POSITIONAL REGULATION AND EVOLUTION OF MATING TYPE GENES IN HETEROTHALLIC AND HOMOTHALLIC SPECIES OF *NEUROSPORA*

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Biotechnology Laboratory and Department of Botany)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1998

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Date Sept. 28/98

Abstract

The mating type genes of *Neurospora crassa* were shown to function abnormally when located at ectopic chromosomal positions. Crosses involving strains with ectopic mating type genes produce defective perithecia. Ascus number is reduced. The hypothesis that perithecial development requires physical proximity of opposite mating type homologs during meiosis was tested. The sterility of the crosses made between strains with both mating type regions relocated to the same ectopic position failed to support the hypothesis. To test the hypothesis that normal expression levels of the mating type genes require distant *cis*-acting sequences not present on ectopic fragments, autoradiograms of mRNA from wild type and ectopic-*mt* strains were compared. Differences between expression levels in ectopic-*mt* and wild type cells were observed, but their significance cannot be assessed without additional studies. The hypothesis that nuclear identity is disrupted in ectopic-*mt* strains was tested. Strains with disturbed nuclear identity (dual mating type) were crossed to wild type. The appearance of the reduced ascus number phenotype suggested that the affected function in ectopic-*mt* strains is nuclear identity.

The homothallic species *N. terricola* contains *mt A*- and *mt a*-like sequences. The genes were cloned and sequenced to determine whether or not they were functional. The genes specifying identity, *mt A-1* and *mt a-1*, are more than 95% similar at the amino acid level to the *N. crassa* homologs, but the putative MT A-2 polypeptide is truncated. *N. terricola mt A-1* and *mt a-1* genes induced mating and vegetative incompatibility in *N. crassa* mating type mutants. Expression in *N. terricola* of *mt A-1* and *mt a-1* was detected by reverse transcriptase PCR, upholding the hypothesis that the genes are functional.

To determine the pattern of evolution of homothallism, a phylogeny of *Neurospora* was reconstructed from *mt A-1* DNA and amino acid sequences. Homothallic *Neurospora* species that carry both mating type genes are more closely related to heterothallic species than they are to the *A*-only homothallic species, suggesting that either heterothallic species are derived from a homothallic ancestor or that homothallism arose twice.

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Acknowledgement

Gratitude-plus to Louise Glass to whom I am permanently bonded for accompanying me through a bewildering number of defining moments in my life. Thank you so much for keeping faith that I would eventually finish. Buckets of thanks laced with profound admiration to Tony Griffiths for your appreciation of life in all its subtle and bizarre refractions; for your wisdom, profound insights, broad-mindedness, kindness, humanity, impish sense of humour, integrity, excellent editing skills, creativity and for providing a living example of the rich, balanced life available to the scientist. Thanks to Mary Berbee for always making time to teach me science, even when you were insanely busy. I am very grateful for your gift of the map to the fascinating world of evolutionary thought. I am grateful for the kindly presence of Carl Douglas. Thank you for emotional support; you were there to champion me and my scientific ability when my confidence was wilting. Thanks also for your gentle humanity to whose level I aspire. Universe-encompassing gratitude to Rik Myers for sharing with the world (me included) your profound understanding of life. Thank you for sharing your spirit, friendship, love and for accepting mine. Unending thanks to Maline Vellani Campbell and Shaelin Campbell Vellani for being your phenomenally wonderful selves, for your love, your anger, your laughter, your tears, in short, your everything! Inexpressible thanks to Mishu Vellani for your constant and unconditional support, love, friendship and understanding. Thankyouthankyouthankyou! My gratitude to Jasswanti Vellani cannot possibly come close to being expressed in words, but I'll try...thanks, Mum, you're unquestionably THE BEST!!! I would like to express my deep, if belated appreciation for the late Nazeer Vellani for teaching me the value of education, spirituality, clan, culture, heritage, questioning and iconoclasm. Many many thanks to Sven Saupe for engaging into all of our discussions with your full mind, for understanding my half-formed ideas and sharing in their development and for the heart-warming sound of your laughter. Soul-felt thanks to Alexandra Ball for white-hot-core-communion. Warm thanks to Mishtu Banerjee for almost a decade of unconditional friendship and for sharing the

Commonwealth. Chocolate babies *can* fly. Thanks to Rod Campbell for many years of laughter, friendship, love and support. Thanks to Lane Ferreira for your friendship and collegiality. Thanks to Sally Otto for the loads of time and energy you so willingly donated to help me understand what I was doing. I feel honoured to acknowledge the friendship and support of Hugh Brock, Maria da Silva, Lorraine Graves, Frans Huijing, GAK (Gretchen Kuldau), Phoolmatteea Lutchmun, the late Roopnarain Luctchmun, Sandra Millard, Debbie Neufeld, Kenn Rudd, Myron Smith, Carol Thomas, Richard Todd, Louise Wheeler, Ugh (Sandra Wiese), my lab-mates from 1991-1997 and the Botany Department from 1991-1997.

Dedication

This thesis is dedicated to Moodle and Panwald, whom I can never thank enough.

General Introduction

This work addresses the genetic phenomenon called position effect and how it may have influenced the evolution of filamentous fungi. Organisms from various kingdoms have genes that must occupy a particular chomosomal position for normal function. A gene said to exhibit a position effect imparts, when displaced from its usual locus, an abnormal phenotype. Mechanistically, position effects are not all the same. The mating type genes of *Neurospora crassa* are used here as a model to study the mechanistic underpinnings of one type of position effect. The mating type genes of *N. crassa* carry out some of their functions, but not all, when they are separated from their usual locus. In this work, competing hypotheses regarding the molecular mechanism of the *N. crassa* mating type gene position effect are tested genetically. One model, the *cis*-acting regulator model, is pitted against the transvection model. The *cis*-acting regulator model proposes that the position-sensitive genes are under the control of a cis-acting regulator and that when the genes lose the connection to the regulator, the functions are disturbed. The transvection model claims that the position-sensitive functions of the mating type genes depend on the synapsis of the opposite mating type genes during meiosis.

Additionally, dual mating type transformants of *N. crassa* exhibit both vegetative and, as demonstrated here, sexual incompatibility. To explore further the workings of the mating type gene position effect, another species of *Neurospora*, *N. terricola*, was examined. In *N. terricola*, chromosome I carries both of the mating type genes and even though they are similar to those of *N. crassa*, they appear to be perfectly compatible. If the mating type genes of *N. terricola* can confer sexual interference in the appropriate genetic background, then they would have to be inactivated for the incompatibility functions in *N. terricola*. Since morphological differences from *N. crassa* are not evident, suppressor alleles may be present or the mating type genes themselves, although similar to *N. crassa*'s may be unable to confer incompatibility. In this work, the genes are cloned, sequenced and tested for function to gain insight into the biological significance and the evolution of the position effect.

The interplay between *Neurospora* evolution and the position effect is tackled directly in a phylogenetic analysis. The sequences of the mating type genes are used to infer relationships in the genus. The data are analyzed and discussed in terms of the evolution of mating strategies.

The three chapters are united in a evolutionary model that draws connections between the evolution of mating type genes and mating strategies in filamentous fungi and how the mating type gene position effect may have impacted on these processes in the genus *Neurospora*.

Backgound

Populations of some haploid microorganisms (algae and fungi) are divisible into genetically distinct interfertile/intrasterile groups, said to be of different mating type. Fertile crosses, in which the participants differ in their mating type, result in the formation of meiotic products. Fertility groups were shown to be defined in the fungi by different alleles at one or more chromosomal loci which encode putative or proven transcriptional regulators that top a cascade of genes required for sexual processes (Kües and Casselton, 1992).

N. crassa is a haploid heterothallic (self-sterile, cross-fertile) fungus. Its classification tends to shift, but a recent one follows. *N. crassa* belongs to the kingdom Myceteae; division Amastigomycota; subdivision Ascomycotina, characterized by the production of sexual spores in a sac or "ascus"; class Pyrenomycetidae, characterized by the activation of sexual spores by heat; order Sphaeriales; family Sordariaceae; genus *Neurospora*, the group of species producing football-shaped ribbed (as opposed to pitted or smooth) ascospores (Moore-Landecker, 1990).

N. crassa sexual development has been reviewed by Raju (1980; 1992). Mating type is controlled by a single locus with two types, *A* and *a* (a list of *N. crassa* gene symbols is provided in Appendix A). Each *A* or *a* mycelium has both male and female structures. Male gametes, macroconidia, microconidia and hyphae, form part of the vegetative mycelium while female structures develop in response to nitrogen deprivation. The trichogyne, the receptive hypha emanating from the protoperithecium, perceives the presence of an opposite mating type

male cell by a pheromone produced by the latter. The trichogyne grows toward the male cell and fuses with it. The male nucleus migrates into the interior of the protoperithecium, into a structure called the ascogonium. The protoperithecium develops into a perithecium, a black macroscopic flask-shaped structure.

The following parts of sexual development are particularly relevant to this thesis. In the ascogonium, the male nucleus proliferates mitotically along with female nuclei. One nucleus of each type is packaged into the terminal cells of the ascogenous hyphae, which bend to form a structure called the crozier. The last division before karyogamy is co-ordinated so that one male nucleus and one female nucleus divide simultaneously. Many croziers arise in each perithecium, in temporal waves. Karyogamy, meiosis and post-meiotic mitoses occur in the penultimate cell of the crozier, as it enlarges and differentiates to form the ascus, a sac containing the eight linearly ordered products of meiosis. The multinucleate, haploid progeny, ascospores, are forcibly ejected through an opening in the perithecial neck (also called "beak"), and each can germinate and become an independent mycelium.

The three types of spores, microconidia, macroconidia and ascospores, may have different, but overlapping roles. Microconidia are probably not vegetative propagules since they are fragile and not very viable (Perkins and Turner, 1988). They may be exclusively male gametes. Macroconidia may be primarily vegetative propagules (Perkins and Turner, 1988) or primarily male gametes, since they may accumulate too much UV damage to germinate (Taylor, Smolich and May, 1986). Ascospores are UV-resistant, long-lived propagules, quiescent until activated by heat (Hollaender, Sansome and Demerec, 1945).

In heterothallic Ascomycetes, mating type is inherited as a single locus with two alleles (*e.g.* in *S. cerevisiae*, *Schizosaccharomyces pombe*, *N. crassa*, *Sordaria brevicollis*, *Cochliobolus heterostrophus* and *Podospora anserina*) (Kües and Casselton, 1992). The mating type loci of *N. crassa* differ from classical alleles in two ways: they are dissimilar in sequence (and therefore are called "idiomorphs" rather than alleles (Metzenberg and Glass, 1990)), and secondly, the *mt A* idiomorph encodes more than one gene (Glass and Lee, 1992; Ferreira, Saupe and Glass, 1996). The structure of the idiomorphs is shown in Figure 1.1. Genes with

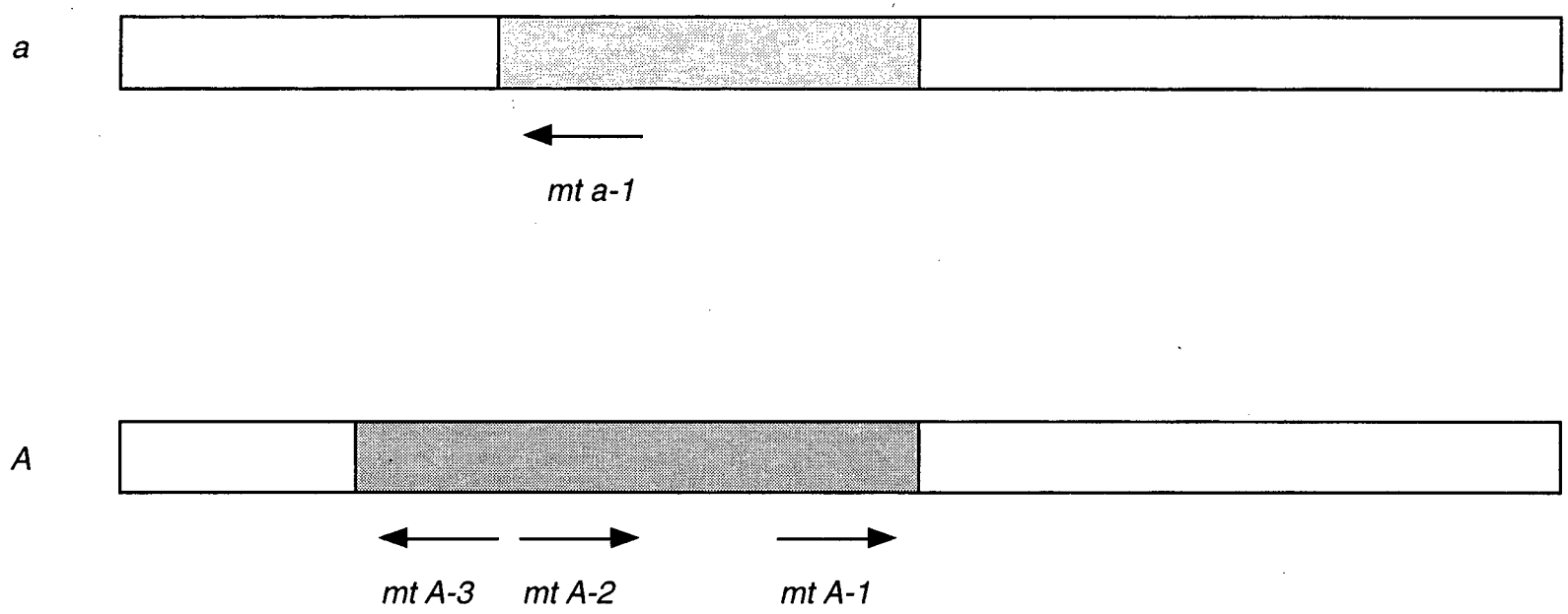


Figure 1.1 Structure of the *N. crassa* mating type idiomorphs. The locus is on the left arm of linkage group I. Centromere is to the right and telomere is to the left of the mating type region as represented in this figure. The open reading frames are represented by arrows and are drawn approximately to scale. The *mt a* idiomorph has one open reading frame and the *mt A* idiomorph has three.

idiomorphic regions are uncommon, and yet other fungal mating type genes have them: *S. cerevisiae* (Astell *et al.*, 1981; Tatchell *et al.*, 1981), *S. pombe* (Kelly *et al.*, 1988), *Magnaporthe grisea* (Kang, Chumley and Valent, 1994), *C. heterostrophus* (Turgeon *et al.*, 1993), *P. anserina* (Debuchy and Coppin, 1992), *U. maydis* (Bölker, Urban and Kahmann, 1992), *S. commune* (Giasson *et al.*, 1989) and *Cryptococcus neoformans* (Moore and Edman, 1993). Possible selective advantages of idiomorphs are discussed in Chapter 3 Results and Discussion.

The *mt A* idiomorph of *N. crassa* is 5301 bp and encodes three genes, *mt A-1*, *mt A-2* and *mt A-3*, all putative transcriptional regulators (Glass, Grotelueschen and Metzenberg, 1990; Ferreira, Saupe and Glass, 1996). Mutants with frameshifts in *mt A-1* are sterile (Griffiths, 1982; Glass, Grotelueschen and Metzenberg, 1990). The *mt a* idiomorph is 3235 bp and encodes one gene, *mt a-1,* which is necessary for mating identity, meiosis and sporulation (Staben and Yanofsky, 1990; Chang and Staben, 1994). Other sequences within the idiomorph, but not in the *mt a-1* gene are required for position-independent perithecial development (C. Staben, personal communication).

N. crassa mating type genes are necessary and sufficient for identity in mating (Shear and Dodge, 1927; Griffiths and DeLange, 1978; Griffiths, 1982; Glass *et al.*, 1988) and heterokaryon incompatibility (Beadle and Coonradt, 1944; Griffiths and DeLange, 1978; Griffiths, 1982; Glass *et al.*, 1988) and for perithecium suppression upon crossing to the same mating type (Griffiths and DeLange, 1978). They are necessary, but not sufficient for secretion and response to pheromones (Bistis, 1981; 1983; Glass, Grotelueschen and Metzenberg, 1990; Staben and Yanofsky, 1990) and perithecial maturation, including meiosis and sporulation (Glass, Grotelueschen and Metzenberg, 1990; Staben and Yanofsky, 1990; Glass and Lee, 1992).

Some mutants in *mt a-1* produce barren perithecia when crossed to either mating type and some only when crossed to *a*. One mutant, a^{m33}, produces abundant perithecia with abundant ascospores when crossed to *A* and a few barren perithecia when crossed to *a* (Griffiths and DeLange, 1978). Since the mutants were selected on the basis of heterokaryon compatibility, they are all heterokaryon compatible (Griffiths and DeLange, 1978).

A heterokaryon consisting of one nuclear type which carried the null mating type mutation, a^{m1} (Griffiths and DeLange, 1978; Staben and Yanofsky, 1990) and a marker enabling distinction of its ascogenous hyphae and a second nuclear type which carried the wild type *a* allele and a mutation eliminating protoperithecia was crossed to *A*. Perithecia were formed, showing that the mating function of the a^{m1} mutant was rescued by a wild type copy of *mt a*. Ascogenous hyphae did not have the phenotype of the a^{m1} mutant nuclei, showing that the post-fertilization function of the a^{m1} mutant was not rescued by a wild type copy of *mt a* (Raju, 1992). These results suggest that MT a-1 may become nuclear autonomous in ascogenous hyphae, *i.e.* act only on the nucleus from which it came.

Chang and Staben (1994) also showed that *mt a-1* is involved in post-fertilization events by demonstrating that asci developed when *mt a-1* integrated at the mating type locus, but not at an ectopic position. A strain transformed with the entire *mt a* idiomorph produces perithecia that are more mature than *mt a-1* transformants, but they are still barren (C. Staben, personal communication).

Most mutants in *mt A-1* are unable to induce perithecia when crossed to either mating type (Griffiths and DeLange, 1978; Glass, Grotelueschen and Metzenberg, 1990). A strain with a deletion of the *mt A* idiomorph had the same phenotype as the mutants (Ferriera *et al.*, 1998). Since the mutants were selected on the basis of heterokaryon compatibility, they are all heterokaryon compatible (Griffiths and DeLange, 1978). One mutant, A^{m99}, which makes a truncated 85 amino acid polypeptide, was sterile as a male, but produced a reduced number of perithecia with few ascospores when crossed as a female to *a*, suggesting that a post-fertilization role is encoded in the 3' region of the *mt A-1* gene (Griffiths, 1982; Saupe *et al.*, 1996).

Null single mutants of *mt A-2* or *mt A-3* have no phenotype (Ferreira, 1997; Ferreira *et al.*, 1998). Strains appear normal.

A number of mutants affecting more than one of the *mt A* genes were produced by RIP (Glass and Lee, 1992). Several mutants, called $A^{1\,RIP}$ strains, were produced from a cross in which one parent carried a duplication for *mt A-1* and part of *mt A-2*, resulting in the mutation of these genes (as shown by novel restriction fragment patterns). The mutants did not produce

perithecia when crossed as a male or female to a *a* tester strain and were heterokaryon compatible when paired with an *a* tester strain. This phenotype is the same as that seen in *mt A-1* mutants (Griffiths and DeLange, 1978; Glass, Grotelueschen and Metzenberg, 1990), suggesting that *mt A-1* (at least) was mutated.

The normal appearance of the $A^{I\ RIP}$-generating cross (N. L. Glass, personal communication) suggests that the genes mutated in the $A^{I\ RIP}$ mutant, namely *mt A-1* possibly *mt A-2*, are not required after RIP which occurs during premeiotic nuclear divisions in the ascogenous hyphae. The likelihood that normal nuclei were providing normal MT A-1 protein to the mutated nuclei in the syncitial tissue is low since the product is probably nuclear limited (see Chapter 1 Introduction).

A solitary mutant, called $A^{II\ RIP}$, was generated from a cross in which one parent was duplicated for *mt A-2* and part of *mt A-3* (Glass and Lee, 1992). Sequencing showed that the mutant is missing the C-terminal 244 amino acids of *mt A-2* and has 3 amino acid substitutions in *mt A-3* (Ferreira, 1997). This mutant produced abundant perithecia, but very few ascospores when crossed to a *a* tester, as did its *A* progeny. Perithecial development proceeded normally until approximately four to five days post-fertilization. At this point fewer asci than normal were seen, but they matured normally and produced ascospores (Glass and Lee, 1992).

The phenotype of this double mutant contrasts to the non-existent phenotypes of *mt A-2* and *mt A-3* null mutants. The two genes could act in parallel pathways or in a complex that fails to function only when both gene products are missing. The genes could have redundant function. The differences in their constellations of motifs (see section below entitled "Sequence comparison"), however, suggest that they do not have the same function. Perhaps the genes have independent functions, but can fill each other's roles under certain circumstances.

A post-fertilization role for *mt A-2* and *mt A-3* is supported by the existence of *SMR1* and *SMR2* in *P. anserina*, two genes encoded in the *mat-* idiomorph that code for post-fertilization functions (Debuchy, Arnaise and Lecellier, 1993). The *N. crassa* genes are clearly different, however, given that they cannot complement the meiosis and sporulation functions in *P. anserina* mutants (Debuchy, Arnaise and Lecellier, 1993; Arnaise, Zickler and Glass, 1993)

and the phenotypes of *mt A-2*, *mt A-3* and $A^{II\ RIP}$ mutants differ from the *P. anserina* mutants which produce uniparental progeny (Zickler *et al.*, 1995; Ferreira, *et al.*, 1996; Ferreira, 1997; Ferreira *et al.*, 1998). The fact that *mt A-2* and *mt A-3* share a promoter (Ferreira, Saupe and Glass, 1996) supports the idea that their expression may be co-ordinated. RIP mutants of *mt A-2* and *mt A-3* were difficult to obtain (Glass and Lee, 1992; A. V. B. Ferreira, personal communication) perhaps because the genes are required post-fertilization and if they were excessively mutated, then no ascospores would be recovered. According to this explanation, the one mutant that was obtained may retain partial function of one or both of the genes.

Ascomycete mating type genes encode proteins with transcription factor motifs, suggesting that they regulate transcription. The mating type idiomorph names are presented in Table 1.1

Table 1.1 Mating type gene names.

Species	Gene name
N. crassa	*mt A* and *mt a*
M. grisea	*Mat1-1* and *Mat1-2*
S. macrospora	*Smt A* and *Smt a*
P. anserina	*mat-* and *mat+*
C. heterostrophus	*MAT-1* and *MAT-2*
S. cerevisiae	*MATa* and *MATα*
S. pombe	*mat1-P* and *mat1-M*

The cloning and sequencing of the *S. macrospora* genes took place very recently. *S macrospora* has homologs to all four *N. crassa* genes, all of which are closely linked (Pöggeler *et al.*, 1997a).

N. crassa mt A-1 has a region similar to *MATα1* of *S. cerevisiae* (Glass, Grotelueschen and Metzenberg, 1990), *FMR1* of *P. anserina* (Picard, Debuchy and Coppin, 1991) and *MAT-1* of *C. heterostrophus* (Turgeon *et al.*, 1993). The encoded domain is proposed to be involved in DNA binding and was termed the α-domain (Debuchy and Coppin, 1992).

The product of *N. crassa mt a-1* has two defined regions. One is an *in vitro* DNA-binding activity mediated by an HMG domain (Philley and Staben, 1994), a motif originally

found in high mobility group proteins associated with chromatin and shown to bind DNA (Jantzen *et al.*, 1990). The *mt a-1* HMG box is similar to that in $matM_C$ of *S. pombe* (Staben and Yanofsky, 1990), *FPR1* of *P. anserina* (Debuchy and Coppin, 1992), *MAT-2* of *C. heterostrophus* (Turgeon *et al.*, 1993) and the male determining *SRY* gene in humans (Sinclair *et al.*, 1990) and mice (Gubbay *et al.*, 1990). Its presence correlates with mating ability (Philley and Staben, 1994). The second MT a-1 region is the carboxy terminal half which is similar to transcriptional transactivator proteins and is required for both sexual and vegetative functions (Philley and Staben, 1994).

The product of the *N. crassa mt A-2* gene has 23% overall amino acid identity with *SMR1* product of *P. anserina* and a block of 82% identity extending across 17 amino acids, proposed to be a novel DNA binding domain (Debuchy, Arnaise and Lecellier, 1993; Ferreira, Saupe and Glass, 1996).

N. crassa mt A-3 gene product has 22% amino acid identity with *SMR2* of *P. anserina* (Ferreira, Saupe and Glass, 1996). The MT A-3 protein also possesses an HMG domain. In addition, the protein has a PEST domain which is a motif that correlates with rapid constitutive protein turnover (Rogers, Wells and Rechsteiner, 1986), but not signal-induced degradation (van Antwerp and Verma, 1996) and may be involved in protein-protein interaction (Chu *et al.*, 1996).

N. crassa mt A-1 and *mt a-1* can confer mating identity, but not vegetative incompatibility, to a mating type null strain of *P. anserina* (Arnaise, Zickler and Glass, 1993). *P. anserina FMR1* and *FPR1* can likewise confer mating identity, but not heterokaryon incompatibility to a *N. crassa* strain of the opposite mating type (Arnaise, Zickler and Glass, 1993). Like *mt A-1*, *FMR1* is also required for post-fertilization functions (Debuchy, Arnaise and Lecellier, 1993).

The *P. anserina* homologs to *mt A-2* and *mt A-3*, *SMR1* and *SMR2*, are required for post-fertilization functions (Debuchy, Arnaise and Lecellier, 1993). They are thought to be required for the sorting of one nucleus of each mating type into cells destined to become single asci (Zickler *et al.*, 1995). Uniparental ascospores are formed in mutants of any of the *P. anserina*

mating type genes. The production of ascospores suggests that the mating type genes are not required for meiosis or sporulation (Zickler *et al.*, 1995).

A *P. anserina* mating type null mutant transformed with the *N. crassa mt A* region progressed no further in sexual development than when transformed with *mt A-1* alone, suggesting that while *mt A-2* and *mt A-3* are similar to *SMR1* and *SMR2*, they cannot complement the post-fertilization functions specified by these genes (Debuchy, Arnaise and Lecellier, 1993; Arnaise, Zickler and Glass, 1993). The post-fertilization functions of *mt A-1*, *mt A-2* and *mt A-3* may depend on chromosomal location in *P. anserina* as they do in *N. crassa* (see Chapter 1). Alternatively, the lack of complementation could reflect differences in target genes between the two species.

In contrast to the *SMR1* and *SMR2* phenotypes, crosses of *mt A-2* or *mt A-3* mutants produced no uniparental progeny (Ferreira *et al.*, 1998). The possibility that *mt A-2* and *mt A-3* are required for post-fertilization recognition in *N. crassa* remains viable, however, under the condition that ascus development requires interaction between opposite mating types in *N. crassa*.

S. macrospora genes can induce fruiting body formation in *P. anserina* (Pöggeler *et al.*, 1997a). *C. heterostrophus MAT-1* or *MAT-2* can confer mating identity to the *P. anserina* null strain (S. Arnaise, personal communication to N. L. Glass and M. A. Nelson, cited in Glass and Nelson, 1994). *C. heterostrophus* has no homolog to *mt A-2* or *mt A-3*.

The sequence and functional homologies suggest that barriers to interspecific mating may be specified by mating type target genes, rather than the mating type genes themselves (Arnaise, Zickler and Glass, 1993).

Two mating type-specific transcripts are encoded in the centromere proximal flanks of the idiomorphs (Randall and Metzenberg, 1995). One of the *mt A*-specific predicted proteins is similar to fungal pheromones (T. A. Randall and R. L. Metzenberg, personal communication to N. L. Glass). The transcription of one of the *mt A*-specific transcripts depends on MT A-1 (Randall and Metzenberg, 1995).

A region in the mating type flank in *Neurospora* species appears to be species-specific. It could encode species-specific pheromones (Randall and Metzenberg, 1995). Pheromonal differences are postulated to be an important step in genetic isolation of a group of four *Drosophila* species (Coyne, Crittenden and Mah, 1994). For species in which mating via heterokaryosis is not possible (all those with mating type incompatibility), pheromonal differences may contribute to sexual isolation.

Thesis objective

In this thesis, data was gathered to shed light on the mechanism of the positional regulation of the mating type genes of *N. crassa*. Two competing hypotheses were tested, the transvection hypothesis and the *cis*-acting regulator hypothesis. The research suggested that the mating type position effect is not due to an unmet transvection requirement and may be due to differences in the expression of mating type genes when they are ectopic.

The mating type genes of *N. terricola* were cloned and their functionality tested in order to examine the hypothesis that a species can achieve homothallism by combining mating type genes into single nuclei. Two *N. terricola* mating type genes are very likely functional.

The sequences of a mating type gene from *Neurospora* and three outgroup species were determined or obtained from other sources and used to reconstruct the phylogeny of the *Neurospora* species in order to discover the relationships between homothallic and heterothallic species. The results suggested that homothallic species are long-lived and that a group of homothallic species is more closely related to heterothallic species than to the other group of homothallic species.

Chapter 1

Phenotypic and Mechanistic Analyses of Positional Regulation of *N. crassa* Mating Type Genes

Introduction

The mating type genes in *N. crassa* specify identity during the sexual stage of the life cycle, such that *A* x *a* is a compatible mating and *A* x *A* and *a* x *a* are incompatible (Shear and Dodge, 1927). The genes also specify identity during the vegetative stage, such that *A* + *A* and *a* + *a* form compatible heterokaryons and *A* + *a* form an incompatible heterokaryon (Beadle and Coonradt, 1944). A compatible heterokaryon produces a vigorous mycelium; an incompatible heterokaryon typically grows slowly or not at all.

An *mt A-1* or *mt a-1* mutant produces protoperithecia and conidia before crossing, but it is sterile because it fails to produce perithecia and ascospores. Furthermore, in contrast to wild type, a mating type mutant can form a compatible heterokaryon with a strain of the opposite mating type (Griffiths and DeLange, 1978; Griffiths, 1982). Functional copies of *mt A-1* or *mt a-1* introduced by transformation into the mutants vary in their ability to complement the mutant phenotype. Typically, sterile mating type mutants transformed with cloned mating type genes produced perithecia devoid of ascospores. Reasons for developmental arrest were unknown. Prior to these experiments, mating had never been separated from sporulation. Post-fertilization functions require all four mating type genes, *mt a-1* (Raju, 1992), *mt A-1* (Saupe *et al.*, 1996), *mt A-2* and *mt A-3* (Glass and Lee, 1992; Ferreira, 1997).

In one transformation experiment, a few colonies transformed with *mt A* DNA were able to produce ascospores (Glass, Grotelueschen and Metzenberg, 1990). Because their molecular constitutions were consistent with their having undergone gene replacements, Glass, Grotelueschen and Metzenberg (1990) suggested that *cis*-acting sequences may be important for proper expression of *mt A-1*. Because an ectopically integrated copy of *mt a* into a *tol A* strain

was only partially functional, but the *A* was still fully functional, Staben and Yanofsky (1990) suggested that the *mt a* genes could only function normally when occupying the mating type locus. Because a transformant with *mt a-1* targeted to the mating type locus was fertile, Chang and Staben (1994) suggested that *mt a-1* is controlled by *cis*-acting sequences. In eukaryotic systems, a position effect is defined as the chromosomal position-dependent alteration of the expression of a gene. The definition is limited to only those genes carrying intact coding regions and most or all of the local transcriptional control elements (Wilson, Bellen and Gehring, 1990).

The lack of ascospores in transformants carrying introduced mating type genes can be explained by invoking positional regulation of the mating type genes, but other explanations are also valid. In the transformations of mating type mutants with mating type sequences, genes required for ascospore formation could have been mutated by RIP of duplicated sequences and/or rearrangements caused by premeiotic recombination between duplicated sequences (Selker *et al.*, 1987). Additionally, in the transformations of mating type strains with opposite mating type genes, interference between opposite mating type transcripts or products could have been eliminating ascus development (see Chapter 1 Discussion).

The situation in *N. crassa* contrasts with that in the ascomycetes, *P. anserina* and *C. heterostrophus,* in which mating type deletion strains transformed with mating type genes produced a normal or close to normal number of ascospores (Coppin *et al.*, 1993; Wirsel, Turgeon and Yoder 1996). Why are ectopic mating type genes of *Neurospora* unable to orchestrate sporulation?

The experiments described in this chapter were designed to determine if the *N.crassa mt A* mating type genes are under positional regulation and if so, what is the mechanism of the position effect. Chapter 1 describes experiments designed to identify chromosomal positions that allowed the *mt A* idiomorph to confer both mating and sporulation. The hypothesis being tested was that *mt A-1*, *mt A-2* and/or *mt A-3* are functional only when they reside at the mating type locus. A mating type *a* host was transformed with the *mt A* idiomorph which was presumed to integrate randomly (Fincham, 1989). Two types of transformants were examined

genetically and at the molecular level: those able to mate and sporulate as *A* and those able to mate but not sporulate as *A*. The chromosomal position of the integrated DNA was determined for each of the transformants with normal *A* mating type function. This research differs from previous work in that a large number of transformants was examined and molecular and genetic analyses were performed to test competing hypotheses.

Secondly, Chapter 1 describes the sexual phenotype of a strain constructed to reveal a position effect without the complicating effects of RIP and interference between opposite mating type genes. The results include a genetic analysis of strains in which the mating type idiomorphs were deleted from the mating type locus and integrated at an ectopic chromosomal location. Also, a transvection model is tested. This model proposes that normal sexual development requires close physical proximity of the mating type idiomorphs, *i.e.* "pairing" of non-homologous sequences, but that the genes do not necessarily have to reside at the mating type locus. The model predicts that ascosporogenesis will be restored to a cross between two strains in which the mating type genes have been relocated to homologous chromosomal positions. Additionally, observations are made on a variety of crosses with ectopic mating type genes to answer the following general questions. Are there any consistent sex-specific or mating type-specific differences in the fertility of ectopic-*mt* strains? How does the fertility differ when these strains are crossed to ectopic-*mt* versus wild type strains? Is a cross involving an ectopic mating type gene blocked at one specific developmental step?

Transvection was originally described in *Drosophila* (Lewis, 1954) and most of the homologous pairing-dependent phenomena have been studied in this organism (Wu, 1993). In its older usage, transvection referred to the ability of a normally *cis*-acting sequence to affect expression of the gene on the homologous chromosome (Judd, 1988; Wu and Goldberg, 1989). A broader term, "trans-sensing", was introduced to describe effects that depend on direct interaction between homologs (Tartof and Henikoff, 1991). Models for transvection are numerous and some are specific to certain interactions (Wu, 1993). Judd (1988) describes two classes of models: (1) conformational changes in chromatin brings sites on different DNA molecules into juxtaposition and (2) nuclear messenger molecules with limited range of action

affect only close molecules. Similarly, Henikoff (1994) divided the phenomena into the following two classes: (1) structural, which involve changes in the structure of the genes and (2) kinetic, which involve spreading of regulatory proteins from the area where they normally act.

One type of *Drosophila* trans-sensing (seen in the genes, *ci*, *bx*, *It*, *dpp* and *Scr*) may require continuity between the gene and its centromere (Tartof and Henikoff, 1991). Perhaps significantly, in the fertile *N. crassa* translocation strain *T(I->II) 39311*, the translocated fragment of linkage group I that moves the mating type region carries with it the cluster of genes near the centromere (Perkins, 1972).

Drosophila trans-sensing effects depend on pairing of homologs in somatic cells (Hiraoka *et al.*, 1993). Trans-sensing in *N. crassa* would have to occur in croziers after karyogamy and before meiosis since this is the only diploid cell in the life cycle. Meiotic transvection has been described in *N. crassa*. Alleles of *Asm-1*$^{+}$ require pairing in order to conduct normal ascospore maturation, and the transvection is supposed to occur during the short time when homologs are paired, but before crossing over has begun (Aramayo and Metzenberg, 1996).

Strains for testing the transvection hypothesis were constructed by R. L. Metzenberg and J. Grotelueschen (Figure 1.2). The host of these three strains (RLM 44-02) had the *mt A* idiomorph (on linkage group I) deleted and replaced by the *ad-5* gene of *Schizophyllum commune*, and as a result could neither mate nor produce ascospores. All three of the derivative strains (mt-rel-Ac6, mt-rel-Aflk and mt-rel-Da4) were transformants in which a mating type idiomorph had been targeted to the *am* locus on linkage group V. They all grew and conidiated well. In mt-rel-Ac6 the same fragment of DNA deleted from the mating type locus was targeted to the *am* locus. In mt-rel-Aflk, the introduced fragment contained the deleted sequences plus approximately 2 kbp of flank on either side of the idiomorph, resulting in a duplication of those sequences. In mt-rel-Da4, the introduced fragment contained the *mt a* idiomorph plus a small amount (possibly insignificant with respect to RIP) of flank. Typically sequences greater than 1 kbp are susceptible to RIP (Selker, 1990).

