HOMOTHALLISM IN THE *SORDARIACEAE*: MATING-TYPE LOCI IN SELECTED SPECIES OF *NEUROSPORA*, *ANIXIELLA*, AND *GELASINOSPORA*

by

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ABSTRACT

The mating-type genes in *Neurospora crassa*, called "idiomorphs" and designated A and a, control entry into the sexual cycle and aspects of meiosis. In heterothallic species, nuclei with unlike mating-types must come together before karyogamy and meiosis can occur. Homothallic species, however, can enter the sexual cycle spontaneously and without a mating event *per se*. Two homothallic classes exist in *Neurospora*, those with only the A-specific sequence (the majority) and those with both the A-specific and a-specific sequence (*Neurospora terricola*). *Neurospora terricola* and a number of homothallic *Gelasinospora* and *Anixiella* species are self-fertile but harbor both A and a idiomorphs in a single nucleus. Of what use are mating-types to organisms that do not mate?

A molecular analysis of the mating-type loci of selected *Sordariaceae* homothallics has investigated the degree to which the homothallic A and a genes are similar to those of heterothallic and A-type homothallic species of *Neurospora*. Mapping of the *N.terricola*, *Gelasinospora*, and *Anixiella* mating-type loci has demonstrated that the majority of the *N. crassa* A and a sequences are conserved in these homothallics with the exception of approximately 1.1 kb of A absent in *N.terricola* and approximately 600bp of a absent in all homothallics examined. Further analysis was made of the sequences that flank the mating-type genes themselves to further define the locus. The sequence that in *N.crassa* flanks both A and a idiomorphs to the left is present in the genomes of all homothallic species examined. In most of the homothallics examined, this flank sequence is contiguous with the a idiomorph. A similar right flank sequence is absent in *Sordariaceae* homothallics. The ORF portions of the *N.terricola* A and a genes were amplified by PCR and sequenced. A sequence comparison of *N.crassa* and *N.terricola* A ORFs has

demonstrated an average of 89% DNA identity. Functional analysis has shown that the *N.terricola* ORF A confers function (ability to mate with strains of opposite mating-type and ability to form perithecia) when transformed into *N.crassa* sterile mutant spheroplasts.

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GENERAL INTRODUCTION

This work is a two part investigation of the nature of mating and mating-type loci in homothallic fungi of the *Sordariaceae*. The first part describes the organization of mating-type loci of a *Neurospora* homothallic, *Neurospora terricola*, and of several homothallics in two closely related genera, *Anixiella* and *Gelasinospora*. These mating-type loci are compared to the well described mating-type locus of the heterothallic *Neurospora crassa* and the homothallic *Neurospora africana*. The second part of this investigation is a detailed analysis of one portion of the mating-type locus of *N.terricola*, a portion which in *N.crassa* contains ORFs encoding functional polypeptides, mt A-1 and mt a-1, responsible for regulatory control over mating and meiosis. This latter analysis employs the sequencing of the ORFs of the *N.terricola* A and a idiomorphs, tests of their functionality, and a comparison of these sequences to those of *N.crassa* mt A-1 and mt a-1 and *N.africana* mt A-1. All the information is then utilized in a general discussion of heterothallism and homothallism, their evolution, and common elements in eukaryotic control of mating events.

Neurospora crassa is a filamentous ascomycete that, until the emergence of yeast biology in the last two decades, was a preferred organism for the study of genetics. Beadle and Tatum's now famous work on the first nutritional mutants (6) was done with *Neurospora* and a rich period of genetic research followed, much of it based on *Neurospora* techniques. Currently, a resurgence of interest in filamentous ascomycetes has focused in part on mating, particularly the classical and molecular nature of sexual reproduction. In a number of fungal taxa, reproductive functions have been associated with specific loci and particular genes have been identified that are now known to have mating-specific functions. Some of these genes have been cloned and sequenced and a large body of research has had as its focus their manipulation in mutational and functional analyses.

Neurospora genes that function specifically in mating and sexual cycle events have been cloned and sequenced and we now know that *Neurospora*, like many fungi, has two

