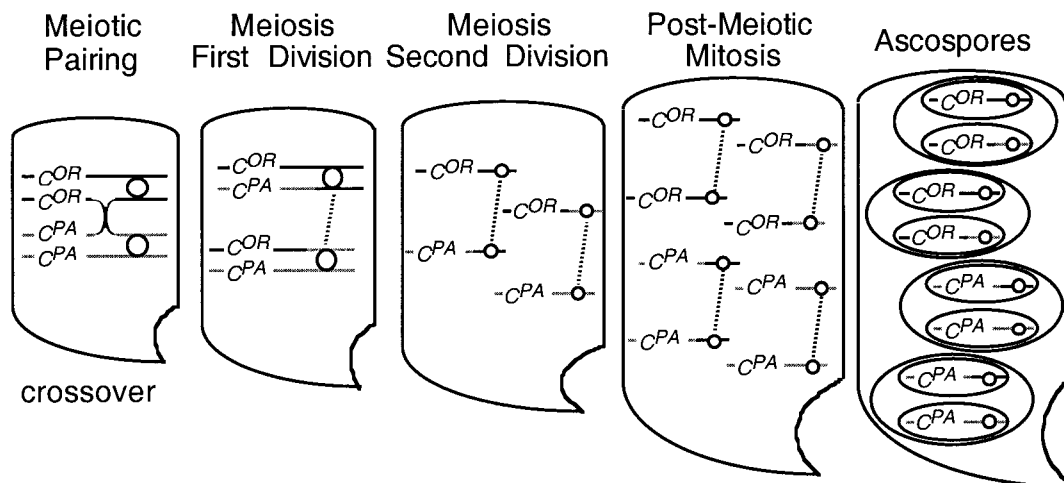


FIG. 2. The mechanism by which crossing-over in *N. tetrasperma* can lead to homoallelism at the *het-c* locus. Normal development of *N. tetrasperma* asci, which includes spindle overlap at the second meiotic division and at a subsequent mitosis, preserves heteroallelism unless crossovers occur. This pattern of development insures that each ascospore will receive a nucleus of each mating type (see Merino *et al.*, 1996). However, when a crossover occurs between a given locus and its centromere, ascospores may become homoallelic. Homoallelism will result in 50% of such crossover asci, with the outcome dependent on the orientation of homologous chromosomes at the first meiotic division.

tiny, however, suggests that multigenic vegetative incompatibility of the type present in diverse ascomycetes provides a formidable barrier to such transitions. This consideration further suggests that genetic control of vegetative incompatibility must differ between these two large fungal groups. The ascomycete allelic vegetative incompatibility system is specifically designed to prevent genetically different nuclei from coexisting in the same mycelium, whereas such coexistence is the normal outcome of mating in basidiomycetes. Basidiomycetes possess vegetative incompatibility systems, but the genetic control of incompatibility is apparently not completely understood (Worrall, 1997). Fundamentally different vegetative incompatibility systems provide an hypothesis to explain why life cycles among members of the two groups differ predictably.

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