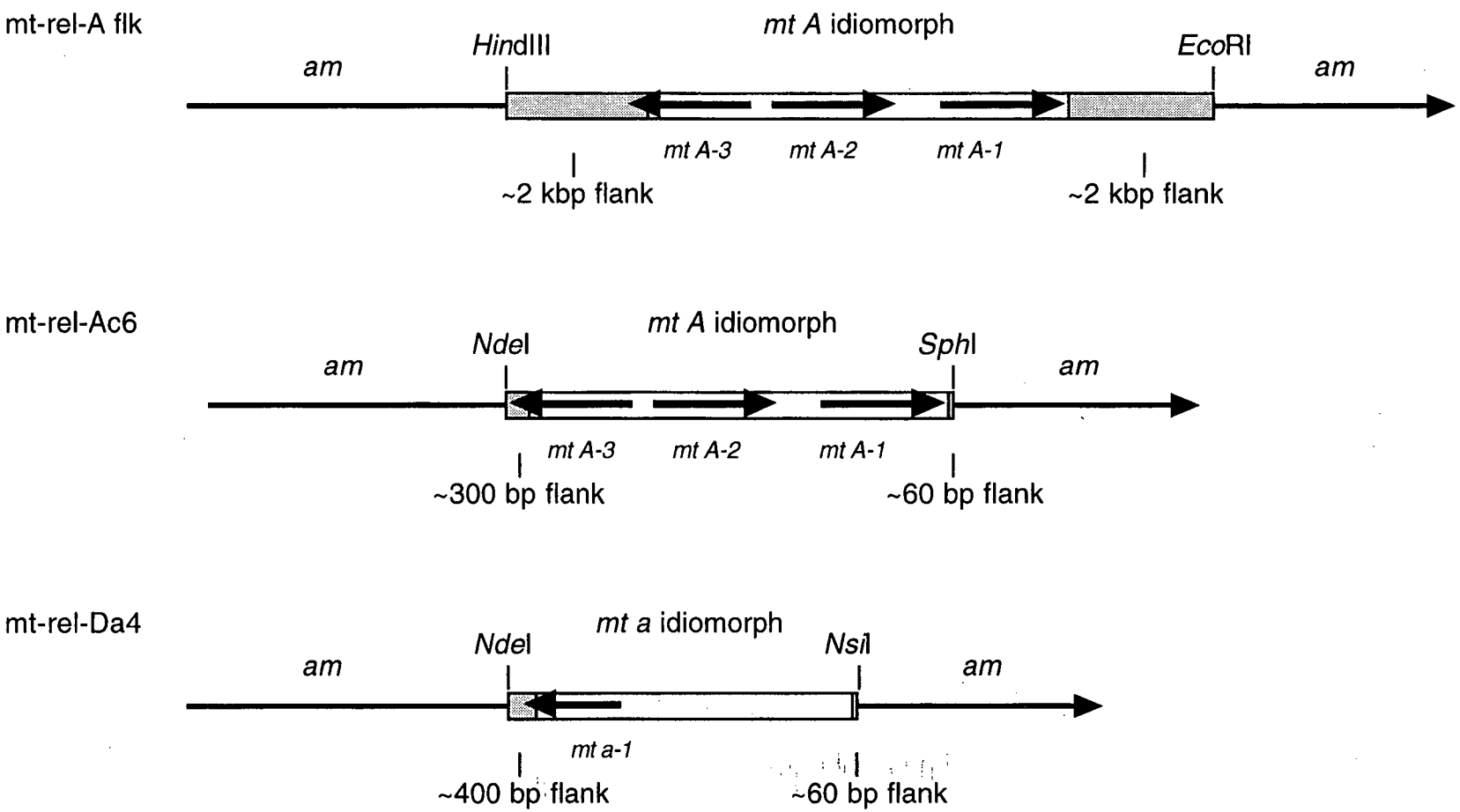


Figure 1.2 Map of *am* locus in ectopic-*mt* strains. Constructs were introduced at the *am* locus of the deleted-*mt* host to make the ectopic-*mt* strains. The strains are *am-* due to the integration of the constructs. They have been made *lys-1+* by site-directed replacement at the *lys-1* locus. All transformation events were confirmed by molecular analysis (J. Grotelueschen and R. L. Metzenberg personal communication).

Thirdly, Chapter 1 describes the examination of a second model for the mechanism of the mating type position effect, the *cis*-acting regulator model. The hypothesis states that the *mt A* idiomorph correctly controls sexual functions only when located at the mating type locus because a *cis*-acting sequence regulates the transcription of the mating type genes. When the genes are separated from it, incorrect regulation leads to the inhibition of ascus development. Evidence exists for altered expression of ectopic genes in *N. crassa* (Versaw and Metzenberg, 1996). To see if the mating type genes were expressed at different levels in ectopic-*mt* versus normal strains, mRNA amounts were compared. Changes in expression of the mating type genes in ascogenous tissue alone would have gone undetected due to the presence of excess perithecial tissue in mRNA preparations, and so early and late vegetative tissue were used.

Finally, Chapter 1 describes the test of the following hypothesis: inhibition of mating type function occurs when the two mating types occupy the same nucleus prior to karyogamy. In the ascomycetes, *P. anserina* and *C. heterostrophus,* mating type genes in ectopic chromosomal locations are able to function as wild type if and only if the resident mating type gene is deleted (Coppin *et al.*, 1993; Wirsel, Turgeon and Yoder, 1996). This phenomenon is called interference and is not due to RIP, since RIP does not occur in these fungi (Selker, 1990). In *P. anserina*, a transformant heterozygous at the mating type locus produced barren perithecia in great excess of fertile ones. These few fertile perithecia produced normal amounts of ascospores (Picard, Debuchy and Coppin, 1991). In *C. heterostrophus*, the interference phenotype is the inhibition of ascus and ascospore development (Turgeon *et al.*, 1993).

Do the opposite mating type genes of *N. crassa* inhibit each other? Since mating type genes in ectopic chromosomal positions in *N. crassa* do not function as wild type (Chapter 1 Results and Discussion), careful observation was required in order to identify interference. The normal fertility of dual mating type heterokaryons may seem to contraindicate interference, but the heterokaryon is an inadequate test of the interference hypothesis. For one thing, homokaryotic sectors may exist within a heterokaryon, and therefore the mating type products may not even be in physical proximity to each other and so interference is not observed. Secondly, the mating type products may not encounter each other frequently in a heterokaryon

as they are probably nuclear-limited. The nuclear localization of the mating type products is inferred by the observation that *A* and *a* nuclei encounter one another in ascogenous hyphae, yet maintain their individual identities, as shown by the fact that one nucleus of each type becomes sequestered into the crozier. Nuclear localization is further supported by the fact that some heterokaryon compatible mutants have reduced transformation efficiency with opposite mating type DNA fragments (Saupe *et al.*, 1996). Finally, a mating type mutant cannot be fully rescued by a fertile heterokaryotic partner (Raju, 1992).

If *mt A* and *mt a* interfere with each other's functions, one might see that deletion of the opposite mating type gene from a heterozygous transformant allows sexual development to proceed normally. To determine whether ectopic mating type genes interfere with the function of resident mating type genes and also whether resident mating type genes interfere with the function of ectopic mating type genes, perithecia from the following crosses were examined. Fertility was compared between crosses of strains with *mt a* at the mating type locus with and without an ectopic *mt A* idiomorph. Fertility was compared between crosses of strains with *mt A* at the *am* locus with and without *mt a* at the mating type locus. The experiments could not be done with ectopic *mt a* and resident *mt A* due to the sterility of the ectopic-*a* strain, mt-rel-Da4.

Chapter 1 aims to answer the following questions. Do the *A* mating type genes function abnormally in ectopic chromosomal positions? If so, what is the phenotype of ectopic-mating type strains? How is this putative position effect mediated?

Materials and Methods

Strains

Strains utilized in Chapter 1 are listed in Table 1.2.

Table 1.2. Strains used in Chapter 1.

Strain name	Genotype	Source
I-10-1	*ad-3A nic-2; tol a*	A. J. F. Griffiths
RLM 44-02	*thi-4 ad-2[ad-5]; lys-1 ΔA*	R. L. Metzenberg
mt-rel-Ac6	*thi-4 ad-2[ad-5]; am ΔA[A]*	R. L. Metzenberg
mt-rel-Aflk	*thi-4 ad-2[ad-5]; am ΔA[A with flank]*	R. L. Metzenberg
mt-rel-Da4	*thi-4 ad-2[ad-5]; am ΔA[a]*	R. L. Metzenberg
	fl a	FGSC 4347
	fl A	FGSC 4317
OR8-1a	*wild type*	FGSC 988
74-OR23-1A	*wild type*	FGSC 987
	tol trp-4 a	FGSC 2337

The *tol* allele allows the compatible heterokaryosis of *A* and *a* strains (Newmeyer, 1970; Vellani, Griffiths and Glass, 1994) and was included in the host strain to allow the integration of *mt A* DNA. Strains with the *ad-3A* gene require adenine for vigorous growth. Strains with the *nic-2* gene require niacinamide for vigorous growth. The two genes are tightly linked and lie 28 map units from the mating type locus. Strains with the *fl* allele make suitable mating type testers because they lack macroconidia and produce abundant protoperithecia. Typically, they were grown on synthetic crossing medium (Westergaard and Mitchell, 1947) for a minimum of 5 days before fertilization. All *N. crassa* strains described in this thesis are of the Oak Ridge genetic background.

Media

Strains were grown on standard media (Davis and de Serres, 1970).

DNA Preparation

The *N. crassa* Orbach/Sachs genomic library was obtained from the Fungal Genetics Stocks Center. Cosmid DNA was isolated from *E. coli* with a Plasmid Kit (Qiagen, Chatsworth, Calif.). Genomic DNA was isolated from *N. crassa* by the method of Lee and Taylor (1990).

Transformation

N. crassa spheroplasts were prepared and transformed according to Schweizer *et al.* (1981), using the modification of Akins and Lambowitz (1985).

Crosses

Hygromycin-resistant transformants were tested for *mt A* mating type function by replica plating. A circle of Whatman 1 filter paper (Whatman International Limited, Springfield Mill, Maidstone, Kent.) was placed onto each transformation plate and transferred the next day to a lawn of *fl a* with visible protoperithecia.

Mating non-sporulating (MNS) transformants were tested for their ability to mate as *a* males by placing 0.25-0.5 mL of conidial suspension of the MNS strain onto a *fl A* lawn with protoperithecia. For testing as *A* and *a* females, the MNS strains were grown on slants of crossing medium (Westergaard and Mitchell, 1947) supplemented with adenine (Eastman Fine Chemicals, Eastman Kodak Company, Rochester, N. Y.) and niacinamide (Sigma Chemical

Company, St. Louis, MO). After the appearance of protoperithecia (8 or 21 days later in the tests of the MNS strains as *A* females, and 21 days in the tests as *a* females) 100-200 μL of conidial suspension the male parent (either OR8-1*a* or 74-OR23-1*A*) were added.

DNA hybridization

Genomic DNA was digested with *Bam*HI for three hours. Digested DNA was partitioned by gel electrophoresis and transferred (Sambrook, Fritsch and Maniatis, 1989) onto Nytran™ Plus nylon membrane (Schleicher and Schuell, Keene, New Hampshire). The blot in Figure 1.3 was probed with a ^{32}P-labelled 9.6 kbp *Bam*HI fragment of pG16/C10.

Probe preparation

Cosmid DNA (pG16/C10) was prepared using a Plasmid Kit (Qiagen, Chatsworth, Calif.) and digested with *Bam*HI. The fragments were separated electrophoretically. The 9.6 kbp *Bam*HI fragment was cut from the agarose gel and purified by centrifugation of the gel slice through Whatman 1 paper (Whatman International Limited, Springfield Mill, Maidstone, Kent.). The fragment was radioactively labelled with Redivue™ α-^{32}P-dCTP (Amersham, Oakville, Ont.) using the random primer method (T7 QuickPrime™, Pharmacia, Baie d'Urfe, Quebec).

Photography

Perithecial contents were squeezed with forceps into a drop of sterile distilled water on a cleaned slide and covered with a cover slip. Slides were viewed with an Olympus BH-2 compound microscope and photographed with TMAX 100 black and white negative film.

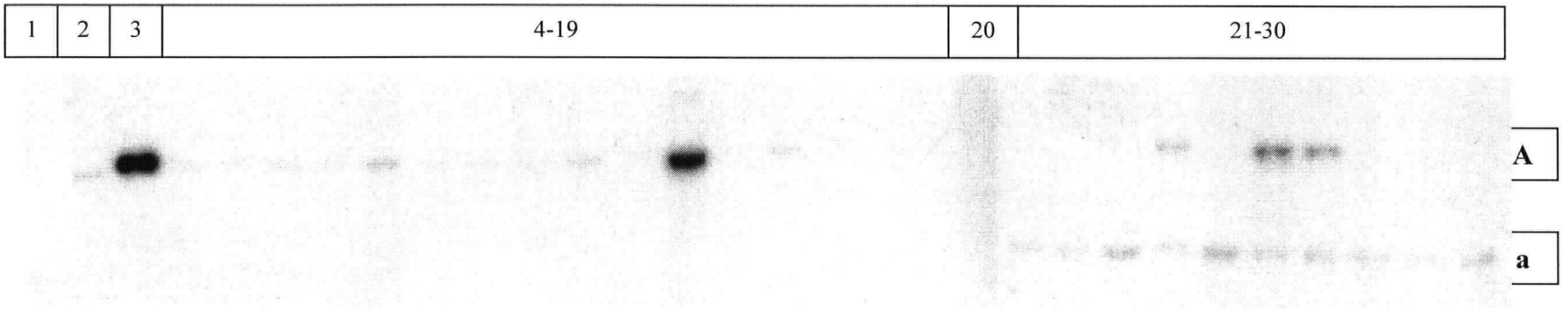


Figure 1.3 DNA blot of MS progeny and MNS strains showing *A* and *a* bands. Lane 1 is untransformed *a* recipient, I-10-1, lane 2 is wild type *A*, lane 3 is pG16/C10 cosmid, lanes 4-19 are representative *A* progeny of the 6 MS transformants, lane 20 is smeared kb ladder and lanes 21-30 are the MNS transformants, MNS-1 to MNS-10 cut with *Bam*HI. DNA was subjected to agarose gel electrophoresis, blotted to Nytran Plus™ membrane and probed with the 9.6 kbp *Bam*HI fragment of pG16/C10 (Figure 1.7). The 9.6 kbp fragment was isolated from an agarose gel and labelled with ^{32}P. The blots were washed in 0.1x saline sodium citrate (SSC) and 0.1% SDS at 60°C and the autoradiogram was exposed at -70°C overnight with an intensifying screen.

RNA Isolation

RNA was isolated from frozen tissue according to Logemann, Schell and Willmitzer (1987). Poly(A)$^+$ RNA was isolated from total RNA using an Oligotex™ mRNA kit (Qiagen, Chatsworth California).

Slot Blots

Slot blots were made on a PR648 Slot Blot (Hoeffer Scientific Instruments, San Francisco, California) according to the manufacturer's instructions. Several different quantities of Poly(A)$^+$ RNA were loaded in each row of slots to enable the estimation of mechanical accuracy (homogeneity of mRNA in solution, pipetting error, *etc.*) and to allow a choice of slots to compare. Each slot blot in Figure 1.4 consists of a set of six slots. The top rows of slots on each of the two slot blots in the left panel were loaded with 125 ng, 250 ng and 500 ng of mt-rel-Ac6 mRNA. The bottom rows were loaded with 125 ng, 250 ng and 500 ng of 74-OR23-1*A* mRNA. The top rows of slots on each of the two slot blots in the right panel were loaded with 135 ng, 270 ng and 540 ng of mt-rel-Ac6 mRNA. The bottom rows were loaded with 130 ng, 260 ng and 520 ng of 74-OR23-1*A* mRNA. In Figure 1.5, the four slots on each of the two slot blots were loaded with 160 ng and 325 ng of mRNA from six-day-old mt-rel-Ac6 mycelia and 180 ng and 365 ng of mRNA from six-day-old 74-OR23-1*A* mycelia.

Slot Blot Probes

Two constitutively expressed gene controls were used: *crp-1*, the *N. crassa* gene with homology to yeast and mammalian ribosomal proteins (Kreader and Heckman, 1987), for the slot blot probed with *mt A-1*, and *β-tubulin* (Orbach, Porro and Yanofsky, 1986) for the slot blot probed with *mt A-2* and *mt A-3*.

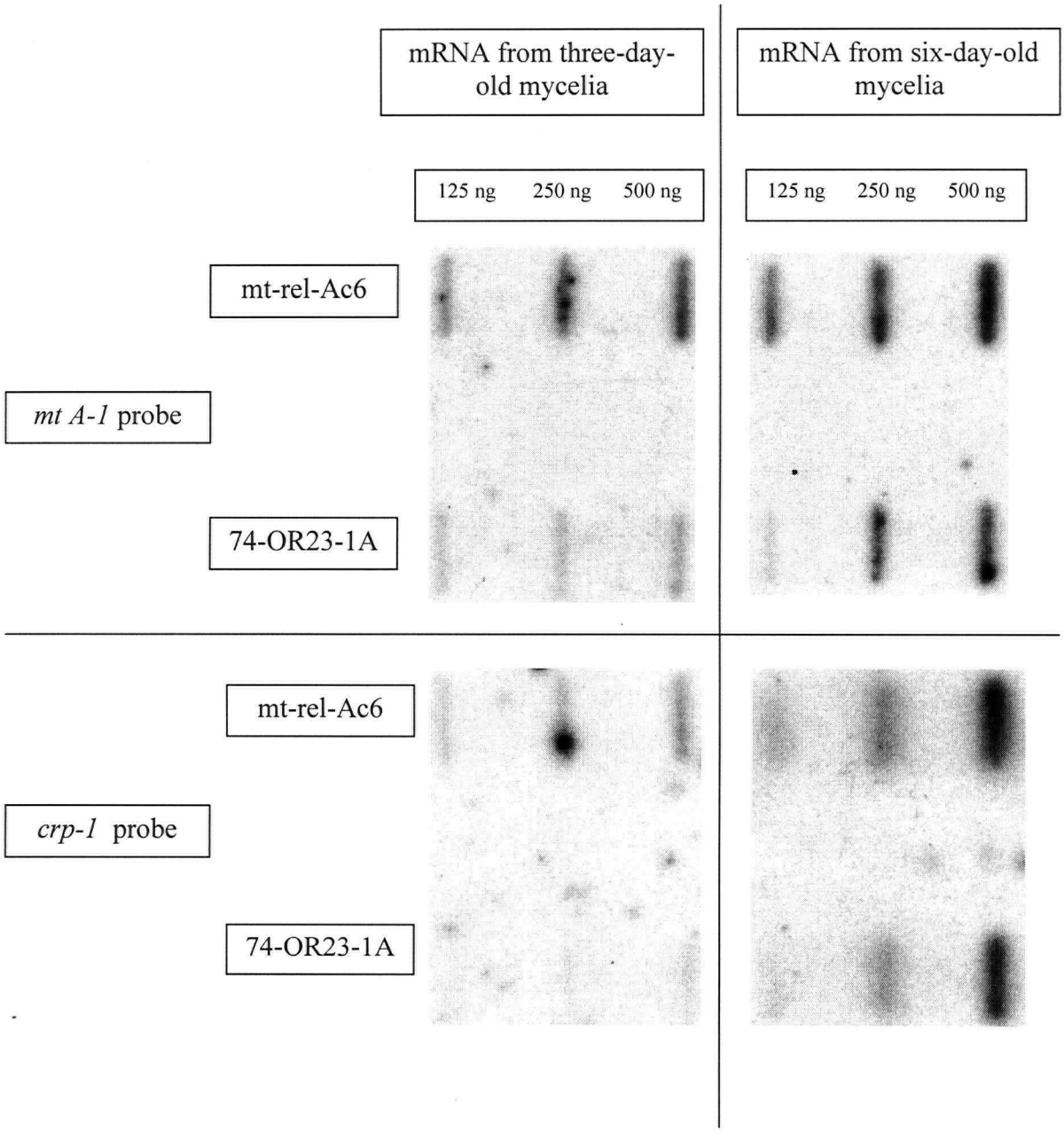


Figure 1.4 Poly $(A)^+$ RNA slot blots of three-day-old (left panels) and six-day-old (right panels) vegetative mycelia from mt-rel-Ac6 and 74-OR23-1A. The *mt A-1* probe was a *Hin*dIII fragment from pBSmt-150 (top panels). The unregulated control probe was an *Eco*RI-cut plasmid (pCC1) containing the small ribosomal subunit protein gene, *crp-1* (bottom panels).

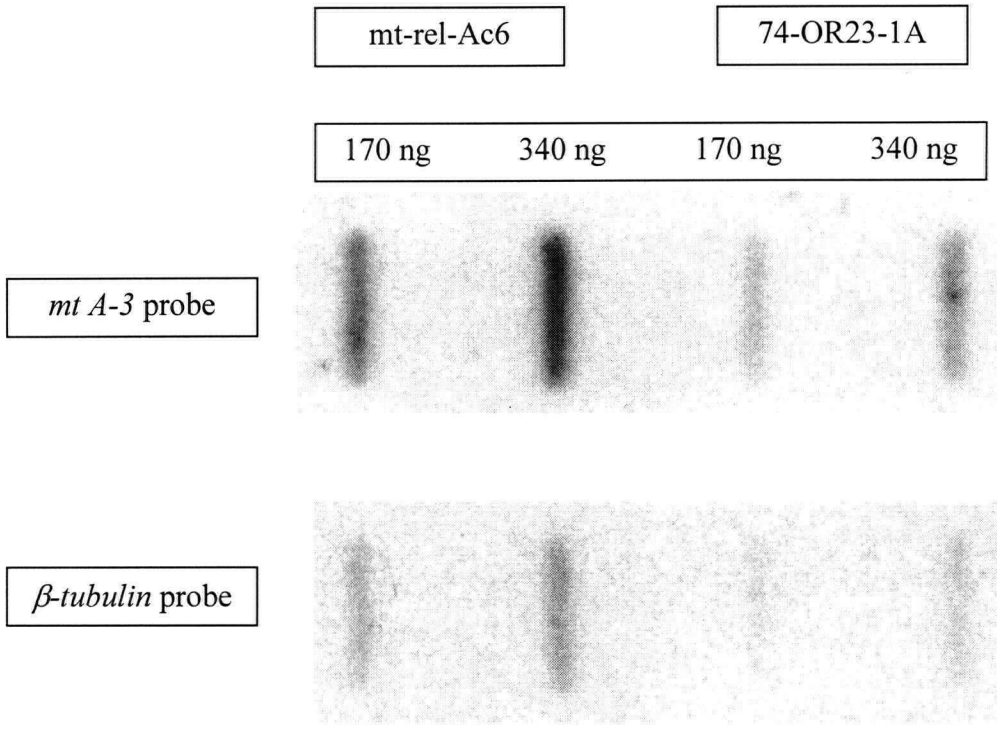


Figure 1.5 Poly $(A)^+$ RNA slot blots of six-day-old mycelia of approximately 170 ng and 340 ng of mt-rel-Ac6 (2 left-most slots in each panel) and 74-OR23-1A (2 right-most slots in each panel) probed with an *mt A-3* probe (top panel) and a *β-tubulin* probe (bottom panel).

The *β-tubulin* probe was a fragment of DNA approximately 500 bp long amplified from *N. crassa* genomic DNA with the primers, BT1a and BT1b whose sequence is listed in Chapter 2 Materials and Methods (Glass and Donaldson, 1995).

The *crp-1* probe was the *crp-1* gene cloned into a plasmid (pCC1) and digested with *Eco*RI.

The *mt A-1* probe was a *Hin*dIII-cut plasmid (pBSmt-150) containing cloned sequences from the *mt A-1* gene.

The *mt A-2* probe was an *Eco*RI-cut plasmid (pNTA2) which contained a 700 bp PCR fragment of the *mt A-2* gene amplified with the primers, rI.1 and rI.2 (Ferreira, Saupe and Glass, 1996).

The *mt A-3* probe was the 1.2 kbp PCR product amplified from *N. crassa* genomic DNA with the primers rII.1 and rII.2 (Ferreira, Saupe and Glass, 1996).

Slot Blot Analysis

Autoradiograms of the slot blots were scanned into a computer using a Fotolook flatbed scanner. The images were analyzed to determine the relative optical densities of the slots using the public domain NIH image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The "uncalibrated O.D." option was chosen. Optical density profiles were generated from slot slices. A representative plot is shown in Figure 1.6. Three independent sets of measurements were made for each row of slots (Appendix B, Table A), with the width of the slice kept constant for each set of measurements. On the optical density plots, baseline gray levels were estimated by eye independently for each slice for the purpose of eliminating background grayness. Then a vertical section of the plot was delineated and the area under the curve within this section was measured and taken to represent the optical density of that slot. Each vertical section was 22 units wide and was positioned to include the highest point of the curve.

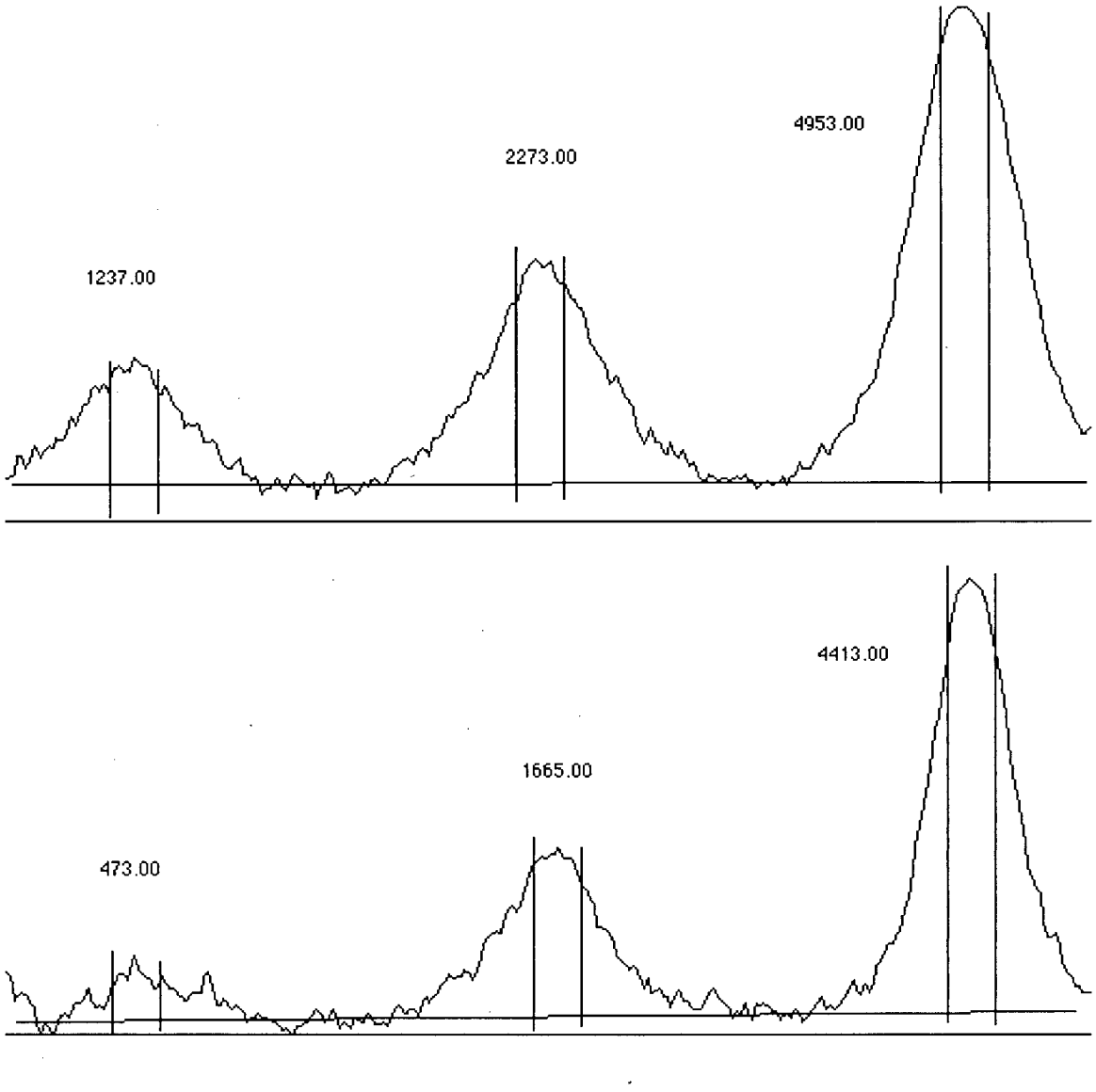


Figure 1.6 Sample of slot blot analysis performed using NIH image. Two horizontal slices were delimited in the image of the autoradiogram, corresponding to three slots per slice. The graph was generated using the gel plotting macro provided with the program. In the graph, the height of the peak is proportional to optical density. Baselines were estimated to eliminate background grayness. Numbers over the peaks indicate area under the peak with a peak-width of 22 units. The plot shown represents 1x, 2x and 4x amounts of mt-rel-Ac6 (top) and 74-OR23-1A (bottom) mRNA probed with the *mt A-1*-specific fragment.

Results

The first question to be addressed was the following: are there any chromosomal locations other than the mating type locus that allow full function of the mating type genes.

Analysis of mating, sporulating strains.

In order to obtain strains in which introduced *mt A* sequences enabled a transformed host to mate and sporulate as *A*, a strain of mating type *a* (*ad-3A nic-2; tol a*) was transformed with a cosmid, pG16/C10. The cosmid contained the *mt A* idiomorph plus 17 kbp of centromere-distal sequences and 13 kbp of centromere-proximal sequences (Figure 1.7). Transformants were selected for hygromycin resistance and 734 colonies were obtained.

The hygromycin resistant transformants were tested for their ability to mate as *A* by replica plating them onto a lawn of *fl a* (see Chapter 1 Materials and Methods). Most of the transformants (640/734) did not produce any perithecia on the tester plates. Some of them (94/734) produced perithecia, indicating that at least *mt A-1* had integrated into the host genome and was functional.

The mating type tester plates were observed for one month and within that period the perithecia from a small number of colonies (6/94) produced ascospores in varying amounts. Four of the six colonies were subcultured to fresh medium. The remaining two transformants did not grow when transferred to fresh medium (presumably because they were too old). The six *m*ating, *s*porulating transformants are hereinafter referred to as MS-1 through MS-6. Although MS-5 and MS-6 did not grow, their progeny were still included in the analyses. Fifteen of the transformants that mated, but did not sporulate were also transferred to fresh culture tubes for further analysis. They are referred to as MNS-1 through MNS-15 for *m*ating, *n*on-*s*porulating.

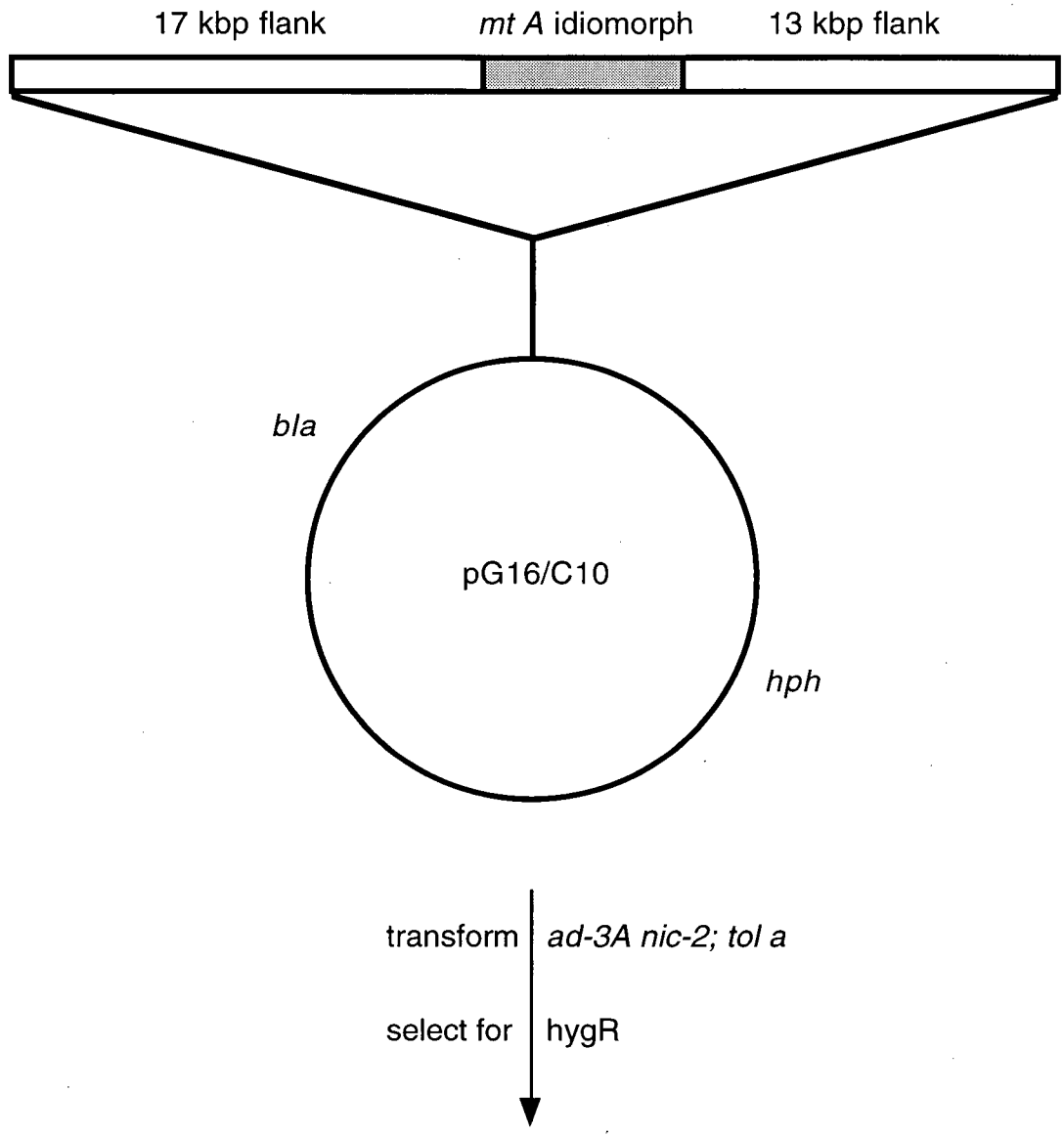


Fig. 1.7 Transformation of *ad-3A nic-2; tol a* strain with cosmid pG16/C10 to generate transformants with *mt A* integrated at various chromosomal positions. *hph* is a bacterial gene encoding hygromycin B phosphotransferase which confers resistance to the protein synthesis inhibitor, hygromycin. *bla* is an *Escherichia coli* gene encoding resistance to the drug, ampicillin.

The fertility of the MS strains varied widely. Ascospore quantities were estimated by eye (Table 1.3).

Table 1.3 Variable fertility of MS transformants.

Transformant	Approx. number of ascospores/plate
MS-1	20 000-50 000
MS-2	10 000
MS-3	1 000
MS-4	200
MS-5	100
MS-6	60

The fertility of the progeny from the crosses of the MS strains to *fl a* was tested by crossing them to *fl a* and *fl A*. The *A* progeny from the crosses of the MS strains to *fl a* did not share the same fertility phenotype as their *A* MS parent. For example, in contrast to the abundant ascospores produced by MS-1 and MS-2, 3/30 *A* progeny from MS-1 and 2/13 *A* progeny from MS-2 produced no ascospores at all. The reasons for the infertility are unknown.

The percent germination of the ascospores from the cross of the MS strains to *fl a* ranged from 81% to 6% (Table 1.4), in contrast to the 95% germination of wild type crosses. The cause of the variable germination frequency is not known.

Table 1.4 Variable percent germination of MS ascospores.

Transformant	# germinated ascospores/ # ungerminated ascospores culled	% germination
MS-1	58/72	81
MS-6	23/56	41
MS-2	25/72	35
MS-3	64/228	28
MS-4	17/72	24
MS-5	4/72	6

To determine whether the introduced *mt A* genes segregated from *mt a* at the mating type locus, the MS strains were crossed to *fl a* and the progeny were scored for their mating type. The MS progeny mated as *A* or *a* with a segregation ratio of 1:1 (Table 1.5). The 1:1 ratio is supported at a statistically significant level ($p=0.05$) in a chi-square test applied to each of the six crosses, individually.

Table 1.5 Segregation of mating type in MS progeny.

Cross	# a progeny	# A progeny
MS-1 x *fl a*	29	30
MS-2 x *fl a*	12	13
MS-3 x *fl a*	36	28
MS-4 x *fl a*	7	10
MS-5 x *fl a*	2	2
MS-6 x *fl a*	14	9

If *mt A* integrated at the mating type locus in the MS strains, then the introduced *mt A* DNA would have become genetically linked to *ad-3A nic-2*, markers that were present in the original recipient strain where they were linked to *mt a*. The normal genetic distance between the mating type locus and *ad-3A nic-2* is 28 map units. The model of 28% linkage between the newly integrated *mt A* idiomorph and *ad-3A nic-2* was applied to the MS progeny (Table 1.6). The MS parent was *ad-3A nic-2* and the other parent was *ad-3A*$^+$ *nic-2*$^+$.

The model of 28% linkage was accepted in a chi-square test applied to each of the six crosses independently at p=0.05, suggesting that the introduced *mt A* sequences had integrated at or near the mating type locus in the MS strains.

Table 1.6 Segregation of markers linked to *mt*

Cross	Parental types		Recombinant types	
	ad nic A	*ad*$^+$ *nic*$^+$ *a*	*ad*$^+$ *nic*$^+$ *A*	*ad nic a*
MS-5 x *fl a*	1	2	1	0
MS-1 x *fl a*	6	4	0	0
MS-2 x *fl a*	3	3	0	0
MS-3 x *fl a*	1	5	3	1
MS-4 x *fl a*	6	1	1	1
MS-6 x *fl a*	3	7	0	0

To determine whether the *mt A* idiomorph alone or parts of the rest of the cosmid had integrated, the hygromycin sensitivity of the MS strains and MS progeny was tested. Two of the MS strains did not grow when transferred from the transformation plate, and so were not

tested. (The progeny were obtained by replica plating from the transformation plates onto lawns of females; see Chapter 1 Materials and Methods)

MS-1 and MS-2 were hygromycin resistant, MS-4 had an intermediate resistance and MS-3 was sensitive. Ten progeny from each cross of the MS strains to *fl a* (four in the case of MS-5) were tested and they were all hygromycin sensitive, suggesting that only the *mt A* idiomorph had integrated.

Occasionally, introduced DNA recombines with endogenous homologous sequences without gene replacement (Miao, Rountree and Selker, 1995). In order to determine whether this event or gene replacement had occurred at the mating type locus, MS progeny strains were assayed for the presence of *mt a* sequences. Since the MS transformants were presumably heterokaryotic and therefore would contain *mt a* sequences, the progeny were examined instead. Progeny of mating type *A* that issued from the cross of MS x *fl a* were examined for the presence of *mt a* mating type sequences.

A Southern blot was made of 16 of the MS progeny that mated as *A* (representing all 6 MS strains), the *ad-3A nic-2; tol a* recipient strain, the transforming cosmid and a wild type *A* strain. The blot was probed with a 9.6 kb *Bam*HI fragment of the cosmid, which contained *mt A-1*, *mt A-2*, part of *mt A-3* and approximately 5 kbp of centromere-proximal flank (Figure 1.8). Probing revealed the presence in the 16 progeny of a 9.6 kbp fragment specific to *mt A* and the absence of the 5.7 kbp fragment specific to *mt a* (Figure 1.3, lanes 4-19). Lanes 4 and 5 were progeny from MS-1; lanes 6, 7, and 8 were progeny from MS-2, lanes 9, 10 and 11 were progeny from MS-4; lanes 12, 13 and 14 were progeny from MS-3; lanes 15, 16 and 17 were progeny from MS-6 and lanes 18 and 19 were progeny from MS-5.

The MS strains were tested for their ability to mate as *a* by inoculating subcultured colonies onto a lawn of *fl A*. MS-1, -2, -3 and -4 mated and sporulated abundantly. MS-5 and -

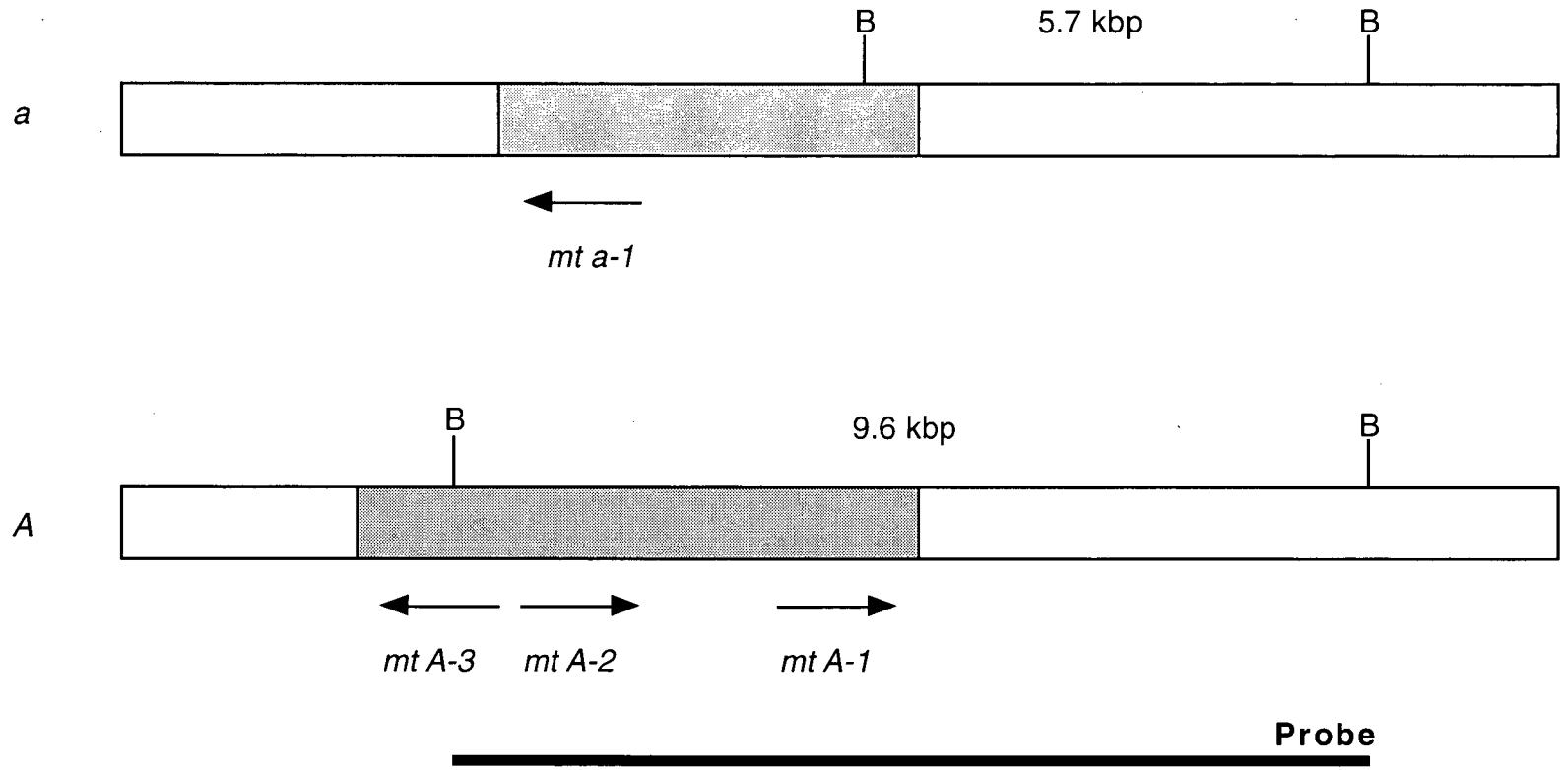


Figure 1.8 Map of *mt A* and *mt a* idiomorphs, showing relevant *Bam*HI (B) sites. The probe was derived from *mt A* and it hybridized to the 9.6 kbp fragment of *mt A* and to the conserved flanking sequences of *mt a*, lighting up a 5.7 kbp fragment.

6 could not be tested because as previously mentioned they could not be subcultured from the transformation plate.

Analysis of the mating non-sporulating strains.

The ability of the MNS strains to mate and sporulate as males and females of both mating types was assessed (Table 1.7). In the crosses testing the ability of the transformants to mate as males and females, the female parents were *fl A* and *fl a*, and the male parents were OR8-1*a* or 74-OR23-1*A*, respectively. The appearance of perithecia showed that mating had occurred.

As *A* males, all of the MNS strains mated, but did not sporulate (hence the name "mating, non-sporulating"). As *A* females crossed to the wild type strain, OR8-1*a*, all of the MNS strains mated in at least one replicate cross. In one of two replicate crosses (the set with the 8-day-old females), five of 14 strains (MNS-2, -4, -7, -9 and -13) produced a small number of ascospores, while the remaining nine strains produced none. As *a* males, Four of five strains (MNS-1, -2, -3 and -5) mated and produced abundant ascospores. MNS-4 mated, but did not sporulate. As *a* females, 11 of 14 strains (MNS-1, -2, -3, -5, -6, -8, -9, -10, -13, -14 and -15) mated and sporulated, while two strains (MNS-4 and -7) mated, but did not sporulate and one strain (MNS-11) neither mated nor sporulated.

A Southern blot of genomic DNA from the MNS strains was probed with the 9.6 kbp *Bam*HI fragment of the cosmid, pG16/C10. Overnight exposure of the Southern blot (Figure 1.3) showed that MNS-4, -6 and -7 contained a 9.6 kbp band of the same intensity as the 5.7 kbp *mt a*-specific band. Longer exposure (not shown) revealed the presence of less intense bands at 9.6 kbp and larger and smaller than 9.6 kbp which were not seen in the MS or MS progeny lanes. Some strains had multiple bands.

Table 1.7 Mating, ascosporogenesis and Southern blot phenotypes of MNS strains.

	As *A* male		As *A* female		As *a* male		As *a* female		9.6 kbp fragment
	mate	asco	mate	asco	mate	asco	mate	asco	
MNS-1	+	0	+, +	0, 0	+	100-500 *	+	30-50, w	faint
MNS-2	+	0	+, +	10, 0	+	10 000-50 000	+	500-1 000	-
MNS-3	+	0	+, +	0, 0	+	5 000-10 000	+	500-1 000	-
MNS-4	+	0	+, +	20, 0	+	0	+	0	+
MNS-5	+	0	+, +	0, 0	+	50 000-100 000	+	500-1 000	-
MNS-6	+	0	+, +	0, 0	n.d.		+	500-1 000	+
MNS-7	+	0	+, +	400-500, 0	n.d.		+	0	+
MNS-8	+	0	+, +	0,0	n.d.		+	500-1 000	-
MNS-9	+	0	+, +	100-200, 0	n.d.		+	10 000-50 000	-
MNS-10	+	0	+, -	0, 0	n.d.		+	500-1 000	-
MNS-11	+	0	+, -	0, 0	n.d.		-	0	n.d.
MNS-12	+	0	died	n.d.	n.d.		n.d.	n.d.	n.d.
MNS-13	+	0	+, +	20, 0	n.d.		+	1 000-5 000	n.d.
MNS-14	+	0	+, +	0, 0	n.d.		+	500-1 000 w	n.d.
MNS-15	+	0	+, -	0, 0	n.d.		+	1 000-5 000	n.d.

asco = approximate number of ascospores/test tube as female; ascospores/plate as male
n.d. = not done
* = may have been a lot more because I may have taken ascospores from this plate before I counted them
w = white ascospores present, see text for discussion

Analysis of ectopic-*mt* strains.

Since well characterized strains known to have a single ectopic insertion of various amounts of the *mt A* idiomorph flanking sequences and no *mt a* idiomorph, were available, I decided to study the effects of ectopic *mt A* sequences with these three strains (Figure 1.2). This strategy also eliminates the complication of both idiomorphs being present in one parent.

The cross of mt-rel-Ac6 (Figure 1.2) to wild type, OR8-1*a*, was analyzed in order to determine the phenotype of a strain in which there was a single ectopic copy of the *mt A* idiomorph. In this cross there were no duplicated sequences (and therefore no RIP) and there was no opposite mating type (and therefore no interference).

Mating efficiency (number of perithecia) was comparable to wild type. Light microscopic observation of cells squeezed with forceps from perithecia in crosses of mt-rel-Ac6 (and its *A* progeny) to various normal-*mt* strains were made at 5, 8, 11, and 12 days post-fertilization. Examination of internal features of perithecia earlier than 5 days post-fertilization requires sectioning of the perithecia. At 5 days post-fertilization, perithecial contents from crosses of the normal-*mt* and ectopic-*mt* strains are already different. Perithecia from crosses of normal strains contain rosettes in which ascospores are just beginning to develop within the few asci. Perithecia from crosses of ectopic-*mt* strains contain rosettes, but the asci have no ascospores yet. At 8 days post-fertilization, perithecia from crosses of normal strains contain rosettes with many asci, many of which contain 8 ascospores, whereas ectopic-*mt* perithecia contain notably fewer asci and significantly fewer ascospores (Table 1.8). The data were not normally distributed and so were analyzed using the non-parametric Mann-Whitney Rank-Sum test which is robust to non-normally distributed data. The two crosses were shown to be significantly different (Appendix C, Table A).

Perithecial contents from 11 days post-fertilization crosses of ectopic-*mt* and normal-*mt* strains clearly show the reduction in ascus and ascospore number of the former compared to the

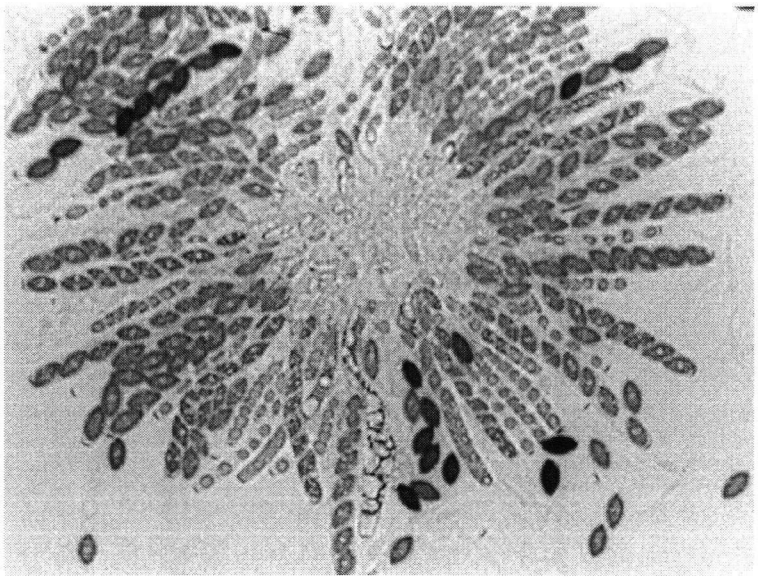
latter (Figure 1.9). The ascospores from both types of crosses germinate and appear to be completely normal (see below).

Table 1.8 Number of ascospores per rosette 8 days post-fertilization

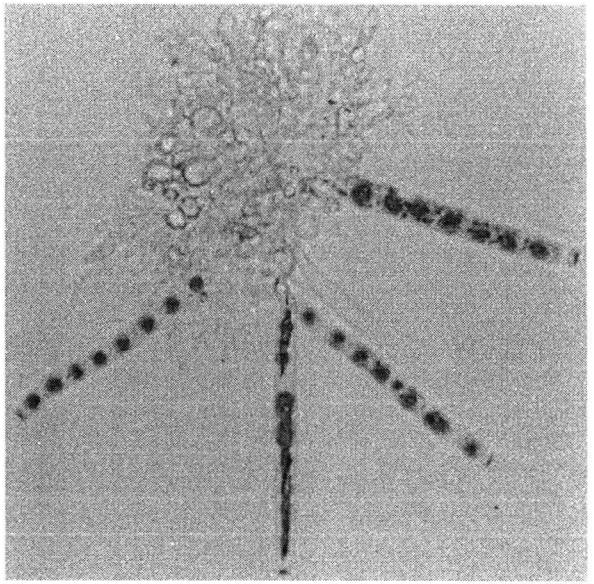
Parameter	normal *A x a*	rel *A x a*
# rosettes examined	8	18
# ascospores/ rosette (range)	24-272	0-16
Median	44	0
25th percentile	29	0
75th percentile	114	2.5

Meiotic non-disjunction leading to chromosome duplication and deletion is a known cause of low ascospore production. To assess whether the correct chromosomal position of mating type is required for meiotic disjunction, the cross of mt-rel-Ac6 x OR8-1*a* was analyzed (Figure 1.10). Only three of the four expected classes of meiotic products were recovered. The unrecovered class was *A/a* self-incompatible progeny. Self-incompatible progeny may have been missing because of a decrease in germination rate, growth rate or viability caused by the incompatibility. Ascospores were collected from the cross on two separate occasions. The first time, ascospores were spread onto petri plates, heat-shocked, allowed to germinate and grow overnight and then were cut out of the agar and placed into separate test tubes. Poorly germinating ascospores may have escaped detection, thus biasing the sample. The second time, ascospores were collected and placed in separate test tubes prior to heat-shock to eliminate bias. The expected number of self-incompatible progeny from this group alone was 10. It is possible that the sample size was small enough to have missed the self-incompatible class of progeny by chance.

Duplication strains are viable in *N. crassa* and the cross was set up to reveal nondisjunction of L.G. V. Strains carrying a duplication of L.G. V would be *am+ A*. No *am+ A* progeny were recovered in which the *A* mating type reaction was normal. There were seven *am+ A* progeny which mated, but produced very few tiny perithecia devoid of ascogenous hyphae.

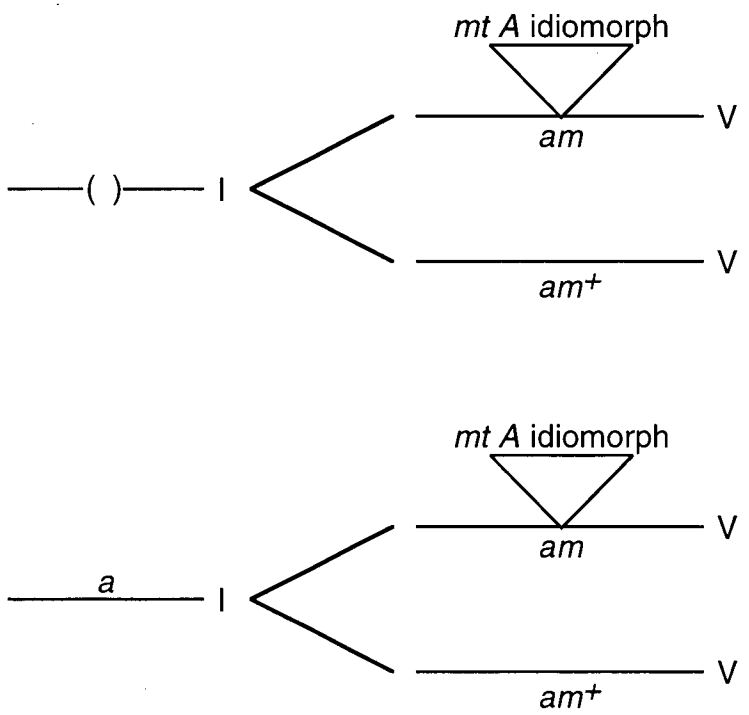


Normal cross, 11 days post-fertilization at 100x magnification.



Ectopic *A* cross, 11 days post-fertilization at 100x magnification.

Figure 1.9 Perithecial contents from 11 days post-fertilization crosses. Control cross (*fl A* x 5*a*) is in the left panel. Cross with ectopic *mt A* (*fl a* x 8*A**) is on the right.



		Expected	Observed
	am A	23	23
	am$^+$ no mating type	23	24
	am A/a	23	0
	am$^+$ *a*	23	38
unexpected class	*am*$^+$ weak *A*	0	7

Figure 1.10 Branch diagram showing the cross of mt-rel-Ac6 x OR8-1a. The *A/a* dual mating type self-incompatible class of progeny was not recovered. There was an unexpected group of *am*$^+$ weak *A* progeny.

Phenotypes of crosses with ectopic-*mt* strains.

Since the ectopic mating type strains generated above were dual mating type and/or heterokaryotic, single mating type strains were used to examine the mating type position effect phenotype and to test the models for the mechanism of action of the position effect.

Various types of crosses were made with mating type ectopic strains in order to describe sexual development (Table 1.9). Wild type perithecia are black and typically have one long neck. On a typical crossing plate, a wild type cross might produce tens or hundreds of thousands of ascospores.

Table 1.9 Crosses with ectopic mating type strains.

	# plates	Female	Male	Perithecia	Perithecial Necks	Ascospores
1	1	*mt-rel-Aflk thi-4 ad-2[ad-5]; am ΔA[A with flank]*	*mt-rel-Da4 thi-4 ad-2[ad-5]; am ΔA[a]*	abundant brown	none	0
2	1	*mt-rel-Aflk thi-4 ad-2[ad-5]; am ΔA[A with flank]*	*OR8-1a wild type*	abundant dark brown	none	100-500
3	1	*fl a*	*mt-rel-Aflk thi-4 ad-2[ad-5]; am ΔA[A with flank]*	abundant dark brown	mostly none; rarely very short	100-500
4	6	*mt-rel-Ac6 thi-4 ad-2[ad-5]; am ΔA[A]*	*mt-rel-Da4 thi-4 ad-2[ad-5]; am ΔA[a]*	abundant brown	none	1-10 (less than 50 in total)
5	6	*mt-rel-Ac6 thi-4 ad-2[ad-5]; am ΔA[A]*	*OR8-1a wild type*	abundant brown	none or short	500-1000
6	1	*fl a*	*mt-rel-Ac6 thi-4 ad-2[ad-5]; am ΔA[A]*	abundant brown	none or short	500-1000
7	7	*mt-rel-Da4 thi-4 ad-2[ad-5]; am ΔA[a]*	*mt-rel-Ac6 thi-4 ad-2[ad-5]; am ΔA[A]*	no ascogenous tissue	none	0
8	7	*mt-rel-Da4 thi-4 ad-2[ad-5]; am ΔA[a]*	*74-OR23-1A wild type*	few no ascogenous tissue	none	0
9	1	*fl A*	*mt-rel-Da4 thi-4 ad-2[ad-5]; am ΔA[a]*	abundant no ascogenous tissue	mostly none; occasionally very short	0

Numbers in parentheses refer to the line numbers in Table 1.9 in the following five paragraphs.

A cross between strains in which the mating type genes have been relocated to the *am* locus is sterile. Comparison of ectopic-*mt* x ectopic-*mt* crosses to ectopic-*mt* x normal crosses ((1) cf. (2), (4) cf. (5), (7) cf. (8)) shows that when *mt A* and *mt a* are in ectopic, but homologous, chromosomal positions, perithecial development and ascospore formation are defective.

Both of the parents in the ectopic-*mt* x ectopic-*mt* crosses, (1), (4) and (7), were *ad-2*. Crosses homozygous for *ad-2* may be sterile (R. L. Metzenberg, personal communication to N. L. Glass). Two *ad-2*$^+$; *A* progeny from mt-rel-Ac6 x OR8-1*a* were isolated and crossed as females to mt-rel-Da4 males. One of the crosses produced no perithecia and the other produced two small, barren perithecia.

Crosses of strains with larger mating type flank duplications are less fertile than those with smaller duplications. Comparison of crosses (1) to (4) and (2) to (5) showed that females with "RIP-invisible" duplications of idiomorph-flanking sequences are more fertile than females with "RIP-visible" duplications of flanking sequences.

Crosses of ectopic-*a* are less fertile than crosses of ectopic-*A*. Comparison of crosses (8) to (5) and of (9) to (6) showed a mating type-specific phenotype. The *a* ectopic-*mt* strain is female and male sterile, whereas the *A* ectopic-*mt* strain is female and male semi-sterile. Crosses (7) and (8) were repeated seven times each. In all cases, ectopic-*mt a* was completely sterile as a female. Perithecial contents were examined under 400x magnification. The perithecia were devoid of asci and ascogenous hyphae.

Reciprocal crosses of ectopic-*mt* strains are equally fertile. Comparison of the pairs of reciprocal crosses, (2) to (3), (5) to (6), (8) to (9), showed no sex-specific differences in fertility. Whether the ectopic-*mt* strain was male or female made no difference to the fertility of the cross.

In the *cis*-acting regulator model, the mechanism for the positional sensitivity of the mating type genes was proposed to be mating type gene expression. The levels of expression of *mt A-1*, *mt A-2* and *mt A-3* in an ectopic-*mt* strain (mt-rel-Ac6) compared to a normal strain (74-OR23-1*A*) were assessed with slot blots loaded with Poly(A)$^+$ RNA from three-day-old and six-day-old mycelia. The experiment was performed once. The slot blots were probed with a constitutively expressed gene, either *crp-1* or *β-tubulin* and with the mating type genes (see Materials and Methods). Upon visual inspection, the levels of expression of all three of the mating type genes, *mt A-1, mt A-2 and mt A-3*, in the ectopic-*mt* strain were very similar to wild type.

The four autoradiograms shown in Figure 1.4 were probed with the *mt A-1*-specific fragment (top panels) and the constitutively expressed control, *crp-1*, (bottom panels). The left-hand panels show three-day-old and the right-hand, six-day-old mycelial mRNA. Figure 1.5 shows the slot blots probed with the *mt A-3*-specific fragment (top panel) and the constitutively expressed control, *β-tubulin* (bottom panel). The slot blots probed with *mt A-2* are not shown because the background signal was high and interpretation was not possible from the scanned computer image (although interpretation was possible from the film).

In order to detect slight but statistically significant differences in expression levels, the slot blots probed with *mt A-1* and *mt A-3* were scanned by computer. The images were analyzed to determine the relative optical densities of the slots (Figure 1.6, see also Materials and Methods). The ratios of mt-rel-Ac6 mRNA to 74-OR23-1*A* mRNA from the control slot blots were averaged and compared to the analogous ratios from the experimental slot blots (Appendix B, Table B). The statistical tests (Appendix B, Table C) show, with two exceptions, no differences between the means.

Two data sets (first and third slots) suggest that the amount of *mt A-1* transcript in three-day-old mt-rel-Ac6 mycelia is not statistically different from wild type, whereas one data set (middle slots) suggests that *mt A-1* is underexpressed in mt-rel-Ac6 relative to 74-OR23-1A. The amount of *mt A-1* transcript in six-day-old mt-rel-Ac6 mycelia is not statistically different from wild type. One data set (first slots) shows that the amount of *mt A-3* transcript in six-day-old mt-rel-Ac6 mycelia is not statistically different than wild type, while the other data set (second slots) shows that *mt A-3* is overexpressed in mt-rel-Ac6.

To determine whether or not mating type genes could interfere with each other's sexual development, various types of strains were crossed and examined for ascospore and ascus number. In order to obtain the requisite strains for the experiments, the cross of mt-rel-Ac6 x *tol trp-4 a* was performed. Four types of progeny were generated from the cross of mt-rel-Ac6 x *tol trp-4 a*: those with no mating type, those with *mt a* at the mating type locus, those with *mt A* at the *am* locus and those with both *mt a* at the mating type locus and *mt A* at *am* (Figure 1.11). To observe the effects of adding the opposite mating type gene to a strain with a mating type

gene already present, the latter three progeny types, (1) *a*; (2) *am A** and (3) *am A*/a*, were crossed to *fl* mating type tester strains. The star symbol designates ectopic mating type genes. Perithecial contents were observed under the microscope. In general, the addition of the opposite mating type idiomorph to a haploid genome reduced the fertility of the cross (Table 1.10). The data shown in Table 1.10 are based on observations from crosses of 8 *a* strains, 6 *A*/a* strains and 7 *A** strains at least 12 days post-fertilization.

Table 1.10 Crosses of strains with and without the opposite mating type idiomorph.

Cross	Phenotype
a x fl A	normal # of asci/perithecium normal # of ascospores
A/a x fl A*	reduced # of asci/perithecium reduced # of ascospores
A x fl a*	reduced # of asci/perithecium reduced # of ascospores
A/a x fl a*	asci and ascospores absent from perithecia

Reduction in fertility of dual mating type strains compared to controls is seen by comparing the fertility of the *a* strains to the *A*/a* strains crossed as *a* (Table 1.10, lines 1 and 2) Figure 1.12 shows the perithecial contents of representative crosses. The cross in which one parent carried a heterozygous duplication of mating type, *fl A* x *A*/a*, had fewer ascospores than the control cross, *fl A* x *a*.

Similarly, fertility reduction is seen by comparing the fertility of the *A** strains to the *A*/a* strains crossed as *A* (Table 1.10, lines 3 and 4). Figure 1.13 shows the perithecial contents of representative crosses. The cross in which one parent carried an ectopic *A* and resident *a, fl a* x 46*A*/a* had fewer asci than the control cross, *fl a* x 8*A**.

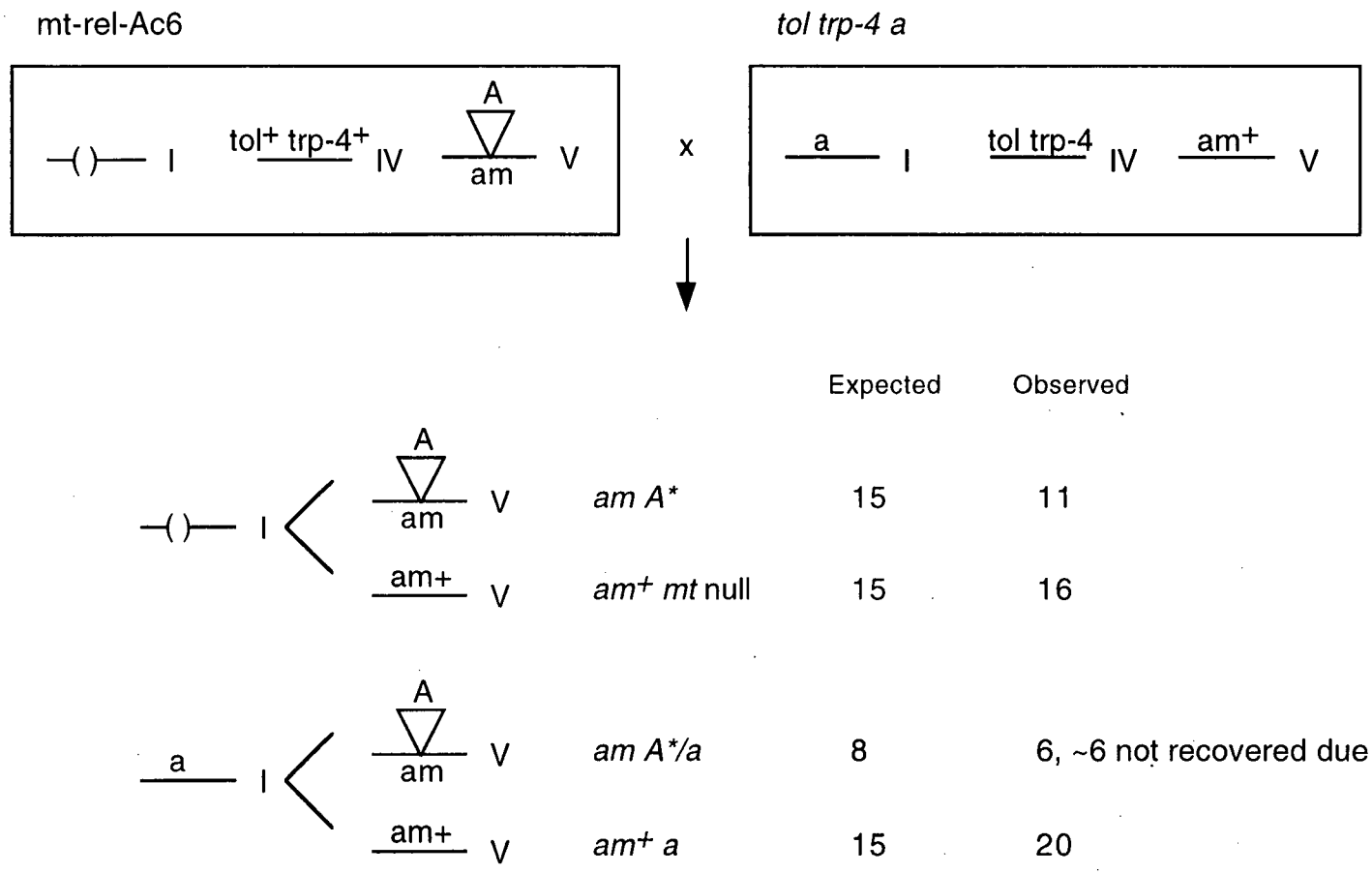


Figure 1.11 Generation of strains used to study interference. The cross of mt-rel-Ac6 x *tol trp-4 a* generated the strains used in the study of interference, namely, strains with ectopic *A*, strains with resident *a* and strains with both resident *a* and ectopic *A*. Approximately half of the *A*/a* progeny would have carried the *tol*$^{+}$ allele which does not suppress incompatibility between *A* and *a*. Strains with the genotype *tol*$^{+}$ *A/a* grow poorly or not at all.

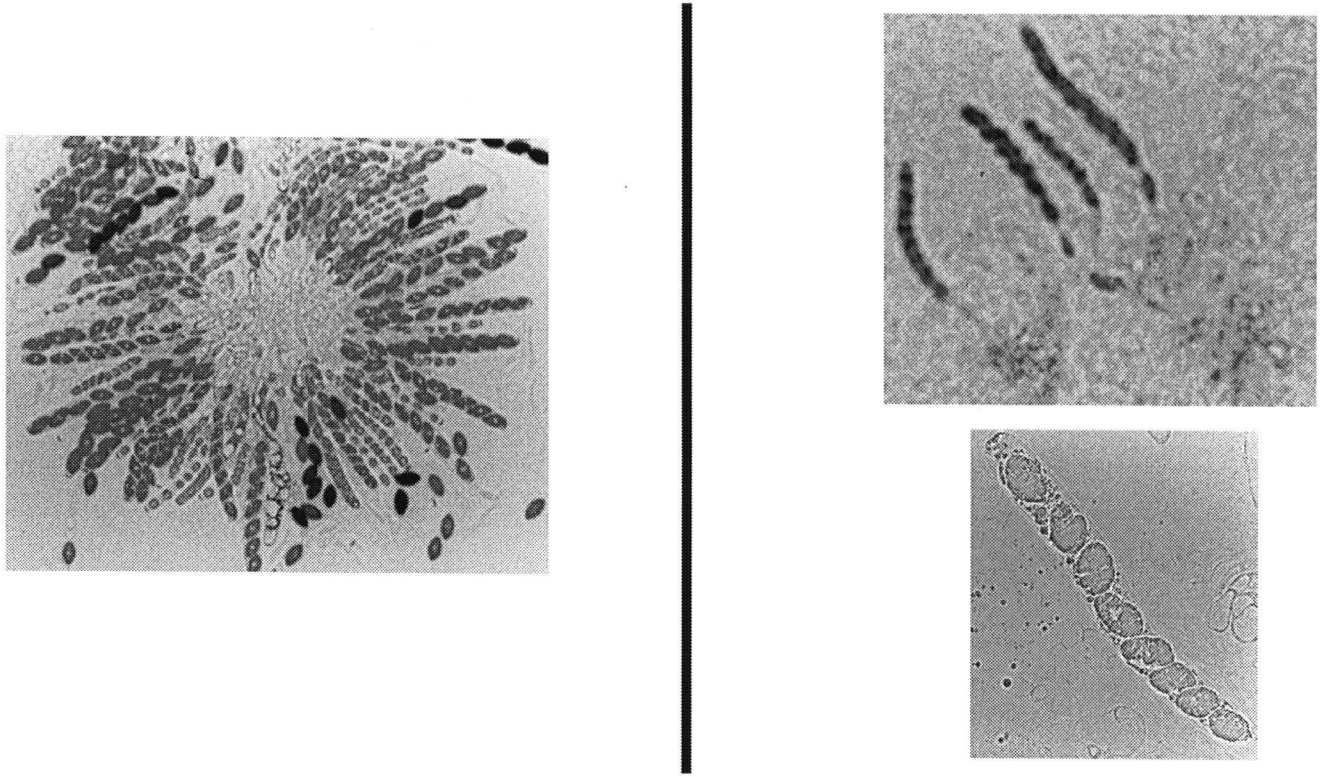
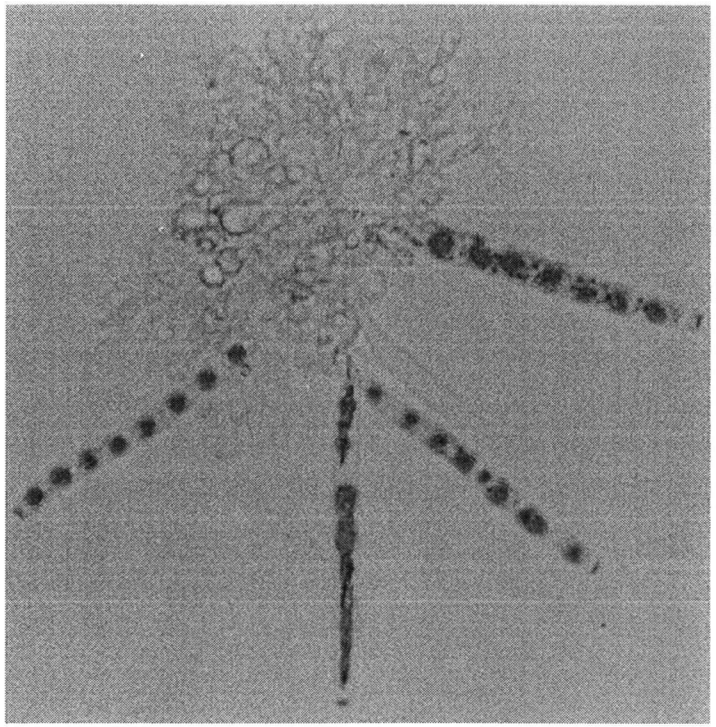
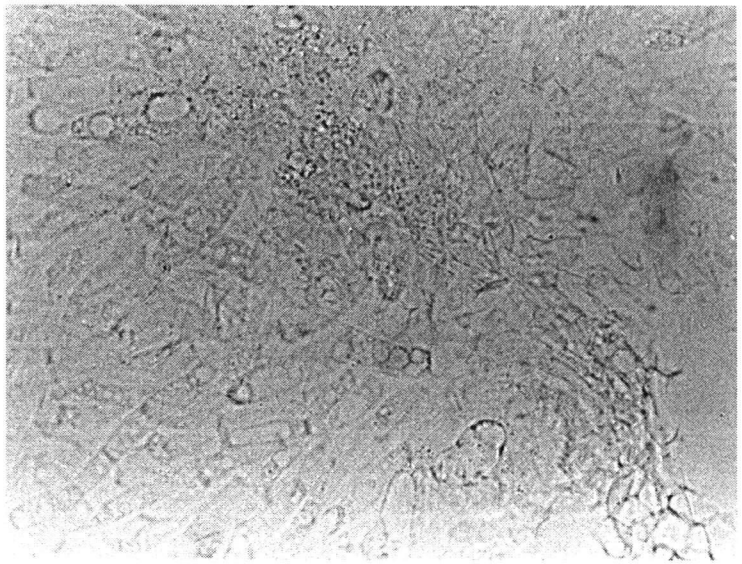


Figure 1.12 Perithecial contents from 11 days post-fertilization crosses. Rosette from wild type cross, *fl A* x 5*a*, is shown as observed under 100x magnification (left). "Rosette" (top right) and ascus (lower right) from cross of a strain containing resident *a* and ectopic *A*, *fl A* x 46*A***/a*, is shown as observed under 100x and 400x magnification, respectively.



Ectopic *A* cross, 11 days post-fertilization at 100x magnification.



Ectopic *A*/resident *a* crossed as *A*, 11 days post-fertilization at 100x magnification.

Figure 1.13 Perithecial contents from 11 days post-fertilization crosses of *fl a* x 8*A** (left) and *fl a* x 46*A**/*a* (right). The cross on the right in which one parent carried a heterozygous duplication for mating type had fewer asci than the control cross on the left.

The differences were statistically significant. Fertility was measured by obtaining data from observing perithecial contents of one cross of each of the four types described in Table 1.10. The fertility parameters were the number of ascospores per rosette eight and 11 days post-fertilization (Table 1.11 and Table 1.12) and the number of asci per rosette 13 days post-fertilization (Table 1.13).

Table 1.11 Number of ascospores per rosette 8 days post-fertilization.

Cross	# ros.	range asco./ros.	median	25th percentile	75th percentile
fl A x a	8	24-272	44	29	114
fl A x A/a*	34	0-4	0	0	0
*fl a x A**	18	0-16	0	0	0.5
fl a x A/a*	18	0	0	0	0

ros. = rosettes
asco. = ascospores

Table 1.12 Number of ascospores per rosette 11 days post-fertilization.

Cross	# ros.	range asco./ros.	median	25th percentile	75th percentile
fl A x a	7	80-328	176	92	212
fl A x A/a*	7	0-8	0	0	0
*fl a x A**	7	0-5	0	0	0
fl a x A/a*	3	0	0	0	0

ros. = rosettes
asco. = ascospores

Table 1.13 Number of asci per rosette.

Cross	# ros.	range asci/ros.	median	25th percentile	75th percentile
*fl a x A**	18	0-13	5.5	0.5	6.5
fl a x A/a*	21	0	0	0	0

ros. = rosettes

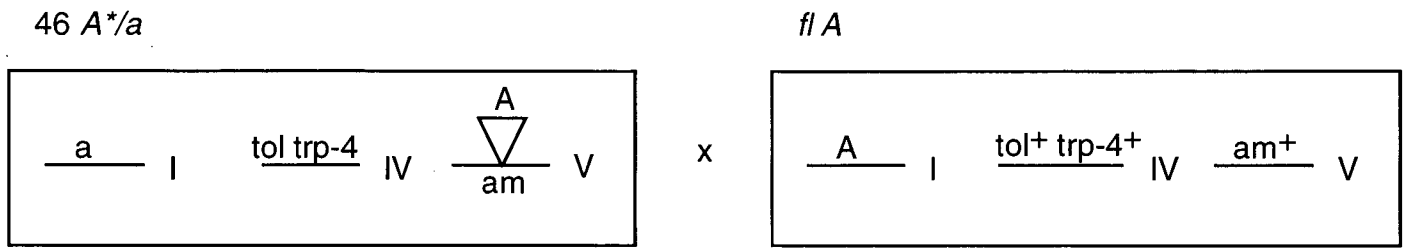
The reduction in the number of ascospores per rosette in crosses of *a* in the presence versus absence of an ectopic *mt A* idiomorph is statistically significant, as shown by Mann-Whitney Rank-Sum tests done on the raw data from eight days post-fertilization (Appendix C,

Table C). The reduction of the number of ascospores per rosette in crosses of *A* in the presence versus absence of a resident *mt a* idiomorph is not statistically significant as shown by Mann-Whitney Rank-Sum tests done on the raw data from eight days post-fertilization (Appendix C, Table D).

Although the number of ascospores per rosette is not significantly reduced, the difference in sexual development was evident in the morphology of the ascogenous tissue. In order to quantify the difference in the sexual development of *A** crosses compared to *A*/a* crosses, the number of asci per rosette (rather than ascospores per rosette) was counted in 13 days post-fertilization perithecia (Table 1.13). Asci were counted if they had pale brown granular filling, mutant- or normal-looking ascospores. The 21 "rosettes" from the cross of *fl a* x *A*/a* were mostly star-shaped masses of cells or masses of an undefined shape. The reduction in the number of asci per rosette is statistically significant as shown by Mann-Whitney Rank-Sum tests (Appendix C, Table E).

To see whether ectopic mating type genes could be interfering with meiosis, several crosses were examined for nondisjunction. Genetic analysis of the cross of mt-rel-Ac6 x OR8-1*a* described earlier revealed no evidence for meiotic nondisjunction, whereas analysis of 46*A*/a* x *fl A* produced possibly disomic progeny. In the cross of 46*A*/a* x *fl A* (Figure 1.14), if linkage group V segregated, then all *A* progeny with normal ascospore-per-rosette number should have been *am+*. All *A/A** progeny should have been *am-* . The *A/A** progeny were identified by reduced ascospore number per rosette (due to RIP or function of the ectopic mating type gene). An internal control was the lack of this phenotype in the *a* progeny.

While ten out of ten *A* progeny with normal ascospore number were *am+* (as expected) and four out of ten *A* progeny with reduced ascospore number were *am-* (as expected), six out of ten *A* progeny with reduced ascospore number were *am+* (unexpected). Additionally, one *A/a* progeny strain, which should have been *am-* had linkage group V segregated, was *am+*.



			Expected	Observed	
a / I / A / am / V		*am A*/a*	4	6	(~half not recovered due to *tol+*)
a / I / am+ / V		*am+ a*	8	9	
A / I / A / am / V		*am A*/A*	8	4	
A / I / am+ / V		*am+ A*	8	10	
unexpected class		*am+ A*/a*	0	1	
unexpected class		*am+ A*/A*	0	6	

Figure 1.14 Cross showing possibly disomic progeny. The cross of 46*A*/a* x *fl A* should have generated two types of progeny that mated as *A*, those with a resident *A*, which should be *am+* and those with two copies of *A*, one at the mating type locus and one at the *am* locus, which should be *am*. See text for a discussion of the unexpected classes.

Discussion

Evidence for a mating type gene position effect in *N. crassa* was accumulated, and several models for the mechanism of its action were tested. In a transformation of a *N. crassa* strains with *A* genes, all of the transformants with fully functional introduced mating type sequences (the MS strains) had those sequences integrated at the mating type locus, suggesting the existence of a position effect. The data supporting gene replacement are the following: most importantly, the MS progeny that mated as *A* are missing a *mt a*-specific restriction fragment, a situation which presumably arose via gene replacement, the MS progeny had only one mating type or the other and they segregated with a 1:1 ratio; the genetic markers that were linked to *mt a* in the *ad-3A nic-2; tol a* recipient strain became linked to the *A* mating type of the transforming cosmid in the MS strains; the MS progeny were all hygromycin sensitive, suggesting that the *A* mating type gene had been integrated without attendant vector sequences.

Since the ectopic strains may have contained copies of both idiomorphs, the presence of the endogenous *a* mating type idiomorph may have eliminated the production of ascospores in crosses as *A* strains, giving a false impression of a position effect. The *N. crassa* mating type genes are known to disrupt each other's vegetative growth, manifested in heterkaryon incompatibility. Mating type sexual interference occurs in other filamentous ascomycetes (Wirsel, Turgeon and Yoder, 1996). If sexual interference were occuring in these experiments, then replacement events would have been favoured over ectopic integration events, thus giving the deceptive appearance of a position effect.

Interference was shown to occur in *N. crassa*. Strains with dual mating identity were constructed and their the sexual development monitored. In order to eliminate vegetative incompatibility, which may have complicated the interpretation of the data, these strains were suppressed for mating type-controlled heterokaryon incompatibility. In a dual mating type strain, the presence of an ectopic *mt A* mating type gene affected the normal functioning of the resident *mt a* mating type gene, resulting in a statistically significant reduction in the number of

ascospores per rosette. In addition, the presence of a resident *mt a* mating type gene in a strain with an ectopic *mt A* idiomorph interferes with the functioning of the ectopic *mt A* mating type genes, resulting in statistically significantly fewer asci per rosette.

The following results verified the existence of a mating type gene position effect independent from interference. Strains were obtained in which the mating type gene (*A*) was introduced into an ectopic chromosomal position and deleted from the normal locus. Crosses made between these and wild type strains developed abnormally and sporulated less abundantly than wild type crosses. This is the first report of a phenotype associated with ectopic mating type genes in the absence of significant sequence duplication and therefore in the absence of RIP.

Two models regarding the cause of the position effect were proposed: the transvection model and the *cis*-acting regulator model. The transvection model proposes that the position-dependent functions of the mating type genes (ascogenous tissue development, meiosis, ascosporogenesis and possibly karyogamy) require close physical proximity of the mating type genes during the diploid phase of the life cycle. The *cis*-acting regulator hypothesis states that the positional regulation of the mating type genes is under the control of a distant *cis*-acting sequence.

The transvection hypothesis was tested and falsified, thus favouring the *cis*-acting regulator model. Crosses were made in which both parents contained a mating type idiomorph that had been relocated to the *am* locus (the two *ad-2*$^{+}$; *A* progeny from mt-rel-Ac6 x OR8-1*a* crossed to mt-rel-Da4). The sterility of these crosses failed to support the transvection hypothesis, with the following caveat. All of the strains used in these experiments were constructed using the same deletion strain as a host with the transforming genes being targeted to the same locus. Since one genetic background and one ectopic locus were used in these experiments, strain-specific or targetted locus-specific effects cannot be ruled out.

Accepting the *cis*-acting regulator until falsified, one can explain the results of the transformation experiments as follows. In the replacement strains of the transformation experiments, the *mt A* genes may have been united with a putative *cis*-acting control region (of

mt a) which was not present on the cosmid. If so, the *cis*-acting region of *mt a* must be sufficient for controlling *mt A* genes in the appropriate way. A second possibility is that the *cis*-acting control region of *mt A* was present on the cosmid, but was integrated only in the replacement strains. In the replacement strains, recombination within the homologous flanks of the resident *mt a* and the *mt A* on pG16/C10 could have allowed integration of the part of the mating type region needed for the production of abundant ascospores. In this model integration of such large fragments would not have occurred in the ectopic integrants. Some of the MNS strains have the 9.6 kbp band of the *mt A* idiomorph, which includes 5 kbp of centromere-proximal flank, so the necessary sequence would lie outside of this boundary or beyond the centromere-distal tip of the idiomorph.

Prior to this work, it was unknown that *N. crassa* mating type genes interfered with each other's functions. A reanalysis of data claiming support for the existence of a mating type position effect shows that the data could be explained by interference and other hypotheses (Table 1.14). Included in the table is the consideration of *mt A-2* and *mt A-3* whose existence was unknown during some of these studies. All of the transformants in Table 1.14 lack the ability to produce ascospores.

In other analyses of the *N. crassa* mating type gene position effect, ectopic integrations of the mating type gene were counter-selected (Chang and Staben, 1994). In this thesis, I examined both ectopic integrants and gene replacements and found a correlation between full *A* mating type function and integration at the mating type locus. The position effect phenotype was characterized in a cross (mt-rel-Ac6 x wild type *a*) lacking the complicating effects of interference and RIP.

A common basis is implied for interference and the position effect by the striking resemblance between their phenotypes. The phenotype of interference (*A*/a* crossed as *a*) resembles that of position effect (mt-rel-Ac6) and the $A^{II\ RIP}$ strain (genotype *mt A-2*^{m1} *mt A-3*^{m1}) (Glass and Lee, 1992). Based on data extrapolated across mating types, interference and the position effect are additive, not epistatic to one another, *i.e.* a strain exhibiting both of these phenomena has fewer asci than a strain with just position effect or a strain with just interference,

Table 1.14 Updated hypotheses for "position effects".

Host strain	Transforming DNA	Hyp.#	Ref.†
mt A-1 mt A-2 mutant *($A^{1\ RIP}$)*	fragment used to generate RIP mutant	pr	1
mt A-1 mt A-2 mutant *($A^{1\ RIP}$)*	*mt A* idiomorph with 1 kbp of flank on either side (pGMT8.1)	pr	2
mt A-1 mutant *(A^{m42}, A^{m44}, A^{m54}, A^{m56}, A^{m64})*	*mt A* idiomorph with a max. of 2 kbp of centromere proximal flank	pr	3
mt a-1 mutant *(a^{m1})*	*mt A* idiomorph with at most 2 kbp of centromere proximal flank (pSV:10A) (24 kbp plus *un-3* on centromere distal side)	pi*r	3, 4
tol a	*mt A* idiomorph	pir	5, 6
mt a-1 mutant *(a^{m33})*	*mt A* idiomorph	pi	5
mt A idiomorph deletion (RLM 41-10)	*mt A-1*	pm	2
mt A-1 mutant *(A^{m64})*	*mt A-1*	pr	3, 4
tol A	*mt a* idiomorph	pi	4, 7
tol A	*mt a-1*	pi	7
mt A-1 mutant *(A^{m44})*	*mt a-1*	pi*	2
mt A idiomorph deletion (RLM 41-10)	*mt a-1*	p	2

# Hypothesis. Ascospore deficiency could be due to:
p=position effect
i=interference (whether mediated via protein or transcripts)
i*=interference (if mediated via transcripts. The mutations are premature stop codons or frameshifts which alter the protein.)
r=RIP
m=missing sequences

† Reference
1 Glass and Lee, 1992
2 Vellani, unpublished data
3 Glass, Grotelueschen and Metzenberg, 1990
4 Glass *et al.*, 1988
5 Chang and Staben, 1994
6 this study
7 Staben and Yanofsky, 1990

suggesting that they may possibly be affecting the same process via a different biochemical pathway.

That opposite mating type genes can each affect the function of the other when they are in the same nucleus provides strong evidence that sexual development requires the mating type genes (or transcripts, gene products or mating-type specific target gene products, *etc.*) to be separate at some or all stages between fertilization and karyogamy. I speculate that the mating identity of nuclei may be affected in strains exhibiting interference. Nuclear identity may be required for one or more of the following three processes: the co-ordination of mitotic proliferation of nuclei in the ascogenous hyphae, co-ordination of the conjugate mitosis that precedes karyogamy and/or sequestration of one nucleus of each type into croziers.

I speculate that in strains exhibiting interference, the identity is reduced by the presence of the opposite mating type idiomorph; in strains exhibiting the position effect, the identity is reduced by the failure to maintain appropriate expression of the mating type genes after fertilization; in the $A^{II\ RIP}$ strain, the *mt A-2* and *mt A-3* genes are mutated (Saupe *et al.*, 1996).

The normal appearance of the cross that generated the $A^{I\ RIP}$ mutants which were mutated in *mt A-1* and possibly *mt A-2* (Glass and Lee, 1992; N. L. Glass, personal communication) favours the idea that identity is not required after RIP occurs. The one process of the three mentioned that precedes RIP, and therefore is most likely the one affected in the position effect and interference, is mitotic proliferation of nuclei in ascogenous hyphae.

This model implies that interference causes an effective reduction in mating type function. If the position effect shares a common cause with interference, in ectopic-*mt* strains, the mating type genes must be inappropriately down-regulated. I specify "down-regulated" rather than "repressed" because the genes are required for the formation of ascogenous hyphae and a small number are formed in mt-rel-Ac6 crosses.

To ascertain whether or not ectopic mating type strains show altered mating type gene expression, expression levels of *mt A-1*, *mt A-2* or *mt A-3*were determined. The autoradiograms of mRNA from mt-rel-Ac6 and 74-OR23-1A vegetative cells show that in mt-rel-Ac6 all three genes, *mt A-1*, *mt A-2* and *mt A-3*, are transcribed. The expression levels of all three genes

appear to be equal in the wild type compared to the ectopic-*mt* strain. The statistical analysis of the autoradiograms bears out this observation in the majority of cases. Two samples suggest that there may be slight differences in the amount of expression of the mating type genes in mt-rel-Ac6 compared to wild type. These results suggest that the reduction in fertility of ectopic mating type strains is not due to large alterations in the expression in vegetative tissue.

The similarity of expression levels in vegetative cells is consistent with the heterokaryon incompatibility phenotype of ectopic mating type strains, assayed by transformation efficiency into same and opposite mating type hosts (Glass *et al.*, 1988). Since ectopic mating type strains can mate, one might expect the expression levels of the mating type genes to be close to normal before fertilization. The phenotype of ectopic mating type strains appears after fertilization.

While the level of expression of the *mt A* genes is not greatly different in mt-rel-Ac6 compared to wild type in vegetative tissue; hypothetically, ascogenous tissue may be sensitive to changes of this magnitude or perhaps expression differences may be increased in ascogenous hyphae. The mating type genes encode putative transcription factors (Glass, Grotelueschen and Metzenberg, 1990; Staben and Yanofsky, 1990). Northern blot analysis of mating type mutant strains suggests that the mating type genes activate and repress some sexual development genes (Ferreira *et al.*, 1998; Ferreira, 1997). The ability of the mating type genes to regulate these genes that are expressed early in sexual development opens the possibility that they may regulate the expression of genes required for ascogenous hyphae development or karyogamy. It may, however be difficult to detect a reduction in expression levels in ascogenous tissue using mRNA extracted from perithecia, given that the perithecial wall presumably would still be expressing the genes. Ascogenous tissue could be extruded from the perithecium, but the amount of material would be too small for RNA blot analysis. It may be possible to perform reverse transcriptase PCR on the ascogenous cells, however, the expression modifications could occur during the entire sexual cycle or during a particular stage of the sexual cycle, in all cell types or with cell-type specificity.

When the *ad-3A nic-2; tol a* recipient strain was transformed with the pG16/C10 cosmid which contained the *A* mating type idiomorph, the percentage of hygromycin-resistant

transformants able to mate as *A* (94/734=13%) was small compared to another study, the transformation of a cosmid containing the *mt A* idiomorph, pSV:10A, into a mating type compatible mutant, a^{m1} (Glass *et al.*, 1988) and to *N. crassa* transformations in general. The use of a mating type mutant as the recipient, rather than a strain with *tol*, as was used here may be significant. Although Glass *et al.* (1988) reported restoration of transformation efficiency of *a* fragments into *A* recipients when the recipient had *tol*, some evidence exists that *tol* may not always suppress incompatibility completely, depending on a strain's genetic background (Vellani, 1991).

An additional explanation for the low transformation efficiency is quelling. Quelling refers to the spontaneously reversible mutant phenotype produced by an excess of wild type genes (Romano and Macino, 1992). Silencing of endogenous and ectopic genes is methylation-independent and correlates with the presence of sense RNA from the ectopic gene or gene fragment (Cogoni *et al.*, 1996). The frequency of quelling is highly variable between genes and could also be variable between fragments of the same gene or between host strains (Romano and Macino, 1992). If multiple copies of *mt A* had integrated into the transformants, as ectopically integrating transforming DNA often does (Miao, Rountree and Selker, 1995), quelling could have eliminated mating reactions and thus reduced the apparent transformation efficiency.

The fertility of the MS strains was variable. This phenotype did not segregate with the integrated *mt A* mating type, suggesting that it was not produced by the specific sequences or position of the integrated *mt A* genes. The reason for the variability is unknown. The hygromycin resistance and the mating phenotypes of the MS strains requires explanation. If the MS strains underwent exact replacements of the *mt a* idiomorph with the *mt A* idiomorph, then they should have been hygromycin sensitive and unable to mate and produce ascospores as *a*. The ability to mate and sporulate as *a* is easily explained because *N. crassa* spheroplasts are multinucleate and so give rise to colonies heterokaryotic for transformed and untransformed nuclei in a transformation experiment. The ability to mate as *a* was probably provided by untransformed nuclei. The MS strains were selected by virtue of their hygromycin resistance. The possibility of *mt A* and *hph* having integrated at the mating type locus in the transformed

nuclei can be discounted because all of the MS progeny were hygromycin sensitive. Also subcultures of MS-3 and MS-4 were hygromycin sensitive.

Although this hypothesis was not tested, one possible explanation accounts for the following observations: the initial hygromycin resistance of the MS strains, the ability of the MS strains to mate and sporulate as *A* and *a*, the variable amounts of ascospores produced by the MS strains when crossed as *A*, the loss of hygromycin resistance in some MS subcultures and the hygromycin sensitivity of the MS progeny. Most likely, the MS strains were heterokaryons with three nuclear types: untransformed nuclei (conferring fertility as *a*), transformed nuclei with ectopic insertions (providing hygromycin resistance through the phosphotransferase activity) and transformed nuclei with homologous integrations (providing fertility as *A*). Random sampling of nuclei during subculturing or shifts in nuclear ratios could explain the appearance of hygromycin sensitive subcultures in some of the MS strains and the hygromycin resistant subcultures in others. The observation that all of the MS progeny were sensitive can be explained by proposing that only nuclei in which *mt A* had replaced *mt a* were able to undergo karyogamy and give rise to progeny. The presence of many nuclear types, only one of which could produce ascospores could explain why the ascospore number was lower than wild type for most of the MS strains crossed as *A*.

Much variability was observed in the mating and sporulation phenotypes of the MNS strains. The causes are unknown. Most of the MNS strains were fully fertile as *a*, likely due to the presence of untransformed nuclei and/or cells in the colony (Table 1.7). One strain (MNS-4), however, failed to sporulate as a male or female, one strain that was tested only as a female (MNS-7) mated, but failed to sporulate and another strain that was tested only as a female (MNS-11) failed even to mate. The cause of the infertility of these strains is unknown. The possibility of rearrangements having occurred is supported by the appearance of hyaline ascospores (indicating aneuploid progeny) in two of the 11 fertile crosses of the MNS strains as females (MNS-1 and MNS-14).

Southern blotting of the MNS strains revealed two classes of integrants. The strains in the first class, MNS-4, -6 and -7, contained the 9.6 kbp *Bam*HI fragment in its entirety. The

cause of the lack of sporulation in this class is not known, but is not likely due to missing sequences since the 9.6 kbp fragment contained all of the genes except the 3' portion of *mt A-3*.

The strains in the second class, MNS-1, -2, -3, -5 and -8, contained portions of the 9.6 kbp *Bam*HI fragment of the pG16/C10 cosmid, but not the whole fragment. The cause of the lack of sporulation in this class is unknown, but could be attributed to the absence of *mt A* sequences required for a normal amount of sporulation (the 3' end of *mt A-1* and/or *mt A-2* and *mt A-3*).

Which of the mating type genes is being affected by chromosomal misplacement in this experiment is unknown. The phenotype of the cross mt-rel-Ac6 x OR8-1*a* is similar to that of $A^{II\ RIP}$ (genotype *mt A-2*^{m1} *mt A-3*^{m1}) x wild type inasmuch as ascus number, but not perithecial number is reduced (Glass and Lee, 1992). The similarity suggests that perhaps *mt A-2* and *mt A-3* are position sensitive. The *mt A-1* gene displays a position effect, as shown by the non-sporulating transformations of A^{m} mutants in which *mt A-2* and *mt A-3* are still at the mating type locus (Glass *et al.*, 1988; Glass, Grotelueschen and Metzenberg, 1990).

Since one of the ectopic-*A* strains, mt-rel-Ac6, had "RIP-invisible" duplications of flanking sequences, the size of the mating type region that must remain intact for sporulation could be estimated. A 5656 bp fragment from the *A* mating type region is sufficient for mating identity and a reduced level of sporulation when located ectopically, suggesting that sequences outside of these boundaries are required for full fertility. The fragment contains all 3 of the *mt A* open reading frames. In addition, it contains 600 bp 3' of *mt A-3* (300 bp between the *mt A-3* stop codon and the centromere-distal end of the idiomorph plus 300 bp of centromere-distal flank) and 220 bp 3' of *mt A-1* (160 bp between the *mt A-1* stop codon and the centromere-proximal end of the idiomorph plus 60 bp of proximal flank).

This fragment likely includes all of the local regulatory elements of *mt A-2*, since that gene is sandwiched between the other two, and the 5' regulatory sequences of *mt A-1* and *mt A-3*, since these genes are transcribed divergently toward the flanks. Position dependence on 3' local regulatory elements of *mt A-1* and/or *mt A-3* is a formal possibility, however it is not

likely because flanking sequences extended at least 200 bp beyond the stop codons at either end of the fragment.

Another transformation experiment suggested that a fragment with extensive flanking sequences is still position dependent. The fragment of *mt A* included up to 2 kbp of sequences flanking the idiomorph on the centromere proximal side and more than 20 kbp on the centromere distal side. A cosmid containing this fragment was used to transform a strain in which *mt A-1* and *mt A-2* were both mutated ($A^{1\ RIP}$ strain of Glass and Lee (1990)). Transformants failed to sporulate when crossed as *A* (Glass, Grotelueschen and Metzenberg, 1990). The lack of sporulation in these transformants could have been due to a position effect or to RIP. Unfortunately, the length of DNA that integrated into the genome of the transformants was not determined.

If the fertility reduction of mt-rel-Aflk relative to mt-rel-Ac6 is not due to RIP of genes residing in the duplicated flanks that are required for ascosporogenesis, then 2 kbp either side of the idiomorph appears to be insufficient to confer position-independent function. The translocation strain, *T(I->II) 39311*, is fully fertile (Appendix C, Table B). In this strain, the translocated fragment includes 35 map units (700-3500 kbp) of sequence centromere-distal to mating type and 18 map units (360-1800 kbp) of sequence centromere-proximal to mating type. To summarize, the smallest possible position-independent fragment identified so far is 700 kbp centromere-distal and 360 kbp centromere-proximal to the mating type locus.

The cross of mt-rel-Ac6 x OR8-1*a* yielded seven *am+ A* progeny which mated, but produced very few tiny perithecia devoid of ascogenous hyphae. Large barren perithecia are sometimes seen in *a* x *a*, a^m x *a* and a^m x *A* crosses, but not in *A* x *a*, A^m x *a* or A^m x *A* crosses (Griffiths and DeLange, 1978; Griffiths, 1982). Apparently, the mating type region may impact on perithecial development.

The origin of the tiny perithecia seen here is unknown, but could be explained by the following hypothesis. If the seven strains were disomic for linkage group V, RIP may have occurred in the duplicated region. If the RIP spread into the *A* idiomorph and mutated a

sequence required for the suppression of perithecial development, then perithecia may have started to develop.

Phenotypes of dual mating type strains in other fungi vary. Like *N. crassa*, some *M. grisea* dual maters (*Mat1-1* transformed with *Mat1-2* and vice versa) make fewer perithecia and the number of perithecia can differ widely, even among dual maters derived from the same strain (Kang, Chumley and Valent, 1994). Like *N. crassa*, one *M. grisea* dual mater produced perithecia in patches. Unlike *N. crassa*, the perithecia that do develop make normal numbers of ascospores. The ascospores are either one or the other mating type, suggesting that the dual-mater has to break down into a heterokaryon before completion of the sexual cycle.

Unlike the bidirectional interference seen in *N. crassa*, the interference in *C. heterostrophus* is unidirectional. The presence of a resident mating type gene interferes with the function of the opposite mating type ectopic gene, resulting in the production of pseudothecia, without ascospores; however an ectopic mating type gene has no effect on the function of the resident gene (Wirsel, Turgeon and Yoder, 1996). Unlike *N. crassa*, self-mating reactions of *C. heterostrophus* dual maters have a normal number of pseudothecia (Turgeon *et al.*, 1993); *N. crassa* has fewer. In contrast to *N. crassa*, *C. heterostrophus* dual mater self-matings are fertile (ascospores are produced), but like *N. crassa*, they have a reduced number of asci. The lack of position effect in *C. heterostrophus* can handily explain this difference.

Similar to *N. crassa* dual maters, *P. anserina mat+mat-** transformants produce sterile perithecia when self-mated (Picard, Debuchy and Coppin, 1991). Unlike any *N. crassa* dual mater, however, one *P. anserina mat+*mat-* transformant produced self-fertile perithecia (Picard, Debuchy and Coppin, 1991). A curious observation, possibly relevant to differences between *mt A-2/mt A-3* and *SMR1/SMR2*, was that some *mat+mat-** transformants using a cosmid that encoded all 3 genes of *mat-* produced a few fully fertile perithecia (Picard, Debuchy and Coppin, 1991).

Unlike in *N. crassa*, in *S. cerevisiae*, heterozygosity of the mating type locus leads to the inability to mate (Haber and George, 1979; Klar, Fogel and MacLeod, 1979).

An unexpected class of progeny was culled from the cross of 46*A*/a* x *fl A* (Figure 1.14). These seven progeny apparently received am^+ from the *fl A* parent and *A* (at the *am* locus) from the dual mating type parent. If nondisjunction were occurring generally, mating type nulls would have appeared in the progeny due to the nondisjunction of linkage group I, provided that such progeny were viable. They did not.

One possible explanation is that in some meioses, *mt A* on linkage group V paired with and segregated from *mt a* on linkage group I, leaving the linkage group V with *am*+ to segregate randomly, rather than away from its homolog. This hypothetical pairing may have been mediated by flanking sequences.

Chapter 2

A Search for Mating Type Gene Activity in a Homothallic *Neurospora* Species

Introduction

The life cycles of heterothallic (obligately outcrossing) and homothallic (self-fertile) *Neurospora* species include a vegetative and a sexual stage. A mycelium can be propagated vegetatively in heterothallic species from a germinated macroconidium or hyphal fragment. Only the latter option is available to homothallic species because they lack conidia (Perkins and Turner, 1988). When nutrients are limited, the sexual cycle initiates in heterothallic species, beginning with the production of protoperithecia from which protrude the trichogynes, the receptive structures. An opposite mating type gamete fuses with the trichogyne, initiating the development of the protoperithecium into ascospore-bearing perithecium. Development from this point, including ascus formation, meiosis and sporulation, is identical in eight-spored heterothallic and homothallic species (Raju, 1978; 1980). In homothallic species, sexual activity occurs via self-reproduction, and so mating type may be irrelevant. It is not known how the sexual cycle initiates in the homothallic species.

Heterothallic *Neurospora* species (*N. crassa*, *N. sitophila*, *N. intermedia* and *N. discreta*) consist of a population of individuals, each having one of two mating types, *A* or *a*. Pseudohomothallic *Neurospora* species (*N. tetrasperma*) are able to self-reproduce by producing ascospores containing nuclei of both mating types. Sexual reproduction in *N. tetrasperma* in nature likely occurs by self-reproduction most often since *A* + *a* protoperithecia lack active trichogynes (Bistis, 1996). Outcrossing could occur since the single mating type progeny that are occasionally produced do have active trichogynes (Bistis, 1996).

Truly homothallic species are those in which two identical nuclei undergo karyogamy. That homothallic species rarely or never outbreed is supported by the following biological and

molecular data. The homothallic *Neurospora* species lack conidia and trichogynes (Nauta and Hoekstra, 1992b) and there is less RFLP variation within homothallic species compared to different isolates of a single heterothallic species (Glass, Metzenberg and Raju, 1990). Even though mating type identity may not be required in these species, they do contain mating type sequences, as detected by Southern blotting (Glass *et al.*, 1988). The homothallic species are divisible into two groups based on their mating type genes (Figure 2.1). In one group (*N. pannonica* and *N. terricola*), each nucleus contains sequences homologous to *N. crassa mt A* and *N. crassa mt a* (T. A. Randall and R. L. Metzenberg, personal communication; Glass *et al.*, 1988; L. Wheeler and N. L. Glass, personal communication). In the other group (*N. africana*, *N. galapagosensis*, *N. lineolata* and *N. dodgei*), only sequences homologous to *N. crassa mt A* have been detected (Glass *et al.*, 1988). The *mt A-1* homolog in *N. africana* has 91% DNA sequence identity with *N. crassa mt A-1*. When used to transform a *N. crassa* mutant, the ectopically located *N. africana mt A-1* gene induced perithecia, but not ascospore-formation (Glass and Smith, 1994). The mating type gene of *N. africana* was unable to induce homothallism in *N. crassa* recipient strains (Glass and Smith, 1994).

In unmated cells of *S. cerevisiae* and *S. pombe*, the mating type proteins regulate mating via the pheromone response pathway (Dolan and Fields, 1991; Egel, Nielsen and Weilguny, 1990). In mated cells, they regulate entry into meiosis (Covitz, Herskowitz and Mitchell, 1991; Egel, Nielsen and Weilguny, 1990). By analogy, the *N. crassa* mating type genes may also regulate entry into meiosis. If so, *N. terricola* and *N. pannonica* may be homothallic because the presence of both mating type genes allows the initiation of the sexual cycle without mating. Perhaps *A*-only homothallic *Neurospora* species have genes that allow them to bypass the mating/pheromone response pathway altogether and begin ascogonial and ascogenous hyphae development directly without the need for *mt a*.

In this chapter, *N. terricola* was selected as a model organism for the study of homothallism since, like *N. terricola*, most homothallic species in the Sordariaceae have both *mt A* and *mt a* sequences (Glass, Metzenberg and Raju, 1990; Pöggeler *et al.*, 1997a). Each nucleus of *N. terricola* has sequences homologous (as shown by Southern hybridization) to *N. crassa mt*

Heterothallic species

A *a*

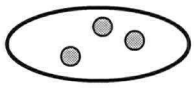
N. crassa
N. sitophila
N. intermedia
N. discreta

Pseudohomothallic species

A+a

N. tetrasperma

Homothallic species (two types)



A/a

N. terricola
N. pannonica

A

N. dodgei
N. lineolata
N. galapagosensis
N. africana

Figure 2.1 Mating types of *Neurospora* species. Oval represents ascospore; circle represents nucleus.

a-1, *mt A-1*, *mt A-2*, but not *mt A-3* (Beatty, Smith and Glass, 1994). In contrast, in *N. pannonica*, all four mating type genes are present, and appear to be linked (L. Wheeler and N. L. Glass, personal communication). The *N. terricola mt A-1* gene has been cloned and partially sequenced (Beatty, 1993). It has a high level of DNA similarity to the *N. crassa mt A-1* gene, and it has been tested for mating type function in *N. crassa* because no transformation protocol has been developed for *N. terricola*. The *N. terricola mt A-1* gene, when used to transform a *N. crassa* mating type mutant, confers mating ability, but not sporulation ability, to the *N. crassa* mutant. These results are comparable to the results of transforming a *N. crassa* mating type mutant with the *N. crassa mt A-1* gene. Transformation usually results in ectopic integration.

As in most other Sordariaceae, the *mt A* and *mt a* regions in *N. terricola* are physically linked. One report states that the opposite mating type probes both bind to a 3.4 kbp restriction fragment (Glass, Metzenberg and Raju, 1990), while another claims that the genes are separated by 12-20 kbp of DNA, as shown by OFAGE gel analysis (M. Smith, personal communication; Beatty, Smith and Glass, 1994).

Is the activation of sexual reproduction in the homothallic species dependent upon the mating type genes? Only mutagenesis could say for certain. It is difficult to study the homothallic species because of a lack of genetic and molecular tools, namely, genetic markers, the ability to construct strains by outcrossing and inefficient transformation protocols that call for the spheroplasting of hyphae.

What is the relationship between the *cis*-acting regulator(s) and the mating type genes? How can the genes function without both being at the mating type locus? Is 3.4-20 kbp close enough for both *mt A* and *mt a* genes to be controlled by the same *cis*-acting regulator or does each have its own? Does *N. terricola* even have a *cis*-acting regulator? Is there interference between *mt A* and *mt a* when they occupy the same nucleus prior to karyogamy, as in *N. crassa*? If so, how does *N. terricola* deal with it?

None of these questions is relevant unless the mating type genes are functional. It is conceivable that *N. terricola* does not need mating identity since all of its nuclei are identical. During compartmentalization of two nuclei into the penultimate cell of the crozier for

karyogamy, *N. terricola* needs to enclose precisely two nuclei, but their identities may be irrelevant. Moreover, homothallic *Neurospora* species do not mate, *per se*, as they do not have trichogynes, conidia or microconidia (Raju, 1978).

The molecular structure and function of the *N. terricola* mating type genes are elucidated in this chapter. First, the DNA and amino acid sequences are compared to those of *N. crassa*. The cloned *N. terricola* mating type genes are then assayed for the functions of the *N. crassa* mating type genes (mating identity and vegetative incompatibility). The effect of a suppressor of the mating type associated vegetative incompatibility is tested on one *N. terricola* gene. Finally, expression of the mating type genes is examined in *N. terricola*.

Materials and Methods

Strains

Strains are shown in Table 2.1.

Table 2.1 Strains used in Chapter 2.

Strain name	Genotype	Source
	arg-2 mtA1.7 (RIP)	N. L. Glass
RLM 41-10	*thi-4 ad-2; lys-1 ΔA*	R. L. Metzenberg
153	*un-3 ad-3A nic-2 cyh-1 A*	A. J. F. Griffiths
7	*ad-3B a*	A. J. F. Griffiths
I-20-41	*arg-1 ad-3B; tol A*	A. J. F. Griffiths
I-20-26	*arg-1 ad-3B A*	A. J. F. Griffiths

Plasmids

Plasmids are shown in Table 2.2.

Table 2.2 Plasmids used in Chapter 2.

Plasmid	Description
pNTa1	*N. terricola mt a-1* in pCR™
pONTa	*N. terricola mt a-1* in pOKE103 *(pan-2; qa-2)*
pONTA	*N. terricola mt A-1* in pOKE103 *(pan-2; qa-2)*
pßC1	*ß-tubulin* in pAT153 (pBR322 derivative)
pGMT8.1	*N. crassa mt A idiomorph* in pGEM™
pCSN4	*N. crassa mt a-1* in SK+ (pBluescript™)
pBSqa-2mt1.7	*N. crassa mt A-1* in pBSqa-2
pMP6	*hph* in pUC18

Plasmid Preparation

Plasmids were prepared by either centrifugation through CsCl (Sambrook, Fritsch and Maniatis, 1989) or with a plasmid kit (Qiagen, Chatsworth, California).

Primers

Primer names, map locations and sequences are shown in Table 2.3. Numbers for the first 11 primers refer to the *mt A* idiomorph sequence (GenBank accession no. M33876). Numbers for the next 12 primers refer to the *mt a* idiomorph sequence (GenBank accession no. M34287). Numbers for the last 2 primers refer to the *ß-tubulin* sequence (GenBank accession no. M13630).

Table 2.3 Primers used to amplify genes in Chapter 2.

Primer name	Map location	Sequence (5'-3')
mt A		
1778	3997-4021	ACCCAAACTTCCCACC
3194	5040-5062	ATGGTACCTCATCTTCCACTAACCC
mint	5000-5020	ATAGCCAGAGCCATGTTGT
orchid	3718-3733	AAGTAGCATTGTTTGT
rI.1	2326-2342	GGTTTCCTTTTCGTCAG
rI.2	3547-3563	TGTAGTCAACGGGGATC
1875	4452-4468	TGTATTCGTCAATCCGG
A2-3200	3182-3199	GCTCGCTGCTGACTTCGTCC
chacha	3301-3317	CGCCAGCACCGACATCC
cactus	2602-2619	GCATAGCAAGAGCTTGGC
condo	4180-4202	CGGCAATGAGAGCTTTCT
mt a		
Y661	3590-3570	GCATGGATGCCCTTGGG
Y663	3535-3550	GCCCGACAGTGTCGTCG
Y694	4615-4590	GAGGTGATATCCTTGGTGACCGGG
Y759	2950-2970	GAATGCTATTCAGGGCCG
Y760	3810-3830	CAGCCGCACATTCGCGAG
Y761	4100-4120	GAAAACTTCGCGCCGCCG
Y762	4370-4390	CCAGGAGGCTAACGAGGC
Y763	4300-4280	GGGTTGGGATTGGGGGGA
Y764	4100-4080	CTAGATCCTGCGACGGGA
Y765	3820-3800	GTGCGGCTGCTCATCACG
Y766	3290-3270	GATATGCGCGACCAAGCC
Y767	3030-3010	GGGGCAACGACGTCGGGA
ß-tubulin		
BT1a	1656-1679	TTCCCCCGTCTCCACTTCTTCATG
BT1b	2168-2192	GACGAGATCGTTCATGTTGAACTC

PCR

The mating type genes of *N. terricola* were amplified from genomic DNA using *N. crassa* primers. The genes were also amplified from *N. crassa* genomic DNA as controls. PCR was

performed on a DNA Thermal Cycler 480 (Perkin Elmer, Foster City, California). Primers for *mt A-1* (1778, 3194) amplified the ORF plus 120 base pairs 5', for *mt a-1* (Y694, Y759), the ORF plus 777 base pairs 5', for *mt A-2* (rI.1, rI.2), the ORF plus 24 base pairs 5'. Primers Y694 and Y759 were gifts from C. Staben (University of Kentucky). Amplification of *mt a-1* was confirmed with a Southern blot probed with a chemiluminescence-labelled *mt a*-specific probe (pCSN4).

Cycling for *mt A-1* was as follows: 97 °C for 5', followed by 30 cycles of 95 °C for 30"/56 °C for 30"/72 °C for 1', followed by 72 °C for 5'.

Cycling for *mt A-2* was as follows: 93 °C for 3', 72 °C for 3', followed by 25 cycles of 94 °C for 1.5'/52 °C for 30"/72 °C for 1', followed by 72 °C for 5'.

Cycling for *mt a-1* was as follows: 95 °C for 2', 55 °C for 1', 72 °C for 1', followed by 32 cycles of 94 °C for 1'/60 °C for 1'/72 °C for 1' (with an automatic 10 seconds/cycle extension).

Cloning

The PCR products were purified from low-melt gel slices with the PCR Magicprep™ (Promega, Madison, Wisconsin). Gel-purified fragments or PCR products direct from the PCR were ligated into the cloning vector, pCR™ (Invitrogen, San Diego, California). The plasmids containing *N. terricola mt A-1*, *mt a-1* and *mt A-2* were named pNTA1, pNTa1 and pNTA2, respectively. *E. coli* was transformed with the plasmids for maintenance. In the case of *mt a-1*, the presence of the 1.6 kbp mating type fragment was confirmed by probing a Southern blot of digested pNTa1 with a chemiluminescence-labelled *mt a*-specific probe. DNA was prepared for sequencing with a plasmid kit (Qiagen, Chatsworth, California) or a CsCl gradient (Sambrook, Fritsch and Maniatis, 1989).

Sequencing

A PCR product was amplified from the *mt A-1* gene for sequencing with the primers 1875 and mint, and from the *mt a-1* gene with the T7 promoter primer, Sp6 promoter primer (synthesized by the NAPS unit at the University of British Columbia.), Y661, Y760-Y767 (gifts from C. Staben), and from the *mt A-2* gene with A2-3200 primer, T7 promoter primer, Sp6 promoter primer, chacha primer and cactus primer. Sequencing was performed using the ABI Taq DyeDeoxi Terminator cycle method (Mississauga, Ont.) on an ABI 373 automated sequencer at the NAPS Unit, Biotechnology Lab., University of British Columbia.

Sequence Comparison

Sequence comparisons were done with the programs of the Wisconsin Genetics Computer Group (Devereux, Haberli and Smithies, 1984).

Transformation

N. crassa spheroplasts were prepared and transformed according to Schweizer *et al.* (1981), using the modification of Akins and Lambowitz (1985).

RT-PCR

RNA was isolated from frozen tissue according to Logemann *et al.* (1987). Poly(A)$^+$ RNA was isolated from total RNA using an Oligotex™ mRNA kit (Qiagen, Chatsworth, California). RNA was reverse transcribed with a First-Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR was done on 100ng of genomic DNA and cDNA of *N. crassa* and *N. terricola* using *ß-tubulin* primers (BT1a and BT1b) and mating type primers, Y663 and Y763 for *mt a-1*, condo and mint for *mt A-1* and chacha and orchid for *mt A-2*. Cycling for *mt a-1* was as follows: 95 °C for 5', followed by 30 cycles of 94 °C for 30"/54 °C for 20"/72 °C for 1', followed by an incubation at 72 °C for 5'. Cycling for *mt A-1* was as follows: 95 °C for 5', followed by 30

cycles of 94 °C for 30"/53 °C for 20"/72 °C for 1', followed by an incubation at 72 °C for 5'. Cycling for *mt A-2* was as follows: 95 °C for 5', followed by 30 cycles of 94 °C for 30"/45 °C for 20"/72 °C for 30", followed by an incubation at 72 °C for 5'.

Results

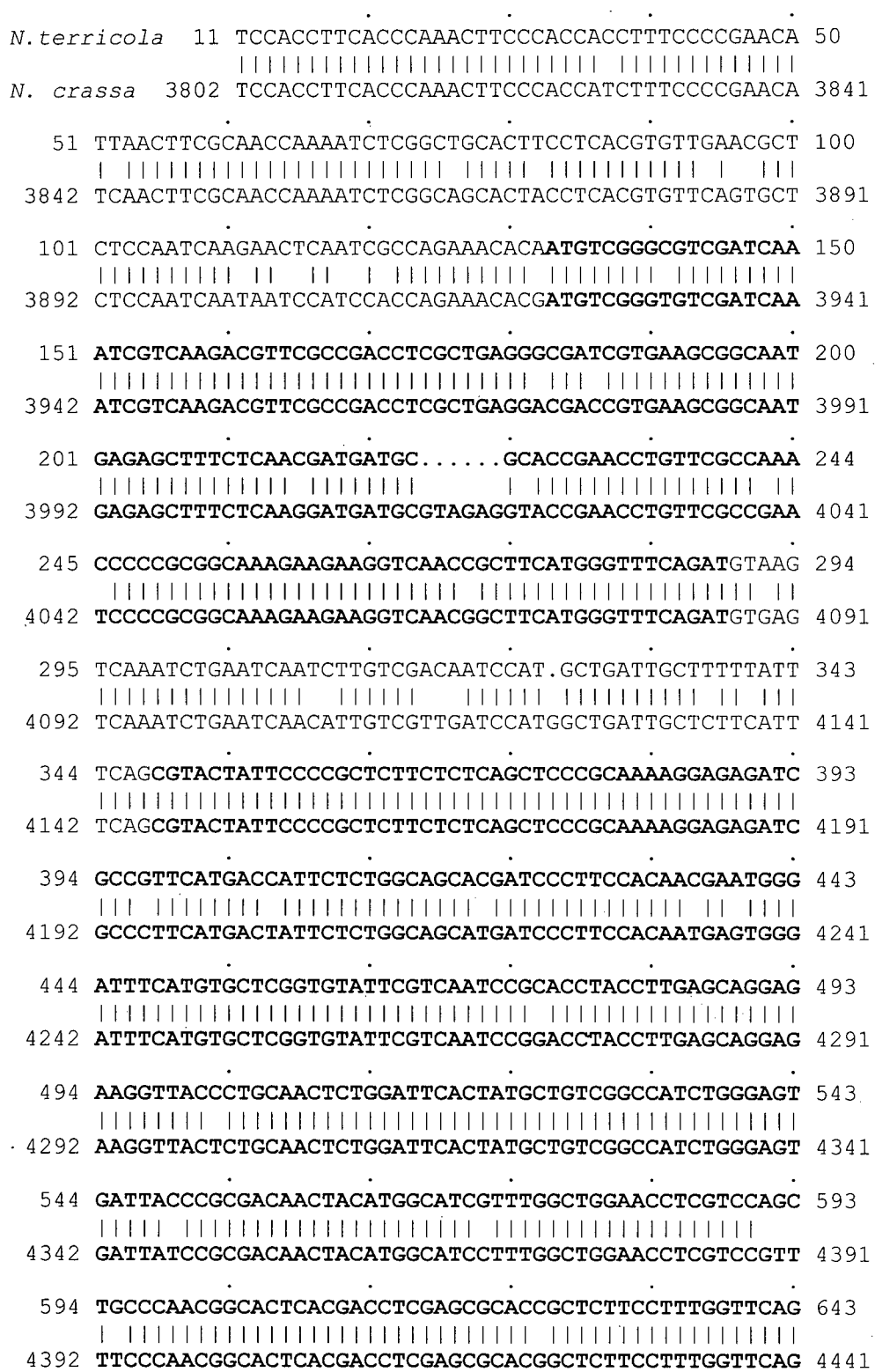
N. terricola mt a-1, but not *mt A-2*, has an open reading frame comparable in length to the *N. crassa* homolog.

The *mt A-1* gene was cloned by PCR with *N. crassa* primers and ligated into a plasmid for use in transformations and for finishing the sequencing begun by Beatty (1993). The sequence alignment of *N. terricola mt A-1* and *N. crassa mt A-1*, as created by the GAP program in the GCG suite (Devereux, Haberli and Smithies, 1984), is shown in Figure 2.2. The two DNA sequences are 93% identical (Table 2.4). *N. terricola* has an open reading frame of a similar size to *N. crassa mt A-1*, with the exception of a six base pair deletion in the first exon. After the *N. terricola* sequencing was finished, *N. crassa mt A-1* was found to encode two transcripts, one giving a 288 amino acid polypeptide and the other giving a 293 amino acid polypeptide (Saupe *et al.*, 1996). It is unknown whether *N. terricola* encodes the second transcription stop since the sequencing did not extend this far. The length of the predicted *N. terricola* MT A-1 protein is 286 amino acids. The *N. crassa* and *N. terricola* MT A-1 products have 93% amino acid identity and 96% amino acid similarity (Table 2.4). The amino acid alignment (Figure 2.3) shows that a region of *N. terricola mt A-1* has similarity with the putative DNA-binding domain of *N. crassa* (α domain). In the region of similarity to *S. cerevisiae MAT*α1 (*N. terricola mt A-1*, position numbers 40-72 in Figure 2.3), *N. terricola* differs from *N. crassa* by one amino acid. *N. terricola* has an arginine in position 47, while *N. crassa*, in the homologous position (49), has a glycine. This substitution is non-conservative.

Table 2.4 Comparison of *N. crassa* and *N. terricola* genes.

Gene	DNA identity	# amino acids in protein (N.t./N.c.)	Amino acid similarity
mt A-1	93%	286/293	96%
mt A-2	90%	118/373	88%
mt a-1	93%	305/382	97%

DNA Sequence Alignment of *mt A-1*



```
                .         .         .         .         .
 644 CATAACCTTCAGCCCATGAACGGCCTATGCCTGCTCACTAGGTGCCTCGA 693
     || || || ||||||||||||||| |||||||||||| | |||||||||
4442 CACAATCTCCAGCCCATGAACGGCTTATGCCTGCTCACCAAGTGCCTCGA 4491
                .         .         .         .         .
 694 GAGCGGATTGCCTCTTCACAATCCTCACCCTGTCATCGCCAAGCTTTCAG 743
     ||||||||||||||||  |||||||||| ||||||||||||||||||||
4492 GAGCGGATTGCCTCTTGCCAATCCTCACTCTGTCATCGCCAAGCTTTCAG 4541
                .         .         .         .         .
 744 ATCCTAGCTACGACATGATCTGGTTCAACAAGCGTCCTCACCGTCAGCAG 793
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4542 ATCCTAGCTACGACATGATCTGGTTCAACAAGCGTCCTCACCGTCAGCAG 4591
                .         .         .         .         .
 794 GGACACGCCGGCCAAACTGACAATTCTGAACTTGGAGTGTCGGCGCTCTT 843
     ||||||||||  ||||||||  | |||||| ||||||| |||||| | ||
4592 GGACACGCCGTTCAAACTGATGAATCTGAAGTTGGAGTTTCGGCGATGTT 4641
                .         .         .         .         .
 844 CCCTCGCAATCACGCAGTCGCTGCAGAGGTAGATGGCATCGCCAATCTTC 893
     |||||||||||| | ||||||||||||||||||||||  ||||||||
4642 CCCTCGCAATCACACGGTCGCTGCAGAGGTAGATGGCATCATCAATCTTC 4691
                .         .         .         .         .
 894 CTCTCTCCCATTGGATTCAGCAGGGAGATTTCGGCACCGAGTCCGGATTC 943
     |||||||||||||||||||||||||| ||||| |||||||| |||| |
4692 CTCTCTCCCATTGGATTCAGCAGGGAGAATTCGGTACCGAGTCTGGATAC 4741
                .         .         .         .         .
 944 TCAGCTCAGTTTGAGACCTTGTTGGATTCGATCCTTGAGAATGGAAACGC 993
     |||||||||||||||||||||||||| || || ||||||||| ||||
4742 TCAGCTCAGTTTGAGACCTTGTTGGATTCAATTCTCGAGAATGGACACGC 4791
                .         .         .         .         .
 994 CTCCAGCAATGATCCCTACAACATGGCTCTGGCTATGGATGTTCCCATGA 1043
     |||||||||||| || ||||||||||||||||||| ||||||||||||
4792 CTCCAGCAATGACCCTTACAACATGGCTCTGGCTATCGATGTTCCCATGA 4841
                .         .
1044 TGGGTTAGTGGAAGATGAGG 1063
     ||||||||||||||||||||
4842 TGGGTTAGTGGAAGATGAGG 4861
```

Figure 2.2 Alignment of *N. terricola* (top) and *N. crassa* (bottom) DNA sequences of *mt A-1* using the GAP program with the default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). Exons are boldface.

N. terricola mt A-2 was cloned by PCR with *N. crassa* primers and sequenced. Sequence and amino acid alignments with *N. crassa* are shown in Figures 2.4 and 2.5, respectively. The two *mt A-2* genes have 90% DNA identity, 83% amino acid identity and 88% amino acid similarity (Table 2.4). More importantly, the *N. terricola* predicted protein has a stop codon at 118 amino acids. The *N. crassa* MT A-2 protein is 373 amino acids (Ferreira, Saupe and Glass, 1996). The putative DNA-binding domain (Ferreira, Saupe and Glass, 1996; Debuchy, Arnaise and Lecellier, 1993) is not present in *N. terricola* MT A-2 (Figure 2.5).

N. terricola mt a-1 was cloned by PCR with *N. crassa* primers and sequenced. Sequence and amino acid alignments are shown in Figures 2.6 and 2.7, respectively. The two *mt a-1* genes have 93% DNA identity, 93% amino acid identity and 97% amino acid similarity (Table 2.4). The *N. terricola* predicted protein is truncated at 305 amino acids (compared to *N. crassa* 382 amino acids (Staben and Yanofsky, 1990; Philley and Staben, 1994)) and has a three amino acid insertion in the third exon. The amino acid alignment (Figure 2.7) shows that the HMG domain (DNA-binding domain, position 117-188) is identical to *N. crassa* except for three conservative and two semi-conservative substitutions. According to the consensus HMG domain (Grosschedl, Giese and Pagel, 1994), three of the changes are at non-consensus positions. Moreover, at one of the semi-conservatively changed positions, *N. terricola* has the consensus residue (serine in *N. crassa*, alanine in *N. terricola*) and at one of the conservatively changed positions (leucine in *N. crassa*, methionine in *N. terricola*), both species differ from the consensus (histidine). The HMG domain of *N. crassa* MT a-1 is required for DNA binding *in vitro* and mating *in vivo*, but not for heterokaryon incompatibility *in vivo* (Philley and Staben, 1994).

The six amino acids absolutely required for heterokaryon incompatibility, 216-220 (Philley and Staben, 1994) and 258 (Staben and Yanofsky, 1990), are present in *N. terricola*. The acidic C-terminus, required for both heterokaryon incompatibility and mating/sporulation in *N. crassa*, is mostly conserved. Sixteen of the acidic residues are conserved, one is missing in *N. terricola* in the three amino acid deletion and five more are missing because of the early translation stop.

Deduced Amino Acid Alignment of MT A-1

```
                .          .          .          .
N.t.  1 MSGVDQIVKTFADLAEGDREAAMRAFSTMMR..TEPVRQTPAAKKKVNRF 48
        |||||||||||||||||:|||||||||| |||  |||||..|||||||| |
N.c.  1 MSGVDQIVKTFADLAEDDREAAMRAFSRMMRRGTEPVRRIPAAKKKVNGF 50
                .          .          .          .          .
     49 MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL 98
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
     51 MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL 100
                .          .          .          .          .
     99 EQEKVTLQLWIHYAVGHLGVITRDNYMASFGWNLVQLPNGTHDLERTALP 148
        ||||||||||||||||||||.||||||||||||||.:||||||||||||||
    101 EQEKVTLQLWIHYAVGHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALP 150
                .          .          .          .          .
    149 LVQHNLQPMNGLCLLTRCLESGLPLHNPHPVIAKLSDPSYDMIWFNKRPH 198
        |||||||||||||||||:|||||||| |||.||||||||||||||||||||
    151 LVQHNLQPMNGLCLLTKCLESGLPLANPHSVIAKLSDPSYDMIWFNKRPH 200
                .          .          .          .          .
    199 RQQGHAGQTDNSELGVSALFPRNHAVAAEVDGIANLPLSHWIQQGDFGTE 248
        ||||||.|||:||:||||:|||||.|||||||| ||||||||||:||||
    201 RQQGHAVQTDESEVGVSAMFPRNHTVAAEVDGIINLPLSHWIQQGEFGTE 250
                .          .          .
    249 SGFSAQFETLLDSILENGNASSNDPYNMALAMDVPMMG* 287
        ||:|||||||||||||||:||||||||||||:|||||||
    251 SGYSAQFETLLDSILENGHASSNDPYNMALAIDVPMMG* 289
```

Figure 2.3 Alignment of *N. terricola* (top) and *N. crassa* (bottom) deduced amino acid sequences of MT A-1 using the GAP program with default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). In *N. crassa*, amino acids 1-111 are sufficient for heterokaryon incompatibility and 99-111 are absolutely required (K. T. Shiu, personal communication). The α-domain, thought to be involved in DNA binding is in boldface (Debuchy and Coppin, 1992).
Colon indicates a conservative amino acid substitution.
Single dot indicates a semi-conservative amino acid substitution.

DNA Sequence Alignment of *mt A-2*

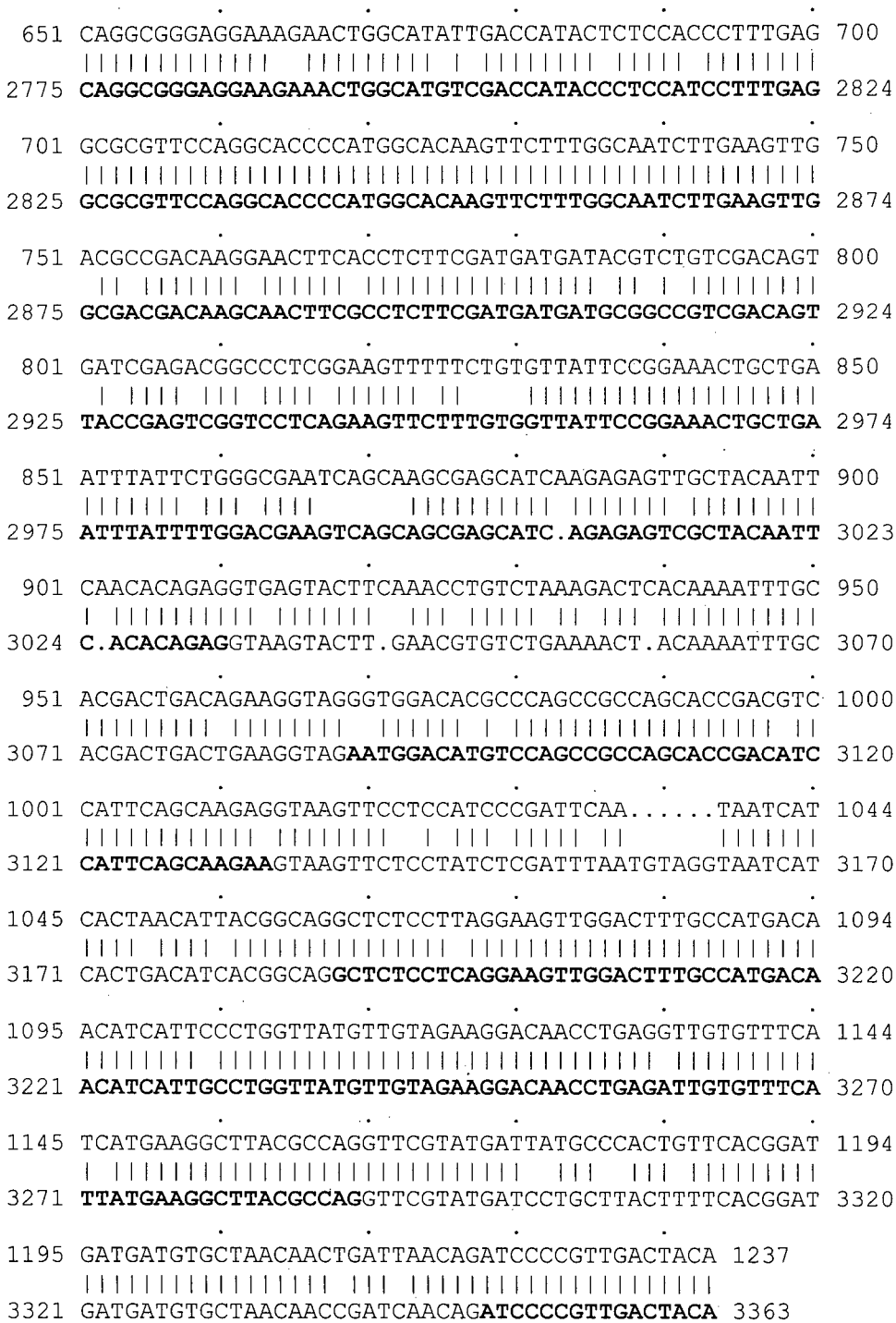


Figure 2.4 Alignment of *N. terricola* (top) and *N. crassa* (bottom) DNA sequences of *mt A-2* using the GAP program with default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). Exons are in boldface.

Deduced Amino Acid Alignment of MT A-2

```
                .         .         .         .         .
N.t.   1 MNLINMQPRRSEQPVMLEENRTSSQEGQDLEVMYKKLHQLQARLSRSVLS  50
         |||:||||:||||||.|:|||||.||||||||||||||||||||||||||
N.c.   1 MNLLNMQPKRSEQPAMFEENRASSQEGQDLEVMYKKLHQLQARLSRSVLS  50
                .         .         .         .         .
      51 EAIKEFEENLQCLFHETKLLLCTKRTKYRQSWFGSSNEFGSSNESRIIKA 100
         ||||| |||||.|||||.||||||..|||||||||||||||||:|.|||||.
      51 EAIKENEENLRCLFHEAKLLLCSTRTKYRQSWFGSSNEFGSSDERRIIKT 100
                .
     101 SCCIIESTKHNSQLSLVP* 119
         ||||||||  |. |.::.
     101 SCCIIEST..NTILNFLSF...383
```

Figure 2.5 Alignment of *N. terricola* (top) and *N. crassa* (bottom) amino acid sequences of MT A-2 using the GAP program with the default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). Note the frameshift at amino acid 109 and the premature stop codon in *N. terricola*. The shortened product does not contain the putative DNA-binding domain of *N. crassa* (Debuchy, Arnaise and Lecellier, 1993).

DNA Sequence Alignment of *mt a-1*

```
N.t. 2950   GAATGCTATTCAGGGCCGTGTCAATAACCGTCAATAGACAAAGCGGT 2999
            ||||||||||||||||||||||||| || ||||||||||||||||
N.c. 2950   GAATGCTATTCAGGGCCGTGTCAATAGCCATCAATAGACAAAGCGGT 2999

    3000 TCCACCCAAAAATCCCGACGTCGTTGCCCCTCCTTATAATCTCCCTCCCT 3049
         ||||||||||||||||||||||||||||||||||||||||||||||||||
    3000 TCCACCCAAAAATCCCGACGTCGTTGCCCCTCCTTATAATCTCCCTCCCT 3049

    3050 TCCAATTCTTCCTTCTTCCCACTCCTTACATTCCTCCGTCAACTTCGCAA 3099
         |||||||||||| |||||||||| |||||||||||||| ||||||
    3050 TCCAATTCTTCCTCCTTCCCACTCCTAACATTCCTCCGTCAACATCGCAA 3099

    3100 CAATAGTCGACAATCACGACCAATCTTCGCCATCAACTCATTTCTCCTTC 3149
         |||||||||||||||||||| |||||||||| |||||||||||||
    3100 CAATAGTCGACAATCACGACCGATCTTCGCCATCTACTCATTTCTCCTTT 3149

    3150 CCACAATCAATCGCCCAAGTCTTTGACAATCCCAAGCATCAGCCTTCTTC 3199
         |||||| || |||||||||||||||||||| ||||||||||||||||||
    3150 CCACAACCAGTCGCCCAAGTCTTTGACAATCTCAAGCATCAGCCTTCTTC 3199

    3200 ATCGTCAACGAACAATCAATCGAAACA**ATGGACGGCAACTTGACGCACCC** 3249
         ||| || |||| ||||| |||||||||||||| |||| ||| |||||
    3200 ATCACCAGAGAACGATCAACCGAAACA**ATGGACGGTAACTCGACACACCC** 3249

    3250 **CGCTCCGGACGTCAAGACCACCTTGGCTTGGTCTCGCATCTCCAACCAAC** 3299
         |||||| .|| ||||||| || |||||||||| ||||| || |||||||
    3250 **CGCTCCAAACCTCAAGACTACTATGGCTTGGTCGCGCATATCAAACCAAC** 3299

    3300 **TCGGACACTGGAACGACCGCAAGGTCATTGCCATTCCTCTGAGCGACTTC** 3349
         |||| |||||||| ||||||||||||||||||||||||||||||||||||
    3300 **TCGGTCACTGGAATGACCGCAAGGTCATTGCCATTCCTCTGAGCGACTTC** 3349

    3350 **CTTCACACCGACCCTGTCATTCAGTCTGGCATCATCGCCAGCTTCAAG**TA 3399
         ||| ||||| |||||| ||||||||||||||||||||  ||||||||
    3350 **CTTAACACCCACCCTGACATTCAGTCTGGCATCATCGCCGAGTTCAAG**TA 3399

    3400 AGTGTCCTCACCCATTTCTCACCTTACCTTGTACTGACCATTTGCACCAG 3449
         |||||||||||||||||||||| |||||||||||||||||||||| ||
    3400 AGTGTCCTCACCCATTTCTCACCCTACCTTGTACTGACCATTTGCACTAG 3449

    3450 G**AAAGCGACTGGTGAAGAGGGCATGTTTGCCCGCGATCCCGAGTCACTGG** 3499
         |||||||||||| |||||||||||||||||||||||||| || ||| |||
    3450 G**AAAGCGACTGGCGAAGAGGGCATGTTTGCCCGCGATCCTGAATCATTGG** 3499

    3500 **GAATCATGCTTCTTGGCCCCGCCAAGCTGTTCAAGCCCGACAGTGTCGTC** 3549
         ||||||||||||||| |||| ||||||||||||||||||||||||||||
    3500 **GAATCATGCTTCTTGGTCCCGTCAAGCTGTTCAAGCCCGACAGTGTCGTC** 3549

    3550 **GTCGACGGCAACCTTTTCTGGGATCCCAAGGGCATCCATGCTTCGGCACC** 3599
         |||||||||||||| ||||||||||||||||||||||||||||||||||
    3550 **GTCGACGGCAACCTGTTCTGGGATCCCAAGGGCATCCATGCTTCGGCACC** 3599
```

```
3600 CAAGGAGCAGCAGAAGAAGGCCAAGATTCCTCGCCCCCCCAACGCCTACA 3649
     |||||||||||||||||||||||||||| |||||||| ||||| |||||||
3600 CAAGGAGCAGCAGAAGAAGGCCAAGATCCCTCGCCCTCCCAATGCCTACA 3649

3650 TCTTGTACCGAAAGAACCATCATCGTGAGATCCGCGAGCGGAACCCCGGA 3699
     |||||||||| ||| ||||||||||||||||||||||| ||| ||||||
3650 TCTTGTACCGTAAGGACCATCATCGTGAGATCCGCGAGCAGAATCCCGGA 3699

3700 CTCCACAACAACGAGATTTGTAAGTTTCTTGTCACCATGATCTATAATGT 3749
     || |||||| |||||||||||||||||||||||| ||||||| | ||| |
3700 CTTCACAATAACGAGATTTGTAAGTTTCTTGTCATCATGATCGAAAATCT 3749

3750 TTGACCCTGAGACTAACCTCACTTAGCGGTCATCGTCGGCAACATGTGGC 3799
     ||| || ||||||||||||||||||||||||||||||||||||||||||
3750 TTGGCCTTGAGACTAACCTCACTTAGCGGTCATCGTCGGCAACATGTGGC 3799

3800 GTGATGAGCAGCCGCACATTCGCGAGAAATATTTCAACATGGCCAATGAG 3849
     |||||||||||||||||||||||||||||||||||||| ||||||||
3800 GTGATGAGCAGCCGCACATTCGCGAGAAATATTTCAACATGTCCAATGAG 3849

3850 ATCAAGACTAGAATGTTGCTGGAGCATCCCGACTATCGCTACAATCCACG 3899
     |||||||| ||| |||||||||| |||||||||||||||||||| ||
3850 ATCAAGACCAGACTGTTGCTGGAGAATCCCGACTATCGCTACAATCCGCG 3899

3900 TCGGTCTCAAGACATTCGCAGGCGCGTCTCGCCGTATCTCAAAATCAAGC 3949
     |||||||||||||||||||||||||||||||||||||| |||||||
3900 TCGGTCTCAAGACATTCGCAGGCGCGTCTCGCCGTATCTCAAGATCAAGC 3949

3950 TCCTCAACTATGACGCTAATGGCAACCTTCTTTGGGGCACCGTCAACGCC 3999
     |||||||||| |||| ||||||||||||||||||||||||||||||||
3950 TCCTCAACTACGACGTTAATGGCAACCTTCTTTGGGGCACCGTCAACGCC 3999

4000 GAGGATGCTGCGCTAATTCGGACTCACTTCCATGGAGTCGTTCGTGTTGA 4049
     |||||||||||||| ||||||||||||||||||||||||||||||||
4000 GAGGATGCTGCGCTGATTCGGACTCACTTCCATGGAGTCGTTCGTGTTGA 4049

4050 GGAAATGGATGATGGCTGCAGAATTGTCTGCCGTCCCGTCGCAGGATCTA 4099
     |||||||||||||| |||||||| ||||||||||||||||||||||||
4050 GGAAATGGATGATGGTTGCAGAATCGTCTGCCGTCCCGTCGCAGGATCTA 4099

4100 GAAAACTTCGCGCCGCCGTTGTCGACACTTGGATGCCTCGCTATACGGTT 4149
     |||||||||||||||||||||||||||||||||||||||| ||||||
4100 GAAAACTTCGCGCCGCCGTTGTCGACACTTGGATGCCTCGCTACACGGTT 4149

4150 GATGCAACCCCCGTCACTGAGGACGACGATGCACAGGCTTTCAACTTCAA 4199
     ||  |||||||||||||| ||||||||||||||||||||||||||||||
4150 GACACAACCCCCGTCACCGAGGACGACGATGCACAGGCTTTCAACTTCAA 4199

4200 CGACCCCATAGACCCCTTGGCCGGCGCTTATTTCCCTATCAATGATCACC 4249
              || ||||||| ||| |||||||||||| | ||||| ||||
4200 .........TGATCCCTTGGGCGGTGCTTATTTCCCTTTGAATGAGCACC 4240

4250 TCTAGGTCACCGTCAACCAAACCCCTCCCTTCAATGCCCCTCCCCCCAAT 4299
     ||| | |||| |||||||||| ||||||||||||||||||||||||||
4241 TCTGGATCACTGTCAACCAAAACCCTCCCTTCAATGCCCCTCCCCCCAAT 4290
```

```
                .         .         .         .         .
4300 CCCAACCCACACCTGGATTTCGTTCACCCCGACGGCATGGACGCAGTTAT 4349
     |||||||||||||||||||||||||||||||||||||||| ||||||  |
4291 CCCAACCCACACCTGGATTTCGTTCACCCCGACGGCATGGAGGCAGTTGT 4340
                .         .         .         .         .
4350 TCACAACGTTCAGGACATGATCGCCCAGGTCCAGGAGGCCAATGAGGCTG 4399
     |||||||||||| |||||||||| |||||||||||||| || |||||||
4341 TCACAACGTTCAGAACATGATCGCTCAGGTCCAGGAGGCTAACGAGGCTG 4390
                .         .         .         .         .
4400 CTGCGCTAACGCCACCTCCGCTACCACCGCTGCGCCTGCCGTCACTCAGG 4449
     |||||||||||| ||| |||| |||||||||||| |||| ||||||||||
4391 CTGCGCTAACGCTACCACCGCCACCACCGCTGCGTCTGCTGTCACTCAGG 4440
                .         .         .         .         .
4450 TTATGGCTGACGATACCATCAACCCAGCGCTCATTCCCACTGTGAACACT 4499
     |||||||||| |||||||| |||||||| ||||||||||||||||||||
4441 TTATGGCTGATGATACCATTAACCCAGCTCTCATTCCCACTGTGAACACT 4490
                .         .         .         .         .
4500 AATGCGACCGTTCTTCCTCACGTCCATACCATTCCTGACAACGCCACCGT 4549
      ||||  | ||||||||  ||||||||||||||||||||||||||||||
4491 CATGCATCTGTTCTTCCCTACGTCCATACCATTCCTGACAACGCCACCGT 4540
                .         .         .         .         .
4550 TACGCCTTCCGCCACTGGAAATTCGGTTCATGTTGTTACACCCGGTCACC 4599
     |||||||||||| |||||||| |||||||| ||||||||||||||||||
4541 TACGCCTTCCGCTACTGGAAACTCGGTTCACGTTGTTACACCCGGTCACC 4590
                .
4600 AAGGATATCACCTCAT 4615
     ||||||||||||||||
4591 AAGGATATCACCTCAT 4606
```

Figure 2.6 Alignment of *N. terricola* (top) and *N. crassa* (bottom) DNA sequences of *mt a-1* using the GAP program with the default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). Exons are in boldface.

Deduced Amino Acid Alignment of MT a-1

```
                .         .         .         .         .
N.t.   1 MDGNLTHPAPDVKTTLAWSRISNQLGHWNDRKVIAIPLSDFLHTDPVIQS  50
         |||| ||||||::|||:|||||||||||||||||||||||||||:|.| |||
N.c.   1 MDGNSTHPAPNLKTTMAWSRISNQLGHWNDRKVIAIPLSDFLNTHPDIQS  50
                .         .         .         .         .
      51 GIIASFKKATGEEGMFARDPESLGIMLLGPAKLFKPDSVVVDGNLFWDPK 100
         ||||.|||||||||||||||||||||||.||||||||||||||||||||||
      51 GIIAEFKKATGEEGMFARDPESLGIMLLGPVKLFKPDSVVVDGNLFWDPK 100
                .         .         .         .         .
     101 GIHASAPKEQQKKAKI**PRPPNAYILYRKNHHREIRERNPGLHNNEISVIV** 150
         |||||||||||||||||||||||||||:|||||||.||||||||||||||
     101 GIHASAPKEQQKKAKI**PRPPNAYILYRKDHHREIREQNPGLHNNEISVIV** 150
                .         .         .         .         .
     151 **GNMWRDEQPHIREKYFNMANEIKTRMLLEHPDYRYNPR**RSQDIRRRVSPY 200
         ||||||||||||||||||.||||||:|||:||||||||||||||||||||||
     151 **GNMWRDEQPHIREKYFNMSNEIKTRLLLLENPDYRYNPR**RSQDIRRRVSPY 200
                .         .         .         .         .
     201 LKIKLLNYDANGNLLWGTVNAEDAALIRTHFHGVVRVEEMDDGCRIVCRP 250
         |||||||||.||||||||||||||||||||||||||||||||||||||||||
     201 LKIKLLNYDVNGNLLWGTVNAEDAALIRTHFHGVVRVEEMDDGCRIVCRP 250
                .         .         .         .         .
     251 VAGSRKLRAAVVDTWMPRYTVDATPVTEDDDAQAFNFNDPIDPLAGAYFP 300
         |||||||||||||||||||||.|||||||||||||||   |||:|||||
     251 VAGSRKLRAAVVDTWMPRYTVDTTPVTEDDDAQAFNFN...DPLGGAYFP 297

     301 INDHL* 306
         :|:||
     298 LNEHLW 303
```

Figure 2.7 Alignment of *N. terricola* (top) and *N. crassa* (bottom) amino acid sequences of MT a-1 using the GAP program with the default settings from the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). The HMG domain (boldface) is very similar (see text for discussion) and the transcription-factor-like acidic, proline-rich C-terminus required for mating and heterokaryon incompatibility (everything C-terminal to the HMG domain) is very similar, except for a three amino acid insertion in *N. terricola* (Philley and Staben, 1994).

A search for consensus sequences revealed that in *N. terricola mt A-1*, *mt A-2* and *mt a-1*, the translation start sites, 5' and 3' splice sites and lariat sites differ from *N. crassa* and published fungal consensus sequences (Edelman and Staben, 1994) by no more than one nucleotide.

N. terricola mt a-1 can induce mating in *N. crassa* mating type mutants and a mating type deletion strain.

N. terricola mt a-1 was first subcloned into the vector, pOKE103. The *N. terricola mt A-1* gene was also subcloned into pOKE103 for use in control experiments. To determine whether or not *N. terricola mt a-1* is able to induce mating, a number of experiments was performed in which sterile *N. crassa* strains were co-transformed with a plasmid containing one of the *N. terricola* mating type genes and a selectable plasmid (with a gene encoding an altered β-tubulin that confers resistance to Benomyl). Transformation with two DNA fragments generally results in the integration of both types of fragments, even if one has no selectable marker (Vollmer and Yanofsky, 1986). The sterile recipient strains were either mating type mutants or had the *mt A* mating type idiomorph deleted. The transformants were crossed to opposite mating type females by replica plating.

The *N. terricola mt a-1* gene was able to induce mating (Table 2.5, rows 1-3). The observed mating reaction was characterized by enlargement and darkening of perithecia which had no or very small beaks and produced no ascospores. The mating reaction proceeded to the same point in development as the control transformants of *N. crassa* mating type mutants transformed with *N. crassa mt a-1* (Table 2.5, row 4).

Table 2.5 *N. terricola mt a-1* confers mating to *N. crassa* mating type mutants or nulls.

N. crassa recipient strain	Transforming plasmids	# mating transformants/ # transformants tested
A mating type mutant *(arg-2 mtA1.7 (RIP))*	*N.t. mt a-1* (pNTa1) Benomyl-R (pßC1)	6/68
mating type deleted *(RLM 41-10thi-4 ad-2; lys-1 ΔA)*	*N.t. mt a-1* (pONTa) Benomyl-R (pßC1)	5/503
mating type deleted *(RLM 41-10thi-4 ad-2; lys-1 ΔA)*	*N.t. mt a-1* (pNTa1) Benomyl-R (pßC1)	11/119
mating type deleted *(RLM 41-10thi-4 ad-2; lys-1 ΔA)*	*N.c. mt a-1* (pCSN4) Benomyl-R (pßC1)	5/333

N. terricola mt A-1 and *mt a-1* can induce mating type incompatibility.

The *N. crassa* mating type genes of opposite mating type cannot coexist for long within the same nucleus. A mycelium containing dual mating type nuclei is phenotypically distinct because the hyphae are short and growth is extremely slow or non-existent. There is one known suppressor (*tol*) of this incompatibility (Newmeyer, 1970; Vellani, Griffiths and Glass, 1994). How are the two mating type genes of *N terricola* able to coexist? Are the mating type genes compatible or is there a suppressor? To test the hypothesis that the *N. terricola* mating type genes are compatible, they were assessed for their ability to induce incompatibility, using reduction in transformation efficiency as an indicator of incompatibility. *N. crassa A* (strain name 153) and *a* (strain name 7) strains were co-transformed with plasmids containing one of the *N. terricola* mating type genes, *mt A-1* (pONTA) or *mt a-1* (pONTa) plus the plasmid containing the gene encoding resistance to hygromycin (pMP6) (Table 2.6).

Table 2.6 Transformation efficiency of mating type genes.

Recipient strain	Transforming plasmids	# replicates	Avg. # colonies ± standard deviation
N.crassa A	*N.t. mt A-1* hygromycin-R	9	129 ± 45
N.crassa a	*N.t. mt A-1* hygromycin-R	9	16 ± 5.7
N.crassa A	*N.t. mt a-1* hygromycin-R	10	41 ± 16
N.crassa a	*N.t. mt a-1* hygromycin-R	10	158 ± 44

Introduction of *N. terricola mt A-1* into *N. crassa a* reduced transformation efficiency 8-fold as compared to the same gene introduced into a *N. crassa A* recipient. Similarly, introduction of *N. terricola mt a-1* into *N. crassa A* versus *a* reduced the number of transformants 4-fold.

The reduction in the number of transformants when *N. crassa A* is transformed by *N. terricola mt a-1* as compared to *N. terricola mt A-1* was statistically significant (as determined by an ANOVA, not shown), suggesting that *N. terricola mt a-1* was able to induce incompatibility against *N. crassa A*. Similarly, the statistically significant reduction in the number of transformants when *N. crassa a* is transformed by *N. terricola mt A-1* as compared to *N. terricola mt a-1* indicated that *N. terricola mt A-1* was incompatible with *N. crassa a*.

Mating type incompatibility is suppressed by *tol*.

In *N. crassa* recessive alleles of *tol* suppress mating type incompatibility. When both components of a mixed mating type heterokaryon have the suppressor allele, then the heterokaryon grows at a wild type rate. If either or both components carry tol^+, then the heterokaryon grows slowly. Similarly, dual mating type partial duplication strains with *tol* grow more vigorously than those with tol^+.

To test the hypothesis that *tol* is able to suppress the incompatibility that occurs between the *N. terricola* and the *N. crassa* mating type genes, the following transformation experiment was performed. Suppressor (I-20-41 which is *arg-1 ad-3B; tol A*) and non-suppressor (I-20-26 which is *arg-1 ad-3B A*) strains of *N. crassa* were co-transformed with one of the *N. terricola* mating type gene plasmid, pONTa, plus a plasmid encoding resistance to hygromycin (Table 2.7).

More transformants were recovered from the transformation of *N. crassa A* cells with *N. terricola mt a-1* in the experiment described in Table 2.7 as compared to a similar experiment described in Table 2.6. One difference was the temperature at which the transformation plates were incubated. In Table 2.6, the cells were *un-3 ad-3A nic-2 cyh-1 A*. Since *un-3* strains are temperature-sensitive, the plates were incubated at room temperature. In Table 2.7, the cells were *arg-1 ad-3B A* and so they were incubated at 30°C, which allowed faster growth. More transformants may have grown to a detectable size by the time the plates were examined.

Table 2.7 Suppression of incompatibility by *tol*.

Recipient strain	Transforming plasmids	# replicates	Avg. # colonies ± standard deviation
tol$^+$ *A*	hygromycin-R	5	223 ± 18
tol$^+$ *A*	*N.t. mt a-1* hygromycin-R	5	163 ± 34
tol A	hygromycin-R	5	60 ± 17
tol A	*N.t. mt a-1* hygromycin-R	5	92 ± 16

Co-transformation of a *N. crassa tol* strain with a plasmid carrying the opposite mating type *N. terricola* gene and the hygromycin plasmid resulted in significantly more transformants (as determined by an ANOVA, p=0.05) compared to transformation with the hygromycin plasmid alone. In contrast, co-transformation of a *N. crassa tol*$^+$ strain with a plasmid carrying the opposite mating type *N. terricola* gene and the hygromycin plasmid resulted in fewer transformants relative to transformation with the hygromycin plasmid alone.

Two of the mating type genes are expressed in *N. terricola.*

In order to determine whether or not the *N. terricola* mating type genes *mt A-1*, *mt A-2* and *mt a-1* were expressed in *N. terricola*, transcripts were amplified by reverse transcriptase PCR (RT-PCR) using *N. crassa* primers. *N. crassa* genomic DNA and cDNA were used at the same time for comparison. The *mt A-1* and *mt a-1* genes are expressed in *N. terricola.* Lanes 2 and 3 show amplification products from *ß-tubulin* primers. Lanes 4 and 5 show DNA fragments produced from the *mt a-1* primers. The bands were of a similar size to the *N. crassa* controls. Lanes 6 and 7 show the bands produced with *mt A-1* primers. Again, the N. terricola fragments were of a similar size to the *N. crassa* controls (Figure 2.8, *N. crassa* controls were not included in the figure).

When *N. terricola* genomic DNA was used as a template with the *mt A-2* primers, a band of the expected size was seen and it hybridized with a pNTA2 probe. When *N. terricola* cDNA was used as a template with the *mt A-2* primers, a band that was several hundred bases smaller than expected appeared. This band did not hybridize with the probe. The band may have been produced through non-specific amplification. The *ß-tubulin* and other mating type primers were able to amplify the expected PCR products from the same preparation of cDNA. These data suggest that *mt A-2* is not expressed in *N. terricola.*

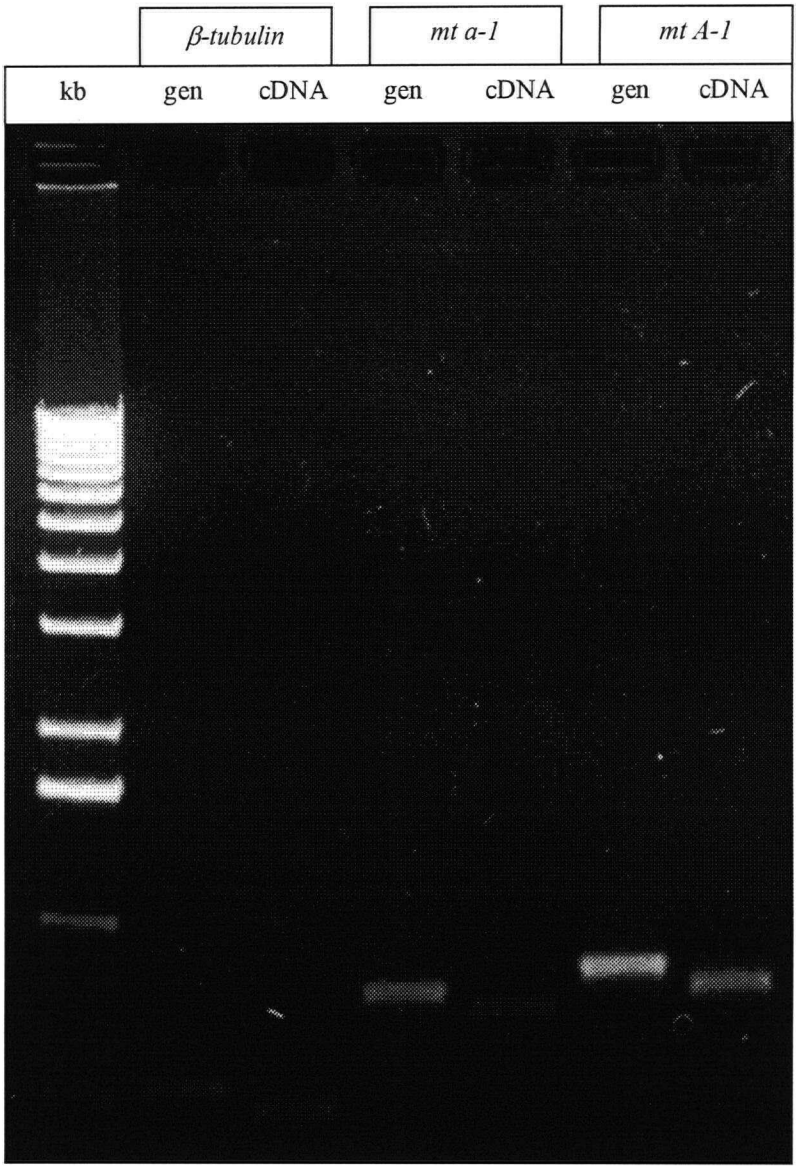


Figure 2.8 Gel electrophoretic analysis of *N. terricola* RT-PCR products. Lane 1 is kb ladder. Lanes 2 and 3 are RT-PCR products amplified from genomic and cDNA with *β-tubulin* primers; lanes 4 and 5, *mt a-1* primers and lanes 6 and 7, *mt A-1* primers.

gen=genomic DNA template
cDNA=cDNA template

Discussion

N. terricola contains two mating type genes (*mt a-1* and *mt A-1*) which tested positive in functional assays. One gene (*mt A-2*) was shown to be non-functional.

The *N. terricola* genes *mt A-1* (Beatty, 1993) and *mt a-1* are able to induce mating in *N. crassa* mating type mutants. The perithecia are similar to those seen when *N. crassa mt A-1* or *mt a-1* DNA fragments are used to transform the same mutants. The mating type genes of *N. terricola* (T. S. Vellani, unpublished data) were unable to induce homothallism in *N. crassa* recipient strains.

The tests done to assess the ability of the mating type genes to induce incompatibility show that the *N. terricola* genes are able to induce incompatibility with the opposite mating type *N. crassa* gene. The incompatibility between *N. crassa A* and *N. terricola a* is suppressed by *tol*, suggesting that the tol^{+} product is necessary for incompatibility. Genomic Southern blots probed with the cloned *N. crassa tol* gene suggest that, in contrast to all other *Neurospora* species, *N. terricola* and *N. pannonica* have no *tol* homolog (K. T. Shiu and N. L. Glass, personal communication). The mating type genes of *N. terricola* and *N. pannonica* probably co-exist without inducing incompatibility.

The *N. terricola* genes *mt A-1* and *mt a-1* are predicted to encode proteins that are highly similar to those of *N. crassa*, they are functional in a *N. crassa* host and they are expressed in *N. terricola*. Taken together, the data suggest that *mt A-1* and *mt a-1* are under the influence of natural selection. Their presumed function could be the specification of identity in ascogenous hyphae or the initiation of sexual development. Sexual reproduction in a haploid self-mating organism produces no recombinant progeny, as shown by the highly similar RFLP patterns found in homothallic strains from a wide geographic distribution (Glass, Metzenberg and Raju, 1990). Meiosis, therefore, must be under selection for some outcome other than recombination. Two such outcomes could be ascospore-production and DNA repair (Perkins and Turner, 1988; Beatty, Smith and Glass, 1994; Glass, Metzenberg and Raju, 1990; see also General Introduction).

All homothallic Sordariaceae examined so far have a homolog to *mt a-1* except for the four *A*-only homothallic *Neurospora* species (Glass, Metzenberg and Raju, 1990; Pöggeler *et al.*, 1997a). Given that the *N. terricola mt a-1* gene appears to be functional, the absence of a homolog from the *N. africana* group of species requires some thought. The presence of an undetected equivalent to *mt a-1* in these species is possible. A *N. crassa mt A* probe hybridized strongly to *P. anserina* genomic DNA, but hybridization with the *mt a* probe was very weak (Debuchy and Coppin, 1992), suggesting that *mt a-1* function is preserved in the absence of strong sequence conservation. One functional region of MT a-1 is the HMG domain, which has few strongly conserved residues (Philley and Staben, 1994). Alternatively, there could be a third mating type allele with no similarity to *mt a-1* or *mt A-1*. Fungal mating type proteins contain any of a number of domains including HMG domains, homoeodomains and α-domains. One final possibility is that the *mt a-1* function is not required in *N. africana*.

The gene, *mt A-2*, is present in the *N. terricola* genome and is similar to the *N. crassa* gene, but its transcript was not detected by RT-PCR and it contains a frameshift followed closely by a stop codon that truncates the deduced polypeptide at 118 amino acids. These data suggest that *mt A-2* is not functional. The *N. crassa mt A-3* gene has no known homolog in *N. terricola* (Beatty, Smith and Glass, 1994). A *N. crassa mt A-2 mt A-3* double mutant (Glass and Lee, 1992; Ferreira, Saupe and Glass, 1996) has a severe phenotype of reduced ascospores, with the developmental block appearing at or around karyogamy and ascus development (Glass and Lee, 1992). In contrast, these same stages appear to be normal in *N. terricola* (Raju, 1978). The functions of *N. crassa mt A-2* and *mt A-3* appear to be dispensable in *N. terricola*.

As shown in Chapter 1, the post-fertilization functions of the mating type genes of *N. crassa* are abnormal when the opposite mating type genes are present in the same nucleus and when the mating type genes reside at ectopic chromosomal locations. Since these post-fertilization functions are intact in *N. terricola*, the mating type genes are either not under positional regulation or else both genes are positioned such that they are under the control of the putative *cis*-acting regulator. The fact that all of the diverse homothallic fungal species examined to date, including *Neurospora sp.* (Glass, Metzenberg and Raju, 1990; Beatty, Smith

and Glass, 1994), *Cochliobolus sp.* (N. L. Glass, personal communication), *Podospora sp.* (Debuchy, personal communication to N. L. Glass) and *Sordaria sp.* (Pöggeler *et al.*, 1997a) have the opposite mating type sequences linked suggests that the mating type genes are under positional regulation (these species did not arise from a single common ancestor). The genes may be linked in order to allow control of both genes by the same *cis*-acting regulator.

Chapter 3

Phylogenetic Analysis of *Neurospora*

Introduction

The phylogenetic study of fungi was difficult prior to the molecular revolution due to the paucity of morphological markers, the prevalence of convergent characters and the difficulty in culturing strains of most species. Traditionally, heterothallic species were defined in *Neurospora* using morphology and fertility, criteria which have corroborated each other more often than not (Perkins, Turner and Barry, 1976). Recently, nucleotide positions have been used as characters in phylogenetic analysis. Some examples in fungi are seen in Gargas *et al.* (1995), Radford (1993), Berbee and Taylor (1992a; 1992b; 1992c), Bowman *et al.* (1992), Hendriks *et al.* (1991), Smith (1989), Hori and Osawa (1987) and Dayhoff (1983).

Relationships in the genus *Neurospora* have been examined by a number of investigators using many types of data, including distribution of mitochondrial plasmids, restriction site differences and restriction fragment length polymorphisms of both mitochondrial and nuclear DNA, DNA and amino acid sequence data (Skupski, Jackson and Natvig, 1997; Lewis and Feldman, 1996; Randall and Metzenberg, 1995; Merrow and Dunlap, 1994; Taylor and Natvig, 1989; Natvig, Jackson and Taylor, 1987; Taylor, 1986; Taylor, Smolich and May, 1986; Russell *et al.*, 1984).

Several of these works include phylogenies, all of which were directed at resolving the issues of species designations or the relationships among the heterothallic and pseudohomothallic species. Some of these relationships remain unresolved. The phylogenies contain some inconsistencies, possibly because some of the heterothallic species may not be reproductively isolated from each other, ancient polymorphisms predating the divergence of some taxa may exist and, finally, tree topology can depend on the type of data or specific strains used to construct it (Skupski, Jackson and Natvig, 1997).

The high similarity of the RFLP patterns in the *A*-only homothallic group of species (*N. africana, N. lineolata, N. dodgei, N. galapagosensis*) suggested that these species are very closely related. In contrast, those of *N. terricola* were different from this group (Glass, Metzenberg and Raju, 1990), raising the question of how these two groups of homothallic *Neurospora* species are related to each other and to the heterothallic group. Estimates of degree of relatedness based on the fraction of shared rDNA restriction sites also suggest that the homothallic species do not form a group, *N. terricola* being distant from the other homothallic species (Verma and Dutta, 1987).

In this chapter, the phylogeny of the entire *Neurospora* genus was reconstructed using sequence data from the mating type gene, *mt A-1* in order to determine the relationship between *Neurospora* homothallic and heterothallic species. The trees thus produced are unique in the literature in the inclusion of all known species of *Neurospora*.

P. anserina, *G. tetrasperma* and *S. macrospora* were chosen as outgroup species. All of them belong to the same family (Sordariaceae) as *Neurospora*, with *Gelasinospora* being the most closely related genus to *Neurospora* (Merrow and Dunlap, 1994). *Gelasinospora* and *Neurospora* are distinguished by their production of pitted versus ribbed ascospores (von Arx, 1981). Their traditional generic separation is supported by phylogenetic analysis based on restriction maps of a nontranscribed spacer region of ribosomal genes (Verma and Dutta, 1987). Both genera include homothallic, pseudohomothallic and heterothallic species.

Mating type genes differ from other genes in the fungal genome in that they do not undergo recombination (Glass *et al.*, 1988). Lack of recombination would speed the evolution of the two alleles away from each other. The evolutionary relationships between species should be accurately reflected in a tree inferred from sequences of any one mating type gene.

Materials and Methods

DNA preparation

Cultures were grown on liquid complete medium (7g/L malt extract, 1g/L soytone, .5 g/L yeast extract). Genomic DNA was prepared from ground lyophilized tissue by the method of Lee and Taylor (1990).

Primers

The primers 1778 and 3194 were chosen because they encompass the open reading frame of *mt A-1*. The primer 2043 is approximately 200 bp downstream of 1778 and was used instead of 1778 in cases where no PCR product was seen with 1778 and 3194. Table 3.1 shows the primer names, sequences and map locations. Numbers refer to the *mt A* idiomorph sequence (GenBank accession no. M33876).

Table 3.1 Primers used to amplify *mt* genes.

Primer	Sequence (5'-3')	Map Location
1778	ACCCAAACTTCCCACC	3997-4021
3194	ATGGTACCTCATCTTCCACTAACCC	5040-5062
2043	GTTCGCCGAATCCCCGC	4227-5143

PCR

The primers used to amplify the *mt A-1* gene were the *N. crassa* primers, 1778 and 3194, for *N. terricola*, *N. pannonica*, and *G. tetrasperma*. Those primers could not amplify a fragment from *N. galapagosensis*, *N. lineolata* or *N. dodgei*, so 2043 and 3194 were used. Neither of those two primer sets could amplify a fragment from either the homothallic ascomycete, *Sordaria*

macrospora or the heterothallic, *S. brevicollis*, mating type *A*. PCR was performed on a DNA Thermal Cycler 480 (Perkin Elmer, Foster City, California).

Cycling for *N. terricola mt A-1* was as follows: 97 °C for 5', followed by 30 cycles of 95 °C for 30"/56 °C for 30"/72 °C for 1', followed by 72 °C for 5'.

Cycling for *N. pannonica mt A-1* was as follows: 95 °C for 5', 72 °C for 2', followed by 25 cycles of 94 °C for 30"/56 °C for 20"/72 °C for 1', followed by an incubation at 72 °C for 5'.

Cycling for *G. tetrasperma*, *N. galapagosensis*, *N. dodgei* and *N. lineolata mt A-1* was as follows: 95 °C for 5', 72 °C for 1', followed by 25 cycles of 94 °C for 30"/56 °C for 20"/72 °C for 1', followed by an incubation at 72 °C for 5'.

Cloning

The PCR products were purified from low-melt gel slices with the PCR Magicprep™ (Promega, Madison, Wisconsin). Gel-purified fragments were ligated into the cloning vector, pCR™(Invitrogen, San Diego, California). Plasmids were named according to the first letter of the genus, the first letter of the species followed by "A-1". *E. coli* was transformed with the plasmids. DNA was prepared for sequencing with a plasmid kit (Qiagen, Chatsworth, California).

Sequencing

The *mt A-1* gene was amplified for sequencing with the primers 1875, the T7 promoter primer and the SP6 promoter primer (synthesized by the NAPS unit at the University of British Columbia). Sequencing was performed using the ABI Taq DyeDeoxi Terminator cycle method (Mississauga, Ont.) on an ABI 373 automated sequencer at the NAPS Unit, Biotechnology Lab., University of British Columbia.

Alignment

The DNA sequences were aligned with the program PileUp from the Wisconsin Genetics Computer Group (Devereux, Haberli and Smithies, 1984) using the default gap penalty. One small gap of 4 or 5 nucleotides was shifted relative to the paired nucleotides to align apparently homologous nucleotides. The sequences were translated into amino acids with the program Translate (Devereux, Haberli and Smithies, 1984), aligned with PileUp and adjusted manually. *S. macrospora* had two large insertions relative to the *Neurospora* and *Gelasinospora* species, one of 21 nucleotides (seven amino acid residues) in the first exon and the other of 18 nucleotides (six amino acid residues) in the second exon. Both of the insertions occur outside of the region of high amino acid conservation among mating type genes. The resulting alignment was 969 nucleotides long.

Phylogeny Reconstruction

Phylogenetic trees were reconstructed from the aligned sequences with two different methods that use optimality criteria, *i.e.* they reconstruct and evaluate many trees to find the most parsimonious or most likely one. Parsimony methods search for the tree with the fewest evolutionary changes (the smallest number of nucleotide changes, in the case of DNA sequence data), while maximum likelihood methods search for the tree that was most likely to have given rise to the data under the evolutionary hypothesis assumed (or specified). Parsimony methods have an advantage in that computational time is shorter. The trees, however, can be inconsistent (not representative of the true phylogeny) even with a very large amount of data and even if all taxa have evolved at the same rate (Penny and Hendy, 1986). Also, they are not based on a model of evolution, although some evolutionary parameters can be specified, *e.g.* different transition/transversion ratios. While maximum likelihood methods consume more computer time, they are based on a model of evolution. They can, therefore, account for multiple hits or estimate the parameters of the model from the data. Furthermore, they are consistent as long as an appropriate model is used, are less affected by sampling error (have lower variance) and are

robust to user-specified parameters that do not match the true parameters (Swofford *et al.*, 1996).

The programs, PAUP 3.0s (Swofford, 1993) and DNAML (Felsenstein, 1993), were used to infer most parsimonious and maximum-likelihood trees, respectively, from the nucleotide DNA sequence alignment. The search algorithm used in PAUP was branch-and-bound. Constraint trees were constructed in MacClade Version 3 (Maddison and Maddison, 1992).

Trees were inferred from the deduced amino acid alignment using PAUP with a step matrix weighting the changes from one amino acid to another and ProtML from the MOLPHY 2.2 package developed by Adachi and Hasegawa and available by ftp from sunmh.ism.ac.jp or from the Internet at http://evolve.zps.ox.ac.uk/PhySoft/PhySoft.html or http://dogwood.botany.uga.edu/malmberg/software.html.

Results and Discussion

The data presented in this chapter suggest that *A*-only homothallic species have a common ancestor and that *A/a*-homothallic species are more closely related to heterothallic species than they are to *A*-only homothallic species. The rooting of the phylogenetic tree of *Neurospora* was not established with strong statistical support. Evolution from homothallism to heterothallism and vice versa is discussed.

The phylogeny of the genus *Neurospora* was reconstructed from DNA and amino acid alignments of the mating type gene, *mt A-1* or its homolog (Appendix D). The species represented in the phylogenetic reconstruction are shown in Table 3.2.

Table 3.2 Species included in the tree.

Mating type gene	Source	GenBank Accession #
N. discreta	T. Randall (pers. comm.)	L42307
N. intermedia	T. Randall (pers. comm.)	L42308
N. sitophila	T. Randall (pers. comm.)	L42309
N. tetrasperma	T. Randall (pers. comm.)	L42310
N. crassa	Glass, Grotelueschen and Metzenberg, 1990	M33876
N. africana	Glass and Smith, 1994	L42301
N. terricola	this study	
N. pannonica	this study	
N. galapagosensis	this study	
N. lineolata	this study	
N. dodgei	this study	
Gelasinospora tetrasperma	this study	
Sordaria macrospora	Pöggeler *et al.*, 1997b	
Podospora anserina	Debuchy and Coppin, 1992	64194.gb-pl

When the tree was determined without outgroups, there was strong support for an all-homothallic clade and an all-heterothallic clade. Specifically, 100/100 (using maximum likelihood) or 500/500 (using parsimony) bootstrap replicate trees had such a division. The topology of the unrooted trees produced by maximum likelihood and parsimony methods were identical (Figure 3.1). Attempts to find a convincing root for the tree with the outgroup *P. anserina* were unsuccessful and so it was removed from the data set.

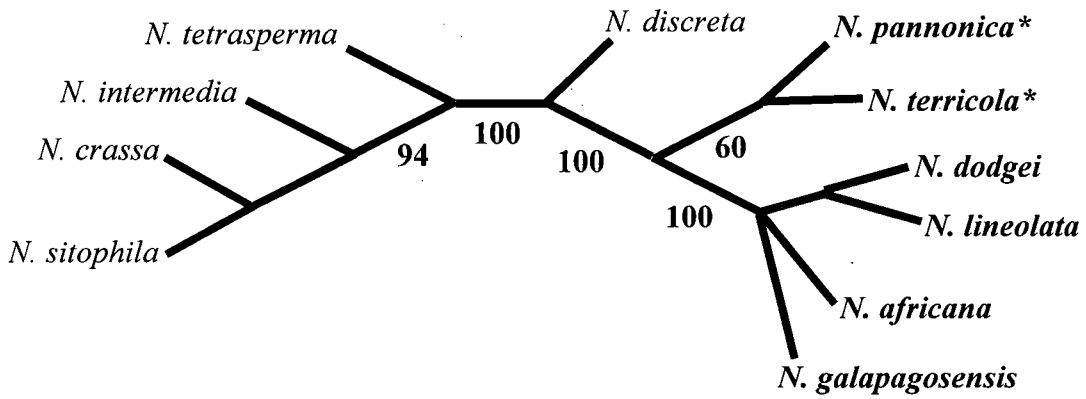


Figure 3.1 Unrooted parsimony tree from DNA sequence alignment. Bootstrap support (%) is shown for 500 replicates. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

A branch-and-bound search in PAUP 3.0s (Swofford, 1993) specifying *G. tetrasperma* and *S. macrospora* as outgroups found three shortest trees of 343 steps. In a consensus of the 343 trees rooted with *S. macrospora*, *Neurospora* was not monophyletic (Figure 3.2). The basal split in the tree separated one cluster comprising the four *Neurospora A*-homothallic species from a second, heterogeneous cluster including the *A/a*-homothallic *Neurospora* species, the heterothallic *Neurospora* species and *G. tetrasperma*. Within the second cluster, the branching order of the *A/a*-homothallic species, the heterothallic group and *G. tetrasperma* was unresolved. No bootstrap support was shown for including *G. tetrasperma* in *Neurospora*, and so there is no reason to include *G. tetrasperma* within the genus *Neurospora*. In 500 bootstrap replicates, the *A*-homothallic species always clustered together, showing that they are more closely related to each other than to any of the heterothallic species. Similarly, the heterothallic *Neurospora* species clustered together in 99% of 500 bootstrap replicates, showing that they are more closely related to each other than to any of the homothallic species.

When *G. tetrasperma* was removed from the data set, the placement of the outgroup, *S. macrospora*, on the tree was supported by 100% bootstrap support (500 bootstrap replicates) relative to the *A*-homothallic group and 80% relative to the *A/a*-homothallic/heterothallic group (Figure 3.3). This tree suggests that the *A/a*-homothallic species are more closely related to the heterothallic species than they are to the *A*-homothallic species. Morever, the tree suggests that homothallism is the ancestral state in *Neurspora*. A direct test of the hypothesis that *Neurospora* evolved from a homothallic ancestor are presented below.

Other trees were constructed from the tree with *G. tetrasperma* as the outgroup to test the statistical support for the rooting. The topology of the trees rooted with alone produced by maximum likelihood and parsimony methods were identical (Figure 3.4). In approximately half of the bootstrap replicate trees, *G. tetrasperma* rooted the tree between the *A*-only homothallic *Neurospora* species and the *A/a*-homothallic/heterothallic group (Figure 3.4). Constraint trees were constructed in which all branches were collapsed except the one that rooted the tree. That

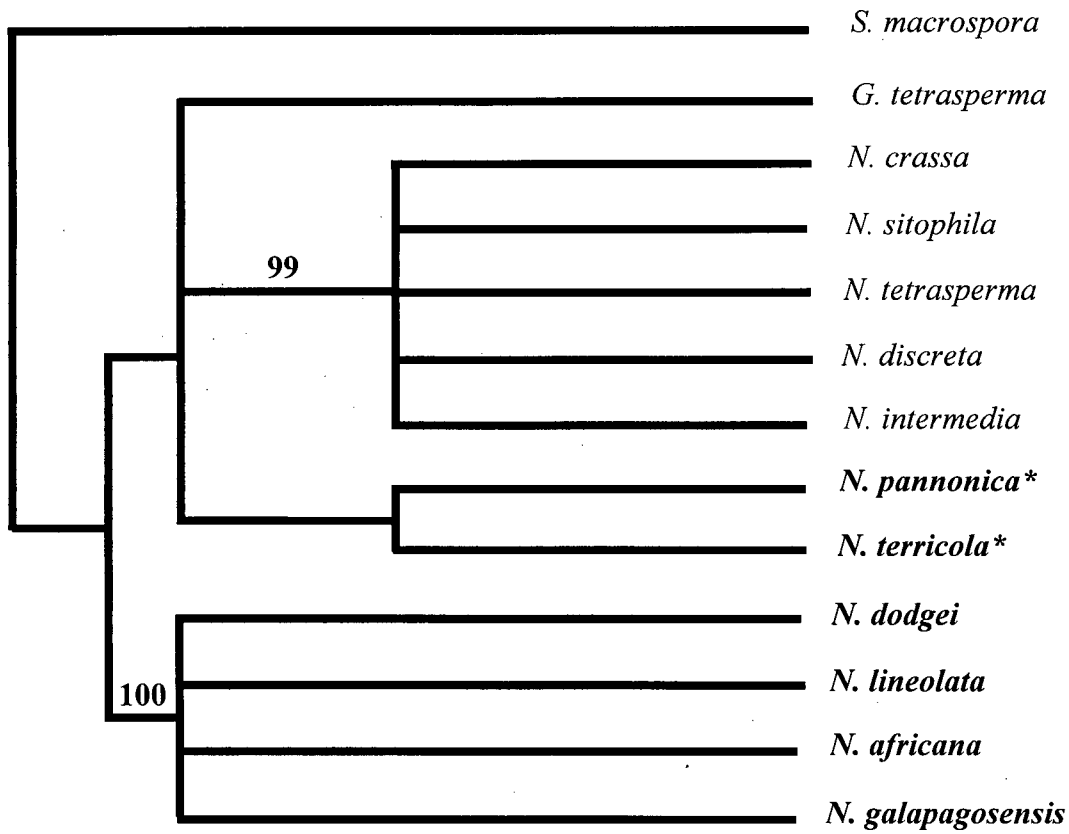


Figure 3.2 Parsimony tree from DNA sequence alignment, specifying *S. macrospora* and *G. tetrasperma* as outgroups. Bootstrap support (%) is shown for 500 replicates. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

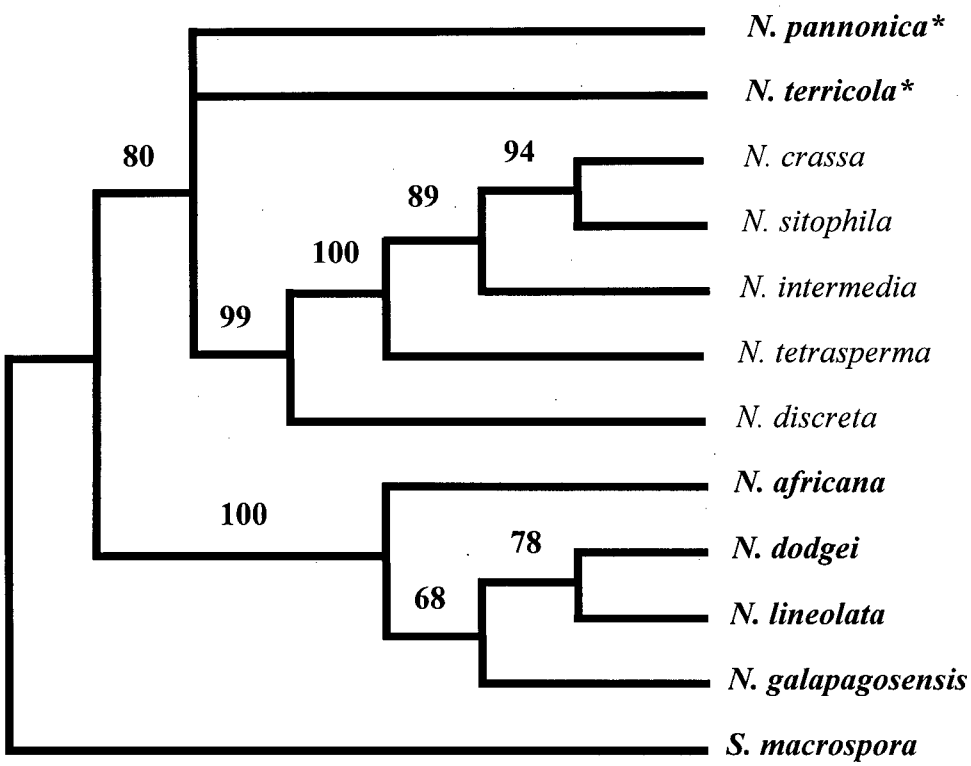


Figure 3.3 Rooted parsimony tree from DNA sequence alignment. Bootstrap support (%) is shown for 500 replicates. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

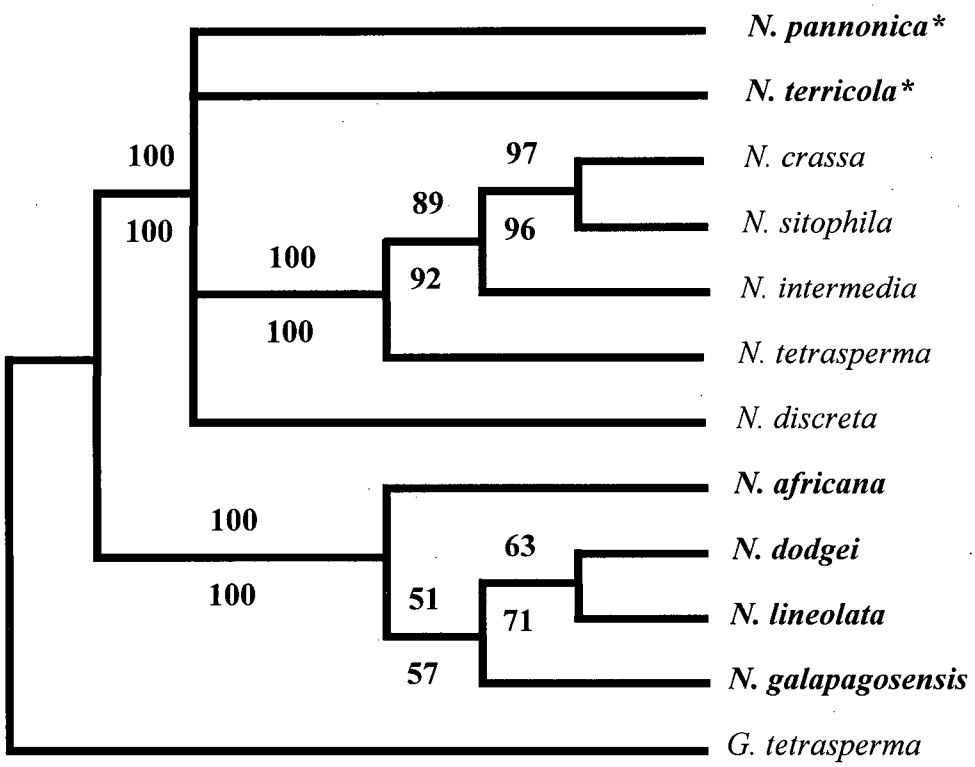


Figure 3.4 Rooted maximum likelihood/parsimony tree from DNA sequence alignment. Bootstrap support (%) is shown on the top for 500 replicates in DNAML; on the bottom for 100 replicates in PAUP. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

branch was moved to see if the competing root placement (between the homothallic and heterothallic species) significantly lengthened the tree. Comparison of constraint trees in which *G. tetrasperma* rooted the tree either before (Figure 3.5) or after (Figure 3.6) the divergence of homothallic species from heterothallic species revealed that the most likely and most parsimonious rooting (Figure 3.5) required only one fewer nucleotide change than did the competing rooting. The rooting, therefore, could not be done with confidence using *G. tetrasperma* since one placement of the root was not significantly better than the other.

Many ascomycete genera and families include both homothallic and heterothallic species, suggesting that there have been multiple independent derivations of homothallism and/or heterothallism (Nauta and Hoekstra, 1992a). Population genetic modeling suggested that evolution from heterothallism to homothallism is much more likely than vice versa (Nauta and Hoekstra, 1992a). The model was based on a hypothetical fungus with the following characteristics: life cycle was haploid with a short diplophase, each individual could form male and female sexual structures and the male gametes could double as asexual spores. Their assumptions seem reasonable for *Neurospora*.

All homothallic species in other fungal genera have both *A* and *a* sequences and they are linked (see Chapter 2 Discussion). The universality of this type of homothallic species suggests that the *A*-only *Neurospora* species arose by a unique evolutionary pathway. If such is the case, the two homothallic mating strategies are independent and thus the ancestral state cannot be determined.

An alternative explanation of the rooting is that it may be affected by the mating strategy of the chosen outgroup. *S. macrospora* is homothallic and *G. tetrasperma* is pseudohomothallic. This hypothesis could be tested by adding the *mt A-1* sequence from a heterothallic *Sordaria* or *Gelasinospora* species or by constructing a tree from the DNA sequence of a non-mating type gene. It was not tested here.

Since the tree was based on sequences of one of the mating type genes, it is a gene tree, not a species tree. If the *mt A-1* gene has a different function in *A/a*-homothallic/heterothallic species than it does in the *A*-only homothallic species, then the clustering could be reflecting

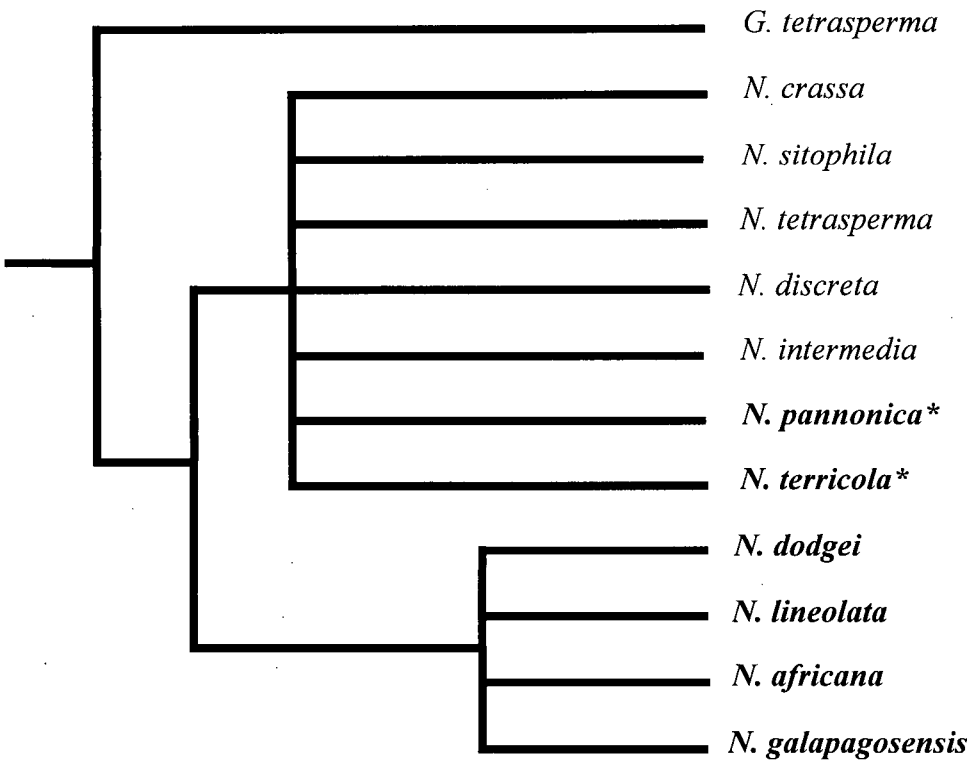


Figure 3.5 Constraint tree with same rooting as maximum likelihood/parsimony tree with branches collapsed to allow comparison to the alternately rooted tree in Figure 3.5. Tree length is 213 nucleotide changes. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

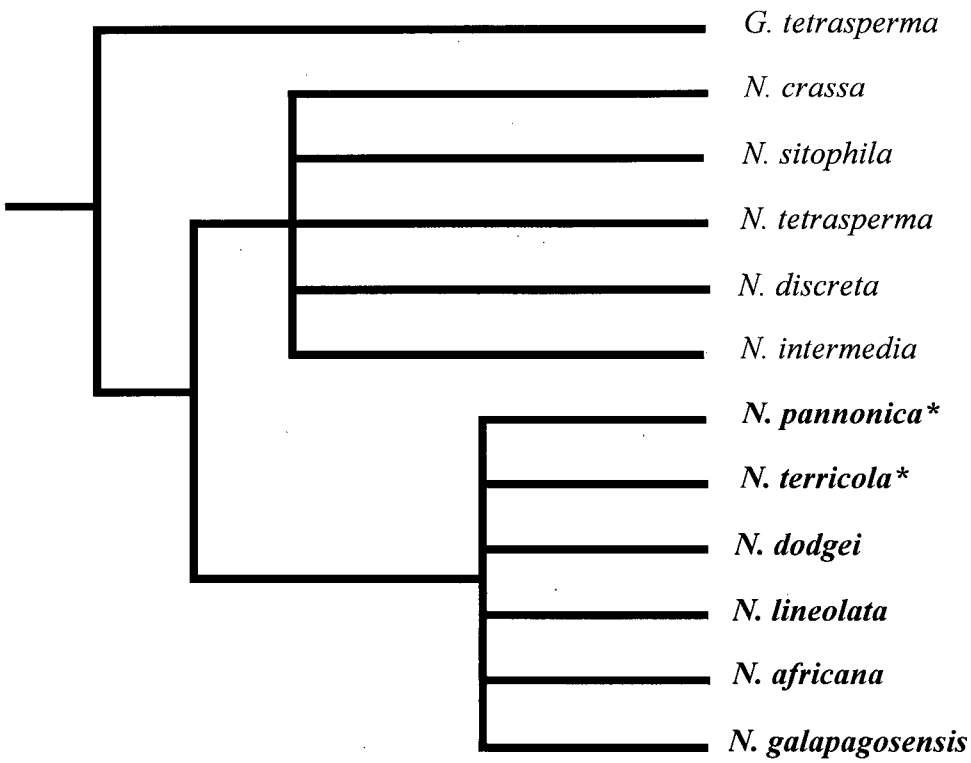


Figure 3.6 Constraint tree with an all-homothallic clade. Tree length is 212 nucleotide changes. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

functional rather than evolutionary similarity. One piece of evidence that counters this idea is that heterothallic- and homothallic-specific sequences have been detected in the mating type idiomorph flanks (Randall and Metzenberg, 1995).

The hypothesis that the heterothallic or the homothallic species are grouping because of the conservation of particular sets of nucleotides required for the possibly different functions of *mt A-1* in heterothallic versus homothallic species was tested. The tree based on an alignment of the amino acids was made using parsimony (PAUP) and maximum likelihood methods (ProtML in the MOLPHY 2.2 package and PAUP with a step matrix weighting the changes from one amino acid to another that makes the method essentially equivalent to the maximum likelihood method). Both methods found trees with the same topology (Figure 3.7). Bootstrap replicate trees were examined to see if there were clusters of amino acids on the branch separating heterothallic from homothallic species. No clusters of three or more amino acids were found, suggesting that there are no large regions of *mt A-1* that are different between homothallic and heterothallic species. Of course, just one or two amino acid differences may be sufficient to confer homothallic- or heterothallic-specific usage of *mt A-1* and these would have gone undetected. The lack of large differences supports the hypothesis that the *mt A-1* gene does not have heterothallic- or homothallic-specific functions and supports its use in this phylogenetic analysis.

In addition, the amino acid sequences were scrutinized to reveal open reading frames (ORFs) or lack thereof. If the homothallic ORFs were truncated, then they are probably not functional, which could account for the grouping of heterothallic species apart from homothallic species. All of the heterothallic species (*N. crassa, N. discreta, N. sitophila, N. intermedia*), pseudohomothallic species (*N. tetrasperma, G. tetrasperma*), *A/a* homothallic species (*N. terricola, N. pannonica*) and one of the four *A*-only homothallic species (*N. africana*) contain an uninterrupted ORF of the same size. The size of the ORF in the 3 *A*-only homothallic species, *N. lineolata, N. dodgei* and *N. galapagosensis*, is unknown because one primer 5' to the ATG failed to amplify a product in a PCR. The 5' primer that amplified a product from which the sequence was derived starts at position #109 in the *N. crassa* ORF. The available sequences for

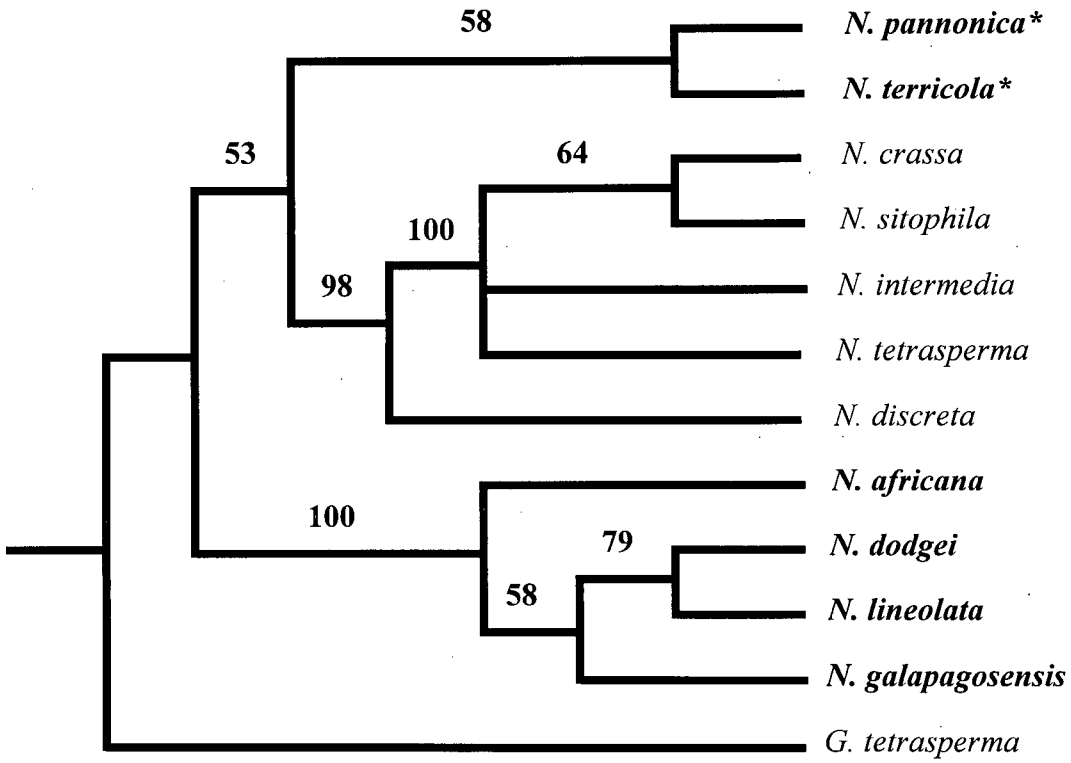


Figure 3.7 Rooted parsimony tree from amino acid sequence alignment. Bootstrap support (%) is shown for 100 replicates. Boldface type denotes homothallic species, with *A/a* homothallic species marked with an asterisk.

the 3 *A*-only homothallic species encode products highly similar to the products from the other species and include a stop codon at the same position.

Support for the use of the mating type gene for the phylogenetic analysis of *Neurospora* is found by comparing the *mt A-1* trees to those based on other characters. While the *mt A-1* trees included all known *Neurospora* species, the latter trees included some, but not all. The latest publication included three different trees of the heterothallic *Neurospora* species, based on restriction maps of eight randomly chosen 40-kbp cosmid probes, a 400-bp sequence of a region upstream of the *al-1* gene and a 500-bp sequence of a region upstream of the *frq* gene (Skupski, Jackson and Natvig, 1997). A tree based on restriction maps of the *frq* gene included the heterothallic *Neurospora* species and one homothallic species (Lewis and Feldman, 1996). Randall and Metzenberg (1995) reconstructed the phylogeny of the heterothallic species and one homothallic species based on the sequence of the *mt A-1* gene. DNA sequence and restriction fragment length polymorphisms of the *frq* gene were used to build a tree of *N. crassa*, *N. intermedia* and *N. discreta* with *Sordaria* and *Gelasinospora* species as outgroups (Merrow and Dunlap, 1994). Mitochondrial RFLPs were used to determine the relationships among *N. crassa*, *N. intermedia*, *N. sitophila* and *N. tetrasperma*, which have proven difficult to resolve (Taylor and Natvig, 1989). The earliest attempt to resolve these relationships was a study that utilized RFLPs of four cloned fragments of nuclear DNA (Natvig, Jackson and Taylor, 1987).

The topology of the trees based on the *mt A-1* sequence is congruent with that based on the sequence and RFLPs of the *frq* gene. The tree shows that *N. crassa* and *N. intermedia* are more closely related to each other than to *N. discreta*, and that *G. cerealis* and *S. fimicola* are outgroups to these three *Neurospora* species (Merrow and Dunlap, 1994). The topology of the tree based on the restriction map of the *frq* gene corroborates the *mt A-1* tree inasmuch as *N. tetrasperma*, *N. sitophila* and *N. crassa* are more closely related to each other than to *N. galapagosensis* (Lewis and Feldman, 1996). Not surprisingly, the tree based on DNA sequence from the mating type region (Randall and Metzenberg, 1995) has the same topology as this *mt A-1* tree.

The relationship between *N. tetrasperma* and three of the heterothallic species, *N. intermedia*, *N. crassa* and *N. sitophila*, is the same in both the mitochondrial RFLP tree (Taylor and Natvig, 1989) and the *mt A-1* tree, upholding the finding that *N. tetrasperma* diverged from the other three species before their divergence from each other. This relationship is contradicted by the *frq* restriction map tree (Lewis and Feldman, 1996), the *frq*-flank sequence tree, the *al-1*-flank sequence tree and the random cosmid tree (Skupski, Jackson and Natvig, 1997). The relationship of *N. sitophila* to *N. tetrasperma* remains unresolved. Some trees show them to be sister taxa while others suggest that *N. tetrasperma* diverged from the heterothallic group prior to the divergence of *N. sitophila* from the other heterothallic species.

Some branches of the mating type tree are not found in trees based on other characters. Uniquely, the mating type tree places *N. crassa* and *N. sitophila* as sister groups at the tip of the tree, with *N. intermedia* diverging prior to the *N. crassa/N. sitophila* split. In contrast, the trees reconstructed from the *frq*-flank sequence, the *al-1*-flank sequence and the random cosmids, show that *N. crassa* and *N. intermedia* are more closely related to each other than to *N. sitophila* and *N. tetrasperma* (Skupski, Jackson and Natvig, 1997). Also, in the *frq* restriction map tree, *N. sitophila* and *N. tetrasperma* are more closely related to each other than they are to *N. crassa* (Lewis and Feldman, 1996), a finding echoed in the nuclear RFLP tree (Natvig, Jackson and Taylor, 1987). Biological data also contrast with the mating type trees. Interspecific crosses between *N. crassa* and *N. intermedia* produce abundant inviable ascospores, whereas *N. crassa* x *N. sitophila* crosses are relatively infertile (Perkins, Turner and Barry, 1976). Multiple isolates of each species were used for the analysis of the mating type (Randall and Metzenberg, 1995) and some of the opposing trees, suggesting that the difference is not strain-specific.

The mating type regions of *N. crassa*, *N. sitophila* and *N. intermedia* have a different evolutionary history than other parts of the genome (Randall and Metzenberg, 1995; this study; Skupski, Jackson and Natvig, 1997), even regions on the same linkage group (Skupski, Jackson and Natvig, 1997). The idea that genes do not necessarily share evolutionary histories is supported by the maximum-likelihood analysis of the *frq*-flank and *mt A-1* trees (Skupski, Jackson and Natvig, 1997; Randall and Metzenberg, 1995). The distinct relationship of the

mating type regions could be explained by lineage sorting of an ancestral polymorphism in the *mt A-1* gene or hybridization between the *N. crassa* and *N. sitophila* that resulted in the transfer of the *mt A-1* region. In support of hybridization, the mating type region of one group of *N. tetrasperma* isolates appears to have been derived from *N. intermedia*-like ancestors while another group appears to have been derived from *N. intermedia a* x *N. sitophila A* or *N. intermedia a* x *N. crassa A* (R. L. Metzenberg, personal communication). Also, the semi-permeability of fertility barriers among all *Neurospora* heterothallic species (Perkins, Turner and Barry, 1976) suggests that occasional hybridization could be occurring in nature.

One relationship worth comparing is that between sexual and asexual species. Asexual filamentous ascomycetes appear to be derived frequently and recently from sexual species (LoBuglio, Pitt and Taylor, 1993; Geiser, Timberlake and Arnold, 1996). Homothallic *Neurospora* species may be similar to asexual species in that both self-reproduce. Homothallic species differ from asexual species in that they undergo sexual processes and may possess outbreeding potential. In contrast to asexual ascomycetes, homothallic *Neurospora* species appear to survive long enough to diverge. They may live longer or simply evolve more quickly than asexual species.

Homothallic species undergo meiosis which may confer advantages through recombination. Recombination is believed to exert is effect through Muller's ratchet, the idea that, in the absence of recombination, each generation possesses no fewer, and possibly more mutations than were possessed by the generation before (Muller, 1964). Experiments designed to show that meiosis confers advantage even without recombination have been difficult to control adequately (*e.g.* Birdsell and Wills, 1996).

In theory, the production of an asexual spore is genetically equivalent to the production of a sexual spore in a self-mating haploid organism (Nauta and Hoekstra, 1992a) in that neither propagule has undergone recombination and therefore does not contain novel deleterious associations of alleles. One cannot discount the possibility that different mutations occur in different nuclei in homothallic species and sex allows recombination. This amount of recombination is unlikely to have a major effect (S. Otto, personal communication).

In addition to the hypothetical advantages that may be conferred to homothallic *Neurospora* species by meiosis and/or ascospores, occasional outbreeding may occur in nature by heterokaryosis (Nauta and Hoekstra, 1992b). Rare outbreeding may be nearly equivalent to frequent outbreeding in terms of long-term selective advantages conferred by recombination (Muller, 1964).

Hypotheses about the evolution of homothallism and heterothallism can be divided into two groups, those which suggest that homothallism evolved first and those that suggest that heterothallism evolved first. Olive (1958) started with the assumption that homothallism is the ancient mating system and heterothallism is the derived one based on intuition: "It is readily apparent that a system permitting karyogamy between any two haploid nuclei in a thallus (homothallism) is less modified than one involving two types of thalli, the nuclei of which are self-incompatible but cross-compatible (heterothallism)". The compound nature of the basidiomycete mating type locus had just been elucidated, leading Olive (1958) to suggest that heterothallic species were derived from a homothallic ancestor by deletion of one gene in a strain and deletion of the other gene in a different strain. Such strains were hypothesized to be self-sterile and cross-fertile. This hypothesis is virtually identical to that proposed by Charlesworth (1994) in which he invokes the same events to account for the evolution of mating type idiomorphs.

Metzenberg and Glass (1990) and Glass, Metzenberg and Raju (1990) suggest that heterothallic species predate extant homothallic species. The *A/a* homothallic species could have arisen after an unequal crossover event led to the co-nuclearization of the mating type genes. When the sequences surrounding the mating type regions are considered, however, a crossover that accounts for all the data cannot be contrived. For a progression such as the one illustrated in Figure 3.8 to occur, either one of two events may have occurred, one which has not been known to happen in any organism and one which contradicts the data. The first event would have been the highly unlikely recombination between non-homologous sequences. The second would have been a DNA end-joining event, in which case the two mating type chromosomes, one from each parent, would have been joined as well. Even if an event had

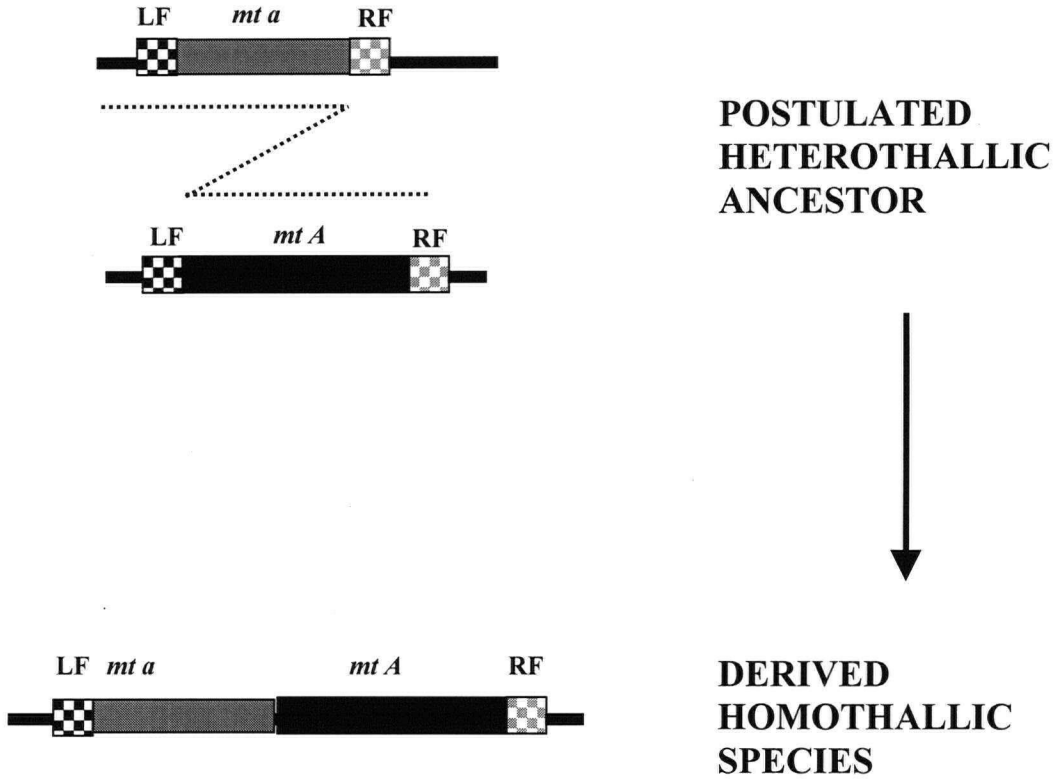


Figure 3.8 Model for the evolution of homothallic species from a heterothallic ancestor. Checkerboard boxes represent sequences homologous to the *N. crassa* left flank, LF (black), and right flank, RF (grey). Solid boxes represent the *mt a* (grey) and *mt A* (black) genes. Dotted line shaped like a Z illustrates the position of the most plausible recombination event.

occurred to accomplish the transition illustrated in Figure 3.8, 12-20 kbp of DNA would have had to insert precisely between the two mating type idiomorphs. Alternative explanations are possible, but they are even less likely.

An alternative model inspired by that proposed by Charlesworth (1994) is introduced in Figure 3.9. Charlesworth (1994) proposed that in an initially self-fertile population, mutation at a recognition locus (*e.g.* a diffusible signal required for the recognition of gametes) results in a strain that cannot self-fertilize, but can still be fertilized. Such a strain might be at a selective advantage, presumably due to outbreeding. A second mutation in another strain at a reciprocal, linked locus (the receptor of the diffusible signal) might also be favoured, as would suppression of recombination between them. Eventually two self-sterile, cross-fertile types would remain. Suppression of recombination between the genes would allow them to evolve independently. Furthermore, the two non-functional alleles could change relatively rapidly by genetic drift. These two processes could result in the formation of idiomorphs.

His hypothesis requires that clustering of recognition genes predate the genesis of idiomorphs, which is not difficult to imagine. Clustering of identity genes may be advantageous to an organism because of the preservation of heterozygosity that is afforded by limiting recombination between the genes. Identity encoded by idiomorphs has advantages over identity encoded by alleles. Distinction is ensured by the elimination of homologous recombination between the idiomorphs. Furthermore, a simple reversion could destroy mating specificity determined by alleles, but not by idiomorphs.

The model I propose in Figure 3.9 provides a plausible explanation for the evolution from homothallism to heterothallism, the evolution of idiomorphs, the existence of a *cis*-acting regulator able to control genes in both mating type regions, *A* and *a*, as we know *N. crassa*'s can (Chang and Staben, 1994) and, finally, the combinations of mating type genes with various flanking sequence blocks.

The ancestral *Neurospora* species is proposed to have been homothallic with a genetic constitution like that of *N. pannonica* (L. Wheeler and N. L. Glass, personal communication), in that it possessed all four mating type genes, *mt A-1*, *mt A-2*, *mt A-3* and *mt a-1* on one

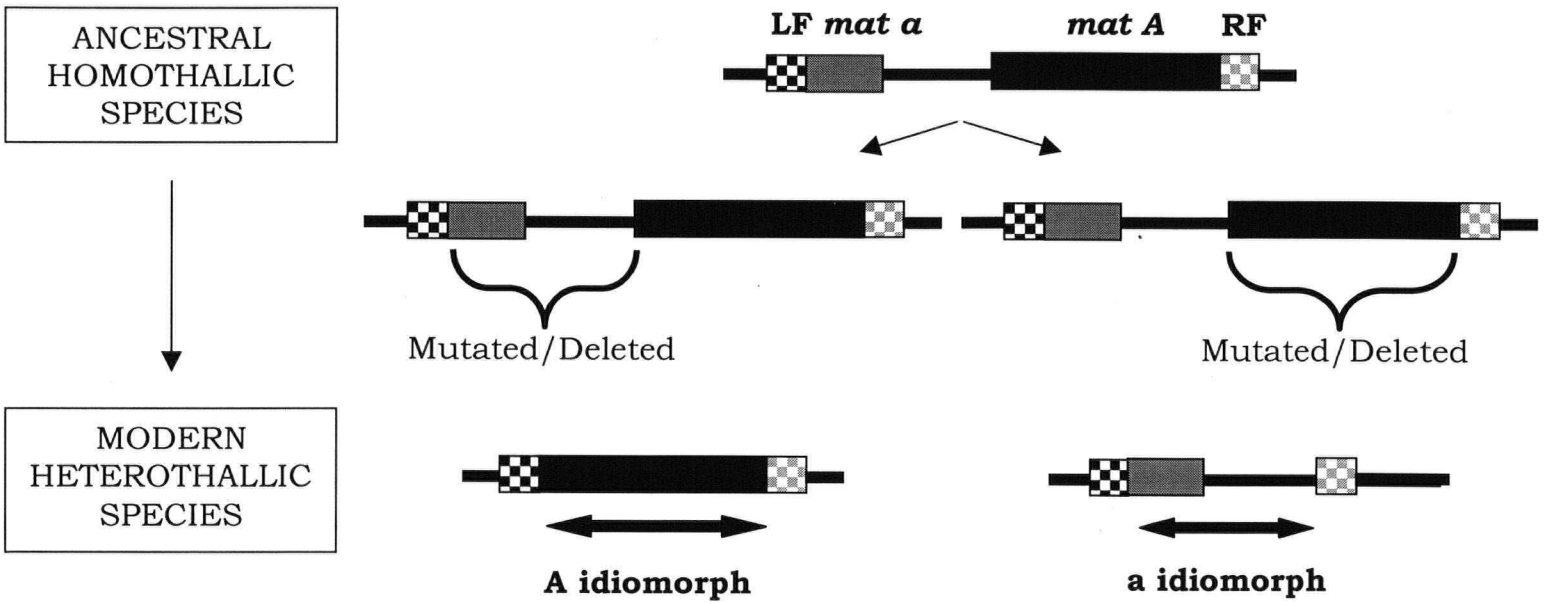


Figure 3.9 Model for the evolution of heterothallic species from a homothallic ancestor. Checkerboard boxes represent sequences homologous to the *N. crassa* left flank, LF (black), and right flank, RF (grey). Solid boxes represent the *mat a* (grey) and *mat A* (black) genes.

chromosome. I speculate that this cluster of genes may have encoded transcription factors that controlled entry into the sexual phase.

I propose that mutations in one gene in one strain and the other gene set in a different strain may have given rise to two self-sterile, cross-fertile individuals. The following events detail this process. I propose that the *a* gene was flanked on the left side by a sequence block (LF) homologous to the block abutting the centromere-proximal side of the *N. crassa* idiomorphs. I propose that the *A* genes resided next to a sequence block homologous to the RF sequence which abuts the *N. crassa* idiomorphs on the centromere-distal (right) side. I propose that a subpopulation arose in which the *mt a* gene was mutated and no longer functional, and was, therefore, free to diverge away from the original sequence. The *mt a* gene and the DNA between the two genes may have been deleted, resulting in a single mating-type strain with a *mt A* idiomorph flanked by RF and LF. Similarly, a mutated *mt A-1* gene could have been free to diverge or be deleted. The resultant strain could have been of mating type *a*, again flanked by RF and LF, just like *N. crassa*. The *N. crassa a* idiomorph contains 2 kbp of sequence that appear to be peripheral to mating type function (Glass *et al.*, 1988) and I suggest that this stretch of DNA may have come from the sequences between the *A* and *a* regions in the ancestral homothallic species.

The putative *cis*-acting regulator may have regulated both *A* and *a* expression by virtue of its position relative to both genes. In all homothallic Sordariaceae containing both *A* and *a* genes, the two genes are linked (Glass, Metzenberg and Raju, 1990). I propose that the *cis*-acting regulator still exists in *N. crassa* and can act on both *A* and *a* genes. The *A*-only homothallic species could have arisen by loss or replacement of *mt a* function.

Thus, the model joins the two main issues addressed in this thesis, namely, positional regulation and evolution of mating type.

Summary

The mechanism of *N. crassa* mating type position effect has been examined. The position effect is likely due to the requirement of the genes to be present on the same molecule of DNA as a *cis*-acting sequence, possibly in a specific orientation or spacing. Many such elements are known to exist, although none has yet been found in a filamentous fungus.

The homothallic species *N. terricola* contains a subset of the *N. crassa* mating type genes. One gene from each mating type, the one specifying mating identity in *N. crassa*, appears to be functional. These results raise the questions of how could the position effect be modified in *N. terricola* to allow opposite mating type genes to express full fertility, something that cannot be done in *N. crassa*? What could mating type genes be doing in a selfing organism?

Homothallic *Neurospora* species that carry both mating type genes appear to be more closely related to heterothallic species than they are to the *A*-only homothallic species, suggesting that the two types of homothallism may have evolved independently. The issue of whether *Neurospora* species evolved from a homothallic or a heterothallic ancestor remains unresolved, but a model has been put forth.

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Appendix A *Neurospora crassa* Gene Symbols

Symbol	Description*
ad-2	adenine
ad-3A	adenine
ad-3B	adenine
al-1	albino
am	amination deficient (NADP-specific glutamate dehydrogenase)
arg-2	arginine
Asm-1	ascospore-maturation deficient
cyh-1	cycloheximide resistant
fl	fluffy (no macroconidia, female hyperfertile)
frq	frequency (disorganized circadian rhythm)
lys-1	lysine
mt	mating type
nic-2	nicotinic acid
pan-2	pantothenic acid
qa-2	quinate catabolism
thi-4	thiamine
tol	tolerant (mating type compatible)
trp-4	tryptophan
un-3	unknown heat-sensitive defect

* Perkins 1996

Appendix B Slot blot Analysis

Table A. Values representing relative optical density of slots.

3-day-old mycelia, *crp-1* probe

1415	3020	4207
1552	3257	4294
1469	3080	4258
439	744	1411
1047	897	1389
921	727	1189

3-day-old mycelia, *mt A-1* probe

2088	3211	4394
2451	4109	4208
1955	3054	4312
964	965	1335
1132	1602	2085
1162	1019	1567

6-day-old mycelia, *crp-1* probe

1150	2199	4852
1180	2336	4921
1220	2223	4886
203	1482	4322
122	1369	4113
377	1583	4253

6-day-old mycelia, *mt A-1* probe

2356	3387	4865
3069	4551	4769
2738	3722	4701
394	2912	3327
559	3241	3940
500	2864	3484

6-day-old mycelia, *β-tubulin* probe

5485	9036	2221	3931
3627	5329	1043	2754
4264	6418	1560	2990

6-day-old mycelia, *mt A-3* probe

501.2	856.0	155.0	337.0
91.84	187.0	29.55	64.02
125.7	249.6	41.03	88.93

N. B. Each set of three numbers represents three measurements of one slot. The layout of this table can be superimposed on the appropriate slot blot. Top numbers within each slot blot are directly comparable, as are middle numbers and bottom numbers, since the width of the slot slice was kept constant for each set of measurements.

Table B. Values representing ratios of mt-rel-Ac6 to 74-OR23-1*A*.

3-day-old mycelia, *crp-1* probe

3.2	4.1	3.0
1.5	3.6	3.1
1.6	4.2	3.6
mean=2.1	mean=4.0	mean=3.2
s^2=.91	s^2=.11	s^2=.11

3-day-old mycelia, *mt A-1* probe

2.2	3.3	3.3
2.2	2.6	2.0
1.7	3.0	2.8
mean=2.0	mean=3.0	mean=2.7
s^2=.09	s^2=.13	s^2=.43

6-day-old mycelia, *crp-1* probe

5.7	1.5	1.1
9.7	1.7	1.2
3.2	1.4	1.1
mean=6.2	mean=1.5	mean=1.1
s^2=11	s^2=.03	s^2=.01

6-day-old mycelia, *mt A-1* probe

6.0	1.2	1.5
5.5	1.4	1.2
5.5	1.3	1.3
mean=5.7	mean=1.3	mean=1.3
s^2=.09	s^2=.01	s^2=.03

6-day-old mycelia, *β-tubulin* probe

2.5	2.3
3.5	1.9
2.7	2.1
mean=2.9	mean=2.1
s^2=.28	s^2=.04

6-day-old mycelia, *mt A-3* probe

3.2	2.5
3.1	2.9
3.1	2.8
mean=3.1	mean=2.7
s^2=.01	s^2=.05

N. B. Each number was derived from Table 10.5 by dividing the mt-rel-Ac6 value by the appropriate 74-OR23-1*A* value.
s^2 = sample variance

Table C. Calculation of differences between control and experimental means of ratios from Table B.

Slots being compared	Pooled variance	Test statistic	Conclusion
3-day *crp-1/mt A-1* 1x	.50	.14	same
3-day *crp-1/mt A-1* 2x	.12	3.6	different
3-day *crp-1/mt A-1* 4x	.27	1.2	same
6-day *crp-1/mt A-1* 1x	5.5	.26	same
6-day *crp-1/mt A-1* 2x	.13	.70	same
6-day *crp-1/mt A-1* 4x	.13	.70	same
β-tubulin/mt A-3 1x	.14	.65	same
β-tubulin/mt A-3 2x	.043	3.6	different

Let $\alpha = 0.05$. The critical values of t are $\pm$ 2.7764, therefore accept H_O if $-2.7764 < t_{calculated} < 2.7764$.

Appendix C Mann-Whitney Rank-Sum Tests

T = sum of ranks in the smaller sample
n_S = number of observations in the smaller sample
n_B = number of observations in the bigger sample
For n_S less than or equal to 8, compare T to the critical values of the Mann-Whitney Rank-Sum Statistic T, for n_S greater than 8, convert T to z_T.

$\mu_T = (n_S (n_S + n_B + 1))/2$

$\sigma_T = \text{sqrt}[(n_S n_B (n_S + n_B + 1))/12]$

$z_T = (| T - \mu_T | - .5)/ \sigma_T$

$t_{.05}$ (df inf.) = 1.9600

If z_T is greater than $t_{.05}$ (df inf.), then the probability that the ranks would have fallen as seen if the two samples were taken from the same population would be 5%. In other words, the two samples are significantly different.

Table A. Number of ascospores per rosette 8 days post-fertilization (*fl A* x 5*a* and *fl a* x 8*A**)

fl A x 5*a*	rank	*fl a* x 8*A**	rank
24	19	0	7
28	20	0	7
30	21	0	7
32	22	0	7
56	23	0	7
96	24	0	7
132	25	0	7
272	26	0	7
		0	7
		0	7
		0	7
		0	7
		0	7
		1	14
		4	16
		4	16
		4	16
		16	18

T	n_S	n_B	μ_T	σ_T	z_T
180	8	18	108	18	3.972>1.96, so populations are different

Table B. Number of ascospores per rosette 8 days post-fertilization (*fl a* x 74-OR23-1A and *fl a* x *T(I-> II) 39311*).

fl a x 74-OR23-1A	rank	*fl a* x *T(I->II) 39311*	rank
112	1	120	2
136	4.5	128	3
152	7.5	136	4.5
168	10	144	6
216	11	152	7.5
240	12.5	160	9
280	14	240	12.5

T	n_S	n_B
60.5	7	7

Critical values (two-tailed) of the Mann-Whitney Rank-Sum statistic T for $n_S = n_B = 7$ are 37 and 68 at P = 0.053, indicating that the two populations are not significantly different.

Table C. Number of ascospores per rosette 8 days post-fertilization (*fl A* x 5*a* and *fl A* x 46*A**/*a*).

fl A x 5*a*	rank	*fl A* x 46*A**/*a*	rank
24	35	0	17
28	36	0	17
30	37	0	17
32	38	0	17
56	39	0	17
96	40	0	17
132	41	0	17
272	42	0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		0	17
		4	34

T	n_S	n_B	μ_T	σ_T	z_T
308	8	34	172	31.22	4.34>1.96, so populations are different

Table D. Number of ascospores per rosette 8 days post-fertilization (*fla* x 8*A** and *fl a* x 46 *A*/a*).

fl a x 8*A**	rank	*fl a* x 46*A*/a*	rank
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
0	16	0	16
1	32	0	16
4	34	0	16
4	34	0	16
4	34	0	16
16	36	0	16

T	n_S	n_B	μ_T	σ_T	z_T
378	18	18	333	31.61	1.41<1.96, not significantly different

Table E. Number of asci per rosette 13 days post-fertilization (*fl a* x 8*A** and *fl a* x 46*A**/*a*).

fl a x 8*A**	rank	*fl a* x 46*A**/*a*	rank
0	13	0	13
0	13	0	13
0	13	0	13
0	13	0	13
1	26.5	0	13
1	26.5	0	13
3	28	0	13
4	29	0	13
5	30	0	13
6	32.5	0	13
6	32.5	0	13
6	32.5	0	13
6	32.5	0	13
7	36	0	13
7	36	0	13
7	36	0	13
10	38	0	13
13	39	0	13
		0	13
		0	13
		0	13

T	n_S	n_B	μ_T	σ_T	z_T
507	18	21	360	35.50	4.13>1.96, so populations are different

Appendix D DNA and Amino Acid Alignments

1 DNA Alignment of *Neurospora* Species, *G. tetrasperma* and *S. macrospora* used to generate Figure 3.3.

```
NCRASSAXXX ATGTCGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NSITOPHILA ATGTCGGGAGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NINTERMEDI ATGTCGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NTETRASPER ATGCTGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NTERRICOLA ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
NPANNONICA ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
GTETRASPER ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
NDODGEIXXX NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NAFRICANAX ATGTCGTGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCACTGAGGG
NGALAPOGOS NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NLINEOLATA NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NDISCRETAX ATGTCGGGCGTCGACCAAATTGTCAAGACGTTCGCCGACCTCGCTGAGGA
SMACROSPOR ATGTCCAGCGTCGATCAAATCGTCAAGACGTTCGCCAAACTCCCTGAGGG

NCRASSAXXX CGACCGTGAAGCGGCAATGAGAGCTTTCTCAAGGATGATGCGTAGAGGTA
NSITOPHILA CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NINTERMEDI CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NTETRASPER CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NTERRICOLA CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
NPANNONICA CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
GTETRASPER CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
NDODGEIXXX NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NAFRICANAX TGATCGTGAAGCGGCAATGAGAGCTTTCTCAATGATGATGC-NNNNNGCA
NGALAPOGOS NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NLINEOLATA NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NDISCRETAX CGACCGCGAA-NNGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
SMACROSPOR CGAGCGCAACGCAGCAGTCAATGCTATCTTAGCCATGATGCCCCCCGGCC

NCRASSAXXX CCGAACCTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NSITOPHILA CCGAACCTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NINTERMEDI CCGAACCTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NTETRASPER CCGAACCTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NTERRICOLA CCGAACCTGTTCGCCAAAC-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NPANNONICA CCGAACCTGTTCGCCAAAC-NNNNNNNNNNNNNNNNNNNNCCCCGCGGTAAAGAAGAAGGT
GTETRASPER CCGAACCTGTTCGCCAAAG-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NDODGEIXXX NNNNNNNTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCGAAGAAGAAGGT
NAFRICANAX CCGAACCTGTTCGCCAAAC-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NGALAPOGOS NNNNNNNTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NLINEOLATA NNNNNNNTGTTCGCCGAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGGCAAAGAAGAAGGT
NDISCRETAX CCGAACCTGTTCGCCAAAT-NNNNNNNNNNNNNNNNNNNNCCCCGCGACAAAGAAGAAGGT
SMACROSPOR CTGGTCCTGTTCGCCAAATCCCCGAACCTGTTTCACAAGCCCCCGCGCCAAAGAAGAAGGT
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NCRASSAXXX CAACGGCTTCATGGGTTTCAGATGTGAGTCAAATCTGAATCAACATTGTCGTT-GATCCA
NSITOPHILA CAACGGCTTCATGGGTTTCAGATGTGAGTCAAATCTGAATCAACATTGTCGTT-GATCCA
NINTERMEDI CAACGGCTTCATGGGTTTCAGATGTAAGTCAAATCTGAATCAACATTGTCGTT-CATCCA
NTETRASPER CAACGGCTTCATGGGTTTCAGATGTAAGTCAAATCTGAATCAACATTGTCGTTTGATCCA
NTERRICOLA CAACCGCTTCATGGGTTTCAGATGTAAGTCAAATCTGAATCAATCTTGTCGAC--AATCC
NPANNONICA CAACGGCTTCATGGGTTTCAGATGTAAGTCAAATCTGAATCAATCTTGTCGAC--AATCC
GTETRASPER CAACGGTTTCATGGGTCTCAGATGTAAGTCAAATCTGAATCAATCTTGTCGAC--AATCC
NDODGEIXXX CAACGGCTTCATGAGTTTCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
NAFRICANAX CAACGGCTTCATGAGTTTCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
NGALAPOGOS CAACGGCTTCATGAGTATCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
NLINEOLATA CAACGGCTTCATGAGTTTCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
NDISCRETAX CAACGGCTTCATGGGTTTCAGATGTAAGTTAAATCTGAATCAATCTTGTCGAT--AATCC
SMACROSPOR CAACGGCTTCATGGGTTTCAGATGTAAGTCAAATCTGAATCAATCTTGACGAC--GATCC

NCRASSAXXX TGGCTGATTGCTCTT-CATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NSITOPHILA TGGCTGATTGCTCTT-CATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NINTERMEDI TGGCTAATTGCTCTT-CAATTTAGCGTACTATTCCCCGCTCTTCTCTCAGC
NTETRASPER TGGCTGATTGCTCTT-CATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NTERRICOLA ATGCTGATTGCTTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NPANNONICA ATGCTGATTGCCTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
GTETRASPER ATGCTAATTGCTTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NDODGEIXXX ATTCTAATTGCTTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NAFRICANAX ATTCTAATTGCCTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NGALAPOGOS ATTCTAATTGCCTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NLINEOLATA ATTCTAATGGCTTTT-TATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGN
NDISCRETAX ATGCTGACTGCTCTT-CATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
SMACROSPOR ATACTGATTGCCTTTCTGTTTCAGCGTACTATTCCCCGCTCTTCTCTCAGT

NCRASSAXXX TCCCGCAAAAGGAGAGATCGCCCTTCATGACTATTCTCTGGCAGCATGAT
NSITOPHILA TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NINTERMEDI TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NTETRASPER TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NTERRICOLA TCCCGCAAAAGGAGAGATCGCCGTTCATGACCATTCTCTGGCAGCACGAT
NPANNONICA TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGGACGAT
GTETRASPER TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTATGGCAGCACGAT
NDODGEIXXX TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGCACGAT
NAFRICANAX TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGCACGAT
NGALAPOGOS TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGCACGAT
NLINEOLATA TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGGACGAT
NDISCRETAX TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTTTGGCAGCACGAT
SMACROSPOR TTCCTCAGAAGGCGCGATCGCCCTTCATGACCATCCTCTGGCAGCACGAT

NCRASSAXXX CCCTTCCACAATGAGTGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NSITOPHILA CCCTTCCACAATGAGTGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NINTERMEDI CCCTTTCACAATGAGTGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
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NAFRICANAX CCCTTCCACAACGAATGGAATTTCATGTGCTCGGTGTATTCGTCGATCCG
NGALAPOGOS CCCTTCCACAACGAATGGAATTTCATGTGCTCGGTGTATTCGTCGATCCG
NLINEOLATA CCCTTCCACAACGAATGGAATTTCATGTGCTCGGTGTATTCGTCGATCCG
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SMACROSPOR CCCTTTCACAACGAATGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
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NINTERMEDI GACCTACCTTGAGC-NNNNNNNNNNNNNNNNNAGGAGAAGGTTACTCTGCAACTCT
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NLINEOLATA CACCTACCTTGAGC-NNNNNNNNNNNNNNNNNAGGAGAAAATTACCCTGCAACTCT
NDISCRETAX TACCTATCTTGAGC-NNNNNNNNNNNNNNNNNAGGAGAAGGTTACTCTGCAACTTT
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NCRASSAXXX GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCCTTT
NSITOPHILA GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCCTTT
NINTERMEDI GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTATCCGCGATAACTACATGGCATCGTTT
NTETRASPER GGATTCACTATGCTGTTCGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
NTERRICOLA GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTACCCGCGACAACTACATGGCATCGTTT
NPANNONICA GGATTCACTATGCTGTCGGCCATCTGGGGGTGATTACCCGCGACAACTACATGGCATCGTTT
GTETRASPER GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTCGCCGCGACAACTACATGACATCGTTT
NDODGEIXXX GGATTCACTATGCTGTCCGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
NAFRICANAX GGATTCACTATCGTGTCCGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
NGALAPOGOS GGATTCACTATGCTGTCCGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
NLINEOLATA GGATTCACTATGCTGTCCGCCATCTGGGGGTGATTATCCGCGACAACTACATGGAATGGATT
NDISCRETAX GGATTCACTATGCTGTCGGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
SMACROSPOR GGCTTCACTTTGCTGTCCCCGACATGGGAGTGCTTGGTCGCGAAAACTACTTGCCCACGCTT

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NINTERMEDI GGTTG--GAACCTCGTCCGTTTTCCCAACGGCACTCACGACCTCGAGCGC
NTETRASPER GGCTG--GAACCTCGTCCGTTTTCCCAACGGCACTCACGACCTCGAGCGC
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NPANNONICA GGCTG--GAACCTCGTCCAGCTGCCCAACGGCACTCACGACCTCGAGCGC
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NGALAPOGOS GGCTG--GAACCTCGTCCAGCTGCCCAACGGCACTCACGACCTCGAGCGC
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SMACROSPOR GGCTG--GGACCTCGTCACGATGCCCAACGGCACTATCGACCTTATGCGC

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NTETRASPER ACGGCTCTTCCTTTGGTTCAGCACAATCTCCAGCCCATGAACGGCTTATG
NTERRICOLA ACCGCTCTTCCTTTGGTTCAGCATAACCTTCAGCCCATGAACGGCCTATG
NPANNONICA ACCGCTCTTCCTTTGGTTCAGCATAACCTCCAGCCCATGAACGGCCTATG
GTETRASPER ACCGCTCTTCCTTTGGTTCAGCAGAACCTCCAGCCCATGAACGGCCTATG
NDODGEIXXX ACCGCTCTTCCTTTGGTTCAGCATAACCTCCAGCCCATGAACGGGCTATG
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SMACROSPOR ATCGCTATGCCTTTGTTTAGAAAGAACCTCCAGCCCATGAACGGCCTATG
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NSITOPHILA CCTGCTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCATT
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NTETRASPER CCTGCTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACT
NTERRICOLA CCTGCTCACTAGGTGCCTCGAGAGCGGATTGCCTCTTCACAATCCTCACC
NPANNONICA CCTGCTCACTAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACC
GTETRASPER CCTGTTCACTAAGTGCCTCGAGAACGGATTGCCTCTTGCCAATCCTCACC
NDODGEIXXX CCTGCTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACC
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NGALAPOGOS CCTGTTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACC
NLINEOLATA CCTGCTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACC
NDISCRETAX CCTGCTCACTAAGTGCCTTGAGAGCGGATTACCTCTTGCCAATCCTCACT
SMACROSPOR CCTGTTCACCAAGTGTCAGGAGGGCGGATTGCAAGTCGACAACCAGCACC

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NSITOPHILA CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
NINTERMEDI CTGTCATCGCCAAGCTTTCAGATCCTAGCTTCGACATGATCTGGTTCAA
NTETRASPER CTGTCATCGGCAAGCTTTCAGATCCTAGCTATGACATGATCTGGTTCAA
NTERRICOLA CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
NPANNONICA CTGTCATTGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
GTETRASPER CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGATGAA
NDODGEIXXX CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
NAFRICANAX CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
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NLINEOLATA CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
NDISCRETAX CTGTCATCGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA
SMACROSPOR TCGTCATTGCCAAGCTTTCAGATCCTAGCTACGACATGATCTGGTTCAA

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NINTERMEDI CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGTCAAACTGATGAATCTG
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NGALAPOGOS CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGCCAAACTTACAATTCTG
NLINEOLATA CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGCCAAACTTACAATTCTG
NDISCRETAX CAAGCGTCCTTACAGTCAGCAGAGACAAGTCGGCCAAACTGACGATTCTG
SMACROSPOR CAAGCGCCCTCACTATCAGCAGAGACACGCCGTCCAAGCTGACAGTTCTG

NCRASSAXXX AAGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTGCAGAG
NSITOPHILA AAGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTGCAGAG
NINTERMEDI AAGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTACAGAG
NTETRASPER AGGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTGCCGAG
NTERRICOLA AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
NPANNONICA AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
GTETRASPER AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
NDODGEIXXX AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
NAFRICANAX AACTTGGAGTGTCGGCGCTCTTCCCTTGCAATCACGCAGTCGCTGCAGCG
NGALAPOGOS AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
NLINEOLATA AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
NDISCRETAX AACTCGAAGTGTCGGCGATGTTCCCTCACAATTACGCAGTCGCCGCAGAG
SMACROSPOR AACTCGGTGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTTGCTGCAGAG
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NSITOPHILA GTAGATGGCATCATCA-NNCTCCTCTCTCCCATTGGATTCAGCAGGGAGA
NINTERMEDI GTAGATGGCATCATCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
NTETRASPER GTAGATGGCATCATCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
NTERRICOLA GTAGATGGCATCGCCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
NPANNONICA GTAGATGGCATCGCCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
GTETRASPER GTAGATGGCATCGCCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
NDODGEIXXX GTAGATGGCATCACCGACCTTCCTCTCTCCCATTGGCTTCAGCAGGGAGA
NAFRICANAX GTCGATGGCATCACCGACCTTCCTCTCTCCCATTGGCTTCAGCAGGGAGA
NGALAPOGOS GTAGATGGCATCACCGACCTTCCTCTCTCCCATTGGCTTCAGCAGGGAGA
NLINEOLATA GTAGATGGCATCACCGACCTTCCTCTCTCCCATTGGCTTCAGCAGGGAGA
NDISCRETAX GCAGATGGTATCGCCAATCTTCCTCTCTCCCATTGGATTCAGCAGGGAGA
SMACROSPOR GCAGATGACGTCGCCACTCTTCAACTCCCTCATTGGATGCAGCAGGGAGA

NCRASSAXXX ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACCTTGTTGGAT
NSITOPHILA ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACCTTGTTGGAT
NINTERMEDI ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACATTGTTGGAT
NTETRASPER ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACCTTGTTGGAT
NTERRICOLA TTTCGGTACCGAGTCCGGATTCTCAGCTCAGTTTGAGACCTTGTTGGAT
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GTETRASPER TTTCGGCACCGAGTCCGGATTCTCGGCTCAGTTTGAGACCTTGTTGGAT
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NLINEOLATA TTTCGGCACCGAGGCCGGATTCTCATCTCAGTTTGAGACCTTGTTGGAT
NDISCRETAX TTTCGGTACTGACCCCGGATACTCAGCTCAATTTGAGACTTTGTTGGAT
SMACROSPOR TTTCGGCACCGAGTCCGGATACTCACCTCAGTTTGAGACCTTGTTGGGT

NCRASSAXXX TCAATTCTCGAGAATGGACACGCCTCCAGCAATGACCCTTACAACATGGC
NSITOPHILA TCAATTCTCGAGAATGGACACGCCTCCAGCAATGACCCTTACAACATGGC
NINTERMEDI TCAATCCTCGAGAATGGACACGCCTCCAGCAATGACCCTTACAACATGGC
NTETRASPER TCAATCCTCGAGAATGGACACGCCTCCAGCAATGACCCCTACAACATGGC
NTERRICOLA TCGATCCTTGAGAATGGAAACGCCTCCAGCAATGATCCCTACAACATGGC
NPANNONICA TCGATCCTTGAGAATGGAAACGCCTCCAACAATGATCCCTACAACATGGC
GTETRASPER TCGATCCTTGAGAATGGAAACGCCACCAGCAATGATCCCTACAACATGGC
NDODGEIXXX TCGATCCTTGAGAATGGAAACGCCTCTAGCAATGAACCCTACAATATGGC
NAFRICANAX TCGATCCTTGAGAATGGAAACGCCTCTATCAATGACCCCTACAATATGGC
NGALAPOGOS TCGATCCTTGAGAATGGAAACGCCTCTAGCAATGACCCCTACAATATGGC
NLINEOLATA TCGATCCTTGAGAATGGAGACGCCTCTAGCAATGACCCCTACAATATGGC
NDISCRETAX TCTATTCTTGAGGATGGACACGCCTCCAGCAATGACCCCTACAACATGGC
SMACROSPOR TCCATACTTGAGAATGGAAACGCCACCAGTAATGATTCCTACAACATGGC

NCRASSAXXX TCTGGCTATCGATGTTCCCATGATGGGTTAG
NSITOPHILA TCTGGCTATCGATGTTCCCATGATGGGTTAG
NINTERMEDI TCTGGCTATCGATGTTCCCATGATGGGTTAG
NTETRASPER TCTGGCTATCGATGTTCCCATGATGG-TTAG
NTERRICOLA TCTGGCTATGGATGTTCCCATGATGGGTTAG
NPANNONICA TCTGGCTATGGATGTTCCCAGGATGGGTTAG
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NDODGEIXXX TCTGGGTATGGGTGTTCCCATGATGGGTTAG
NAFRICANAX TCTTGGTATGGGTGTTCCCATGATGGGTTAG
NGALAPOGOS TCTGGGTATGGGTGTTCCCATGATGGGTTAG
NLINEOLATA TCTGGGTATGGGTATTCCCATGATGGGTTAG
NDISCRETAX TCTGGCTATGGATGTTCCCATGATGGGTTAG
SMACROSPOR TCTGGCTATGGATGTTCCTATGATGGGTTAG
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2. DNA Alignment of the *Neurospora* Species and *G. tetrasperma* used to generate Figure 3.4.

The region of high conservation among mating type genes is underlined.

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NCRASSAXXX ATGTCGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA  50
NSITOPHILA ATGTCGGGAGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NINTERMEDI ATGTCGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NTETRASPER ATGCTGGGTGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGA
NTERRICOLA ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
NPANNONICA ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
GTETRASPER ATGTCGGGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCGCTGAGGG
NDODGEIXXX NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NAFRICANAX ATGTCGTGCGTCGATCAAATCGTCAAGACGTTCGCCGACCTCACTGAGGG
NGALAPAGOS NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NLINEOLATA NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NDISCRETAX ATGTCGGGCGTCGACCAAATTGTCAAGACGTTCGCCGACCTCGCTGAGGA

NCRASSAXXX CGACCGTGAAGCGGCAATGAGAGCTTTCTCAAGGATGATGCGTAGAGGTA 100
NSITOPHILA CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NINTERMEDI CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NTETRASPER CGACCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA
NTERRICOLA CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
NPANNONICA CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
GTETRASPER CGATCGTGAAGCGGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGCA
NDODGEIXXX NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NAFRICANAX TGATCGTGAAGCGGCAATGAGAGCTTTCTCAATGATGATGC-NNNNNGCA
NGALAPAGOS NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NLINEOLATA NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNN
NDISCRETAX CGACCGCGAA-NNGCAATGAGAGCTTTCTCAACGATGATGC-NNNNNGTA

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NSITOPHILA CCGAACCTGTTCGCCGAATCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
NINTERMEDI CCGAACCTGTTCGCCGAATCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
NTETRASPER CCGAACCTGTTCGCCGAATCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
NTERRICOLA CCGAACCTGTTCGCCAAACCCCCGCGGCAAAGAAGAAGGTCAACCGCTTC
NPANNONICA CCGAACCTGTTCGCCAAACCCCCGCGGTAAAGAAGAAGGTCAACGGCTTC
GTETRASPER CCGAACCTGTTCGCCAAAGCCCCGCGGCAAAGAAGAAGGTCAACGGTTTC
NDODGEIXXX NNNNNNNTGTTCGCCGAATCCCCGCGGCGAAGAAGAAGGTCAACGGCTTC
NAFRICANAX CCGAACCTGTTCGCCAAACCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
NGALAPAGOS NNNNNNNTGTTCGCCGAATCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
NLINEOLATA NNNNNNNTGTTCGCCGAATCCCCGCGGCAAAGAAGAAGGTCAACGGCTTC
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NCRASSAXXX ATGGGTTTCAGATGTGAGTCAAATCTGAATCAACATTGTCGTT-GATCCA 200
NSITOPHILA ATGGGTTTCAGATGTGAGTCAAATCTGAATCAACATTGTCGTT-GATCCA
NINTERMEDI ATGGGTTTCAGATGTAAGTCAAATCTGAATCAACATTGTCGTT-CATCCA
NTETRASPER ATGGGTTTCAGATGTAAGTCAAATCTGAATCAACATTGTCGTTTGATCCA
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NPANNONICA ATGGGTTTCAGATGTAAGTCAAATCTGAATCAATCTTGTCGAC--AATCC
GTETRASPER ATGGGTCTCAGATGTAAGTCAAATCTGAATCAATCTTGTCGAC--AATCC
NDODGEIXXX ATGAGTTTCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
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NLINEOLATA ATGAGTTTCAGATGTAAGTCAAATCTGGATCAATCTTGTTGAA--AATCC
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NTETRASPER TGGCTGATTGCTCTTCATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NTERRICOLA ATGCTGATTGCTTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NPANNONICA ATGCTGATTGCCTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
GTETRASPER ATGCTAATTGCTTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NDODGEIXXX ATTCTAATTGCTTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NAFRICANAX ATTCTAATTGCCTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NGALAPAGOS ATTCTAATTGCCTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC
NLINEOLATA ATTCTAATGGCTTTTTATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGN
NDISCRETAX ATGCTGACTGCTCTTCATTTCAGCGTACTATTCCCCGCTCTTCTCTCAGC

NCRASSAXXX TCCCGCAAAAGGAGAGATCGCCCTTCATGACTATTCTCTGGCAGCATGAT 300
NSITOPHILA TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NINTERMEDI TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NTETRASPER TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTCTGGCAGCATGAT
NTERRICOLA TCCCGCAAAAGGAGAGATCGCCGTTCATGACCATTCTCTGGCAGCACGAT
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GTETRASPER TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTATGGCAGCACGAT
NDODGEIXXX TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGCACGAT
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NLINEOLATA TCCCGCAGAAGGAGAGATCACCCTTCATGACCATTCTCTGGCAGGACGAT
NDISCRETAX TCCCGCAAAAGGAGAGATCGCCCTTCATGACCATTCTTTGGCAGCACGAT

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NSITOPHILA CCCTTCCACAATGAGTGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NINTERMEDI CCCTTTCACAATGAGTGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NTETRASPER CCCTTCCACAATGAATGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NTERRICOLA CCCTTCCACAACGAATGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
NPANNONICA CCCTTCCACAACGAATGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG
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NLINEOLATA CCCTTCCACAACGAATGGAATTTCATGTGCTCGGTGTATTCGTCGATCCG
NDISCRETAX CCCTTCCACAACGAATGGGATTTCATGTGCTCGGTGTATTCGTCAATCCG

NCRASSAXXX GACCTACCTTGAGCAGGAGAAGGTTACTCTGCAACTCTGGATTCACTATG 400
NSITOPHILA GACCTACCTTGAGCAGGAGAAGGTTACTCTGCAACTCTGGATTCACTATG
NINTERMEDI GACCTACCTTGAGCAGGAGAAGGTTACTCTGCAACTCTGGATTCACTATG
NTETRASPER GACCTACCTTGAGCAGGAGAAGGTTACTCTGCAACTCTGGATTCACTATG
NTERRICOLA CACCTACCTTGAGCAGGAGAAGGTTACCCTGCAACTCTGGATTCACTATG
NPANNONICA CACCTACCTTGAGGAGGAGAAGGTAAATCTGCAACTCTGGATTCACTATG
GTETRASPER CACCTACTTTGAGCAGGAGAATGTCACCCTGCAACTCTGGATTCACTATG
NDODGEIXXX CACCTACCTTGAGCAGGAGAAAGTTACCCTGCAACTCTGGATTCACTATG
NAFRICANAX CACCTACCTTGAGCAGGAGAAAGTTACCCTGCAACTCTGGATTCACTATC
NGALAPAGOS CACCTACCTTGAGCAGGAGAAAGTTACCCTGCAACTCTGGATTCACTATG
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NDISCRETAX TACCTATCTTGAGCAGGAGAAGGTTACTCTGCAACTTTGGATTCACTATG

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NGALAPAGOS CTGTCCGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
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NDISCRETAX CTGTCGGCCATCTGGGAGTGATTATCCGCGACAACTACATGGCATCGTTT
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NSITOPHILA GGCTG--GAACCTCGTCCGTTTTCCCAACGGCACTCACGACCTCGAGCGC
NINTERMEDI GGTTG--GAACCTCGTCCGTTTTCCCAACGGCACTCACGACCTCGAGCGC
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NLINEOLATA GTTTGGTGGAACTTGTCCAG-TGCCCAACGGCANTCAGGACTTGGAGCGC
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NSITOPHILA ACGGCTCTTCCTTTGGTTCAGCACAATCTCCAGCCCATGAACGGCCTATG
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NTETRASPER ACGGCTCTTCCTTTGGTTCAGCACAATCTCCAGCCCATGAACGGCTTATG
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NTETRASPER CCTGCTCACCAAGTGCCTCGAGAGCGGATTGCCTCTTGCCAATCCTCACT
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NTETRASPER CTGTCATCGGCAAGCTTTC-AGATCCTAGCTATGACATGATCTGGTTCAA
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NSITOPHILA CAAGCGTCCTCACCGTCAGCAGGGACACGCCGTTCAAACTGATGGATCTG
NINTERMEDI CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGTCAAACTGATGAATCTG
NTETRASPER CAAGCGTCCTCACCGTCAGCAGGGACACGTCGGTCAAACTGATGAATCTG
NTERRICOLA CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGCCAAACTGACAATTCTG
NPANNONICA CAAGCGTCCTCACCGTCAGCAGGGACACGCCGGCCAAACTGACAATTCTG
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NDISCRETAX CAAGCGTCCTTACAGTCAGCAGAGACAAGTCGGCCAAACTGACGATTCTG
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NSITOPHILA AAGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTGCAGAG
NINTERMEDI AAGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTACAGAG
NTETRASPER AGGTTGGAGTTTCGGCGATGTTCCCTCGCAATCACACGGTCGCTGCCGAG
NTERRICOLA AACTTGGAGTGTCGGCGCTCTTCCCTCGCAATCACGCAGTCGCTGCAGAG
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NINTERMEDI ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACATTGTTGGAT
NTETRASPER ATTCGGTACCGAGTCTGGATACTCAGCTCAGTTTGAGACCTTGTTGGAT
NTERRICOLA TTTCGGTACCGAGTCCGGATTCTCAGCTCAGTTTGAGACCTTGTTGGAT
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NAFRICANAX TCTTGGTATGGGTGTTCCCATGATGGGTTAG
NGALAPAGOS TCTGGGTATGGGTGTTCCCATGATGGGTTAG
NLINEOLATA TCTGGGTATGGGTATTCCCATGATGGGTTAG
NDISCRETAX TCTGGCTATGGATGTTCCCATGATGGGTTAG
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3. Amino Acid Alignment of the *Neurospora* Species and *G. tetrasperma*

Region of high amino acid conservation among mating types is underlined.

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Npannonica MSGVDQIVKTFADLAEGDREAAMRAFSTMMR--TEPVRQTPAVKKKVNGF  50
Gtetrasper MSGVDQIVKTFADLAEGDREAAMRAFSTMMR--TEPVRQSPAAKKKVNGF
Nterricola MSGVDQIVKTFADLAEGDREAAMRAFSTMMR--TEPVRQTPAAKKKVNRF
Nintermedi MSGVDQIVKTFADLAEDDREAAMRAFSTMMR--TEPVRRIPAAKKKVNGF
Ntetrasper MLGVDQIVKTFADLAEDDREAAMRAFSTMMR--TEPVRRIPAAKKKVNGF
Ncrassaxxx MSGVDQIVKTFADLAEDDREAAMRAFSRMMRRGTEPVRRIPAAKKKVNGF
Nsitophila MSGVDQIVKTFADLAEDDREAAMRAFSTMMR--TEPVRRIPAAKKKVNGF
Nafricanax MSCVDQIVKTFADLTEGDREAAMRAFSMMMR--TEPVRQTPAAKKKVNGF
Ndodgeixxx -???????????????????????????????????????????????????
Ngalapagos -???????????????????????????????????????????????????
Ndiscretax MSGVDQIVKTFADLAEDDRE-AMRAFSTMMR--TEPVRQIPATKKKVNGF
Nlineolata -???????????????????????????????????????????????????

Npannonica MGFRSYYSPLFSQLPQKERSPFMTILWQDDPFHNEWDFMCSVYSSIRTYL 100
Gtetrasper MGLRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYF
Nterricola MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Nintermedi MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Ntetrasper MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Ncrassaxxx MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Nsitophila MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Nafricanax MSFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWNFMCSVYSSIRTYL
Ndodgeixxx MSFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWNFMCSVYSSIRTYL
Ngalapagos MSIRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWNFMCSVYSSIRTYL
Ndiscretax MGFRSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYL
Nlineolata MSFRSYYSPLFSQXPQKERSPFMTILWQDDPFHNEWNFMCSVYSSIRTYL

Npannonica EEEKVNLQLWIHYAVGHLGVITRDNYMASFGWNLVQLPNGTHDLERTALP 150
Gtetrasper EQENVTLQLWIHYAVGHLGVIRRDNYMTSFGWNLVQLPNGTHDLERTALP
Nterricola EQEKVTLQLWIHYAVGHLGVITRDNYMASFGWNLVQLPNGTHDLERTALP
Nintermedi EQEKVTLQLWIHYAVGHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALP
Ntetrasper EQEKVTLQLWIHYAVRHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALP
Ncrassaxxx EQEKVTLQLWIHYAVGHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALP
Nsitophila EQEKVTLQLWIHYAVGHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALP
Nafricanax EQEKVTLQLWIHYRVRHLGVIIRDNYMASFGWNLVQLPNGTHDLERTALP
Ndodgeixxx EQEKVTLQLWIHYAVRHLGVIIRDNYMASFGWKLVQLPNGTHDLERTALP
Ngalapagos EQEKVTLQLWIHYAVRHLGVIIRDNYMASFGWNLVQLPNGTHDLERTALP
Ndiscretax EQEKVTLQLWIHYAVGHLGVIIRDNYMASFGWNLVHLPNGTHDLERTALP
Nlineolata EQEKITLQLWIHYAVRHLGVIIRDNYMEWIVWWNLSSAQRXSGLGAHRLP

Npannonica LVQHNLQPMNGLCLLTKCLESGLPLANPHPVIAKLSDPSYDMIWFNKRPH 200
Gtetrasper LVQQNLQPMNGLCLFTKCLENGLPLANPHPVIAKLSDPSYDMIWMNKRPH
Nterricola LVQHNLQPMNGLCLLTRCLESGLPLHNPHPVIAKLSDPSYDMIWFNKRPH
Nintermedi LVQPNLQPMNGLCLLTKCLESGLPLANPHSVIAKLSDPSFDMIWFNKRPH
Ntetrasper LVQHNLQPMNGLCLLTKCLESGLPLANPHSVIGKLSDPSYDMIWFNKRPH
Ncrassaxxx LVQHNLQPMNGLCLLTKCLESGLPLANPHSVIAKLSDPSYDMIWFNKRPH
Nsitophila LVQHNLQPMNGLCLLTKCLESGLPLANPHSVIAKLSDPSYDMIWFNKRPH
Nafricanax LVQHNLQPMNGLCLFTKCLESGLPLANPHPVIAKLSDPSYDMIWFNKRPH
Ndodgeixxx LVQHNLQPMNGLCLLTKCLESGLPLANPHPVIAKLSDPSYDMIWFNKRPH
Ngalapagos LVQHNLQPMNGLCLFTKCLESGLPLANPHPVIAKLSdpsydmiwfnkrph
Ndiscretax LVRHNLQPMNGLCLLTKCLESGLPLANPHSVIAKLSDPSYDMIWFNKRPY
Nlineolata LVQHNLQPMNGLCLLTKCLESGLPLANPHPVIAKLSDPSYDMIWFNKRPH
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Gtetrasper RQQGHAGQTDNSELGVSALFPRNHAVAAEVDGIANLPLSHWIQQGDFGTE
Nterricola RQQGHAGQTDNSELGVSALFPRNHAVAAEVDGIANLPLSHWIQQGDFGTE
Nintermedi RQQGHAGQTDESEVGVSAMFPRNHTVATEVDGIINLPLSHWIQQGEFGTE
Ntetrasper RQQGHVGQTDESEVGVSAMFPRNHTVAAEVDGIINLPLSHWIQQGEFGTE
Ncrassaxxx RQQGHAVQTDESEVGVSAMFPRNHTVAAEVDGIINLPLSHWIQQGEFGTE
Nsitophila RQQGHAVQTDGSEVGVSAMFPRNHTVAAEVDGIIT-PLSHWIQQGEFGTE
Nafricanax RQQGHAGQTYNSELGVSALFPCNHAVAAAVDGITDLPLSHWLQQGDFGTE
Ndodgeixxx RQQGHAGQTYNSELGVSALFPRNHAVAAEVDGITDLPLSHWLQQGDFGTE
Ngalapagos RQQGHAGQTYNSELGVSALFPRNHAVAAEVDGITDLPLSHWLQQGDFGTE
Ndiscretax SQQRQVGQTDDSELEVSAMFPHNYAVAAEADGIANLPLSHWIQQGDFGTD
Nlineolata RQQGHAGQTYNSELGVSALFPRNHAVAAEVDGITDLPLSHWLQQGDFGTE

Npannonica SGFSAQFETLLDSILENGNASNNDPYNMALAMDVPRMG* 289
Gtetrasper SGFSAQFETLLDSILENGNATSNDPYNMALAMDVPMMG*
Nterricola SGFSAQFETLLDSILENGNASSNDPYNMALAMDVPMMG*
Nintermedi SGYSAQFETLLDSILENGHASSNDPYNMALAIDVPMMG*
Ntetrasper SGYSAQFETLLDSILENGHASSNDPYNMALAIDVPMMV-
Ncrassaxxx SGYSAQFETLLDSILENGHASSNDPYNMALAIDVPMMG*
Nsitophila SGYSAQFETLLDSILENGHASSNDPYNMALAIDVPMMG*
Nafricanax AGFSPQFETLLDSILENGNASINDPYNMALGMGVPMMG*
Ndodgeixxx AGFSPQFETLLDSILENGNASSNEPYNMALGMGVPMMG*
Ngalapagos AGFSPQFETLLDSILENGNASSNDPYNMALGMGVPMMG*
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