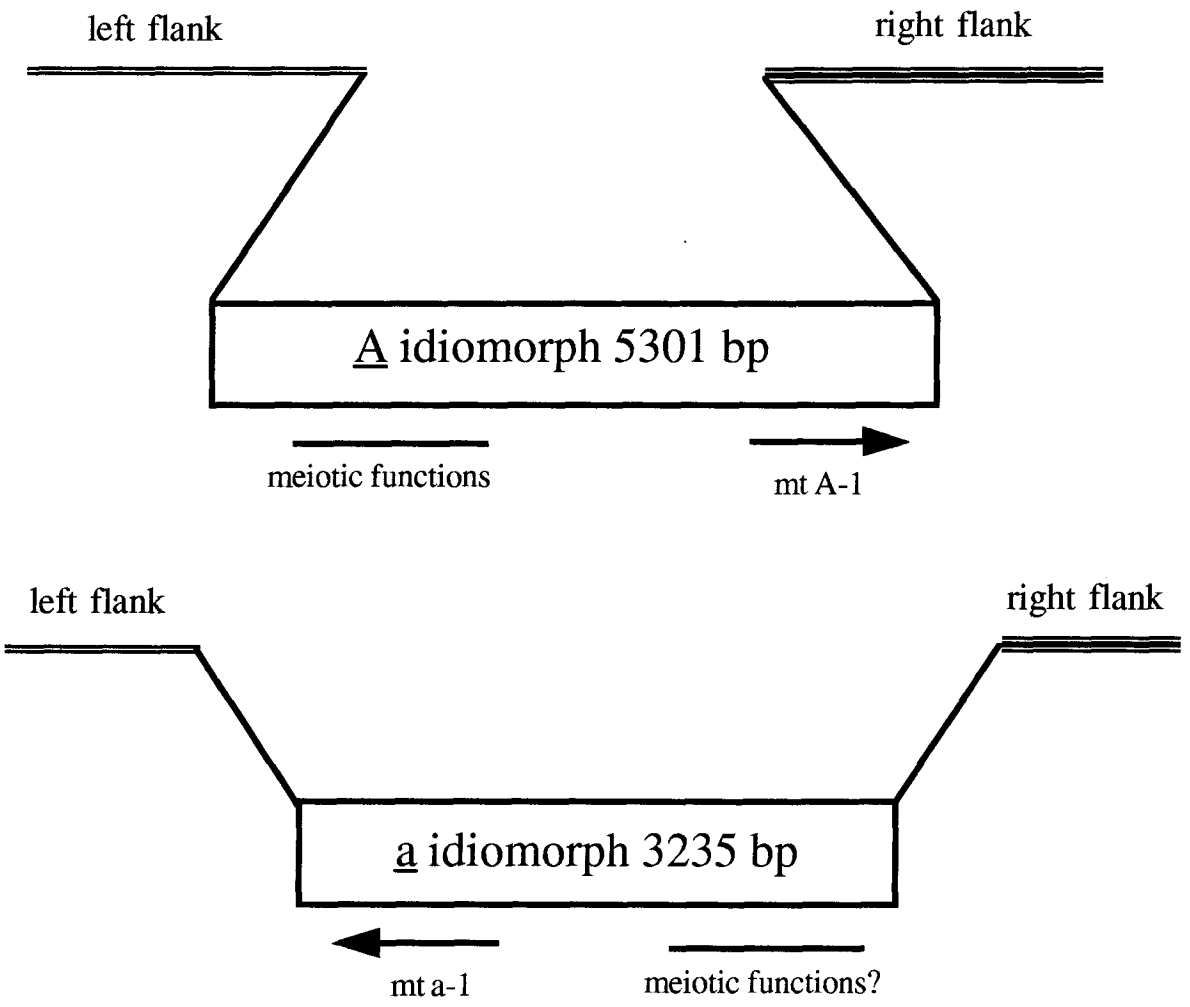

Figure 1 ***N.crassa*** **mating-type idiomorphs and their functions.**
N.crassa idiomorphs are entirely dissimilar. A and a each encode a single polypeptide that is implicated in mating, meiotic, and vegetative incompatibility functions. A second region in A has been associated with meiotic functions and the production of fertile ascospores. A similar region in a has not been confirmed. The sequences flanking the idiomorphs are identical in strains of opposite mating-type.

mating-types, designated A and a [Figure 1] (31). A and a sequences have been termed idiomorphs (51) in *N.crassa* to reflect the fact that the sequences are dissimilar, though resident at the same locus; a single *N.crassa* individual harbors a A or a idiomorph but not both. A and a idiomorphs have several functional regions. One such region of A and a encodes regulatory polypeptides, mt A-1 (26) and mt a-1 (88), that are thought to activate downstream genes, presumably the many genes that effect sexually specific events of the life cycle such as mating. Mt A-1 and mt a-1 also function vegetatively to prevent the formation of mixed mating-type heterokaryons. A x a constitutes a successful cross in the sexual cycle but a A + a union of vegetative mycelia results in a reaction known as heterokaryon incompatibility; contact between hyphae of opposite mating-types results in severely inhibited growth or even cell death at the point of contact (27). The interaction of mt A-1 and mt a-1 is but one factor in vegetative compatibility between individuals. A host of *het* genes define compatibility groups so that individuals with different *het* constitutions are incompatible (27). Heterokaryon incompatibility is also affected by a locus, *tol*, which is unlinked to the mating-type locus. The wild-type *tol* (for "tolerant") allele contributes to the incompatibility between strains with opposite-mating-types during vegetative growth. A mutant form of the allele allows the formation of mixed mating-type heterokaryons by suppressing A/a incompatibility in the sexual cycle (60).

Neurospora mating strategies

In the genus *Neurospora*, there are at least three distinct reproductive strategies, not all of which involve a mating event *per se* [Figure 2]. One is heterothallic mating, the strategy of *N.crassa* described above. Heterothallic individuals are invariably of a single mating-type, containing either a A idiomorph or a a idiomorph but not both. The four heterothallic species of *Neurospora* all have one mating-type idiomorph per nucleus and individual nuclei in multinucleate fertilizing agents (vegetative spores or hyphae) are all of the same mating-type (31). Heterothallic reproduction is truly sexual; individuals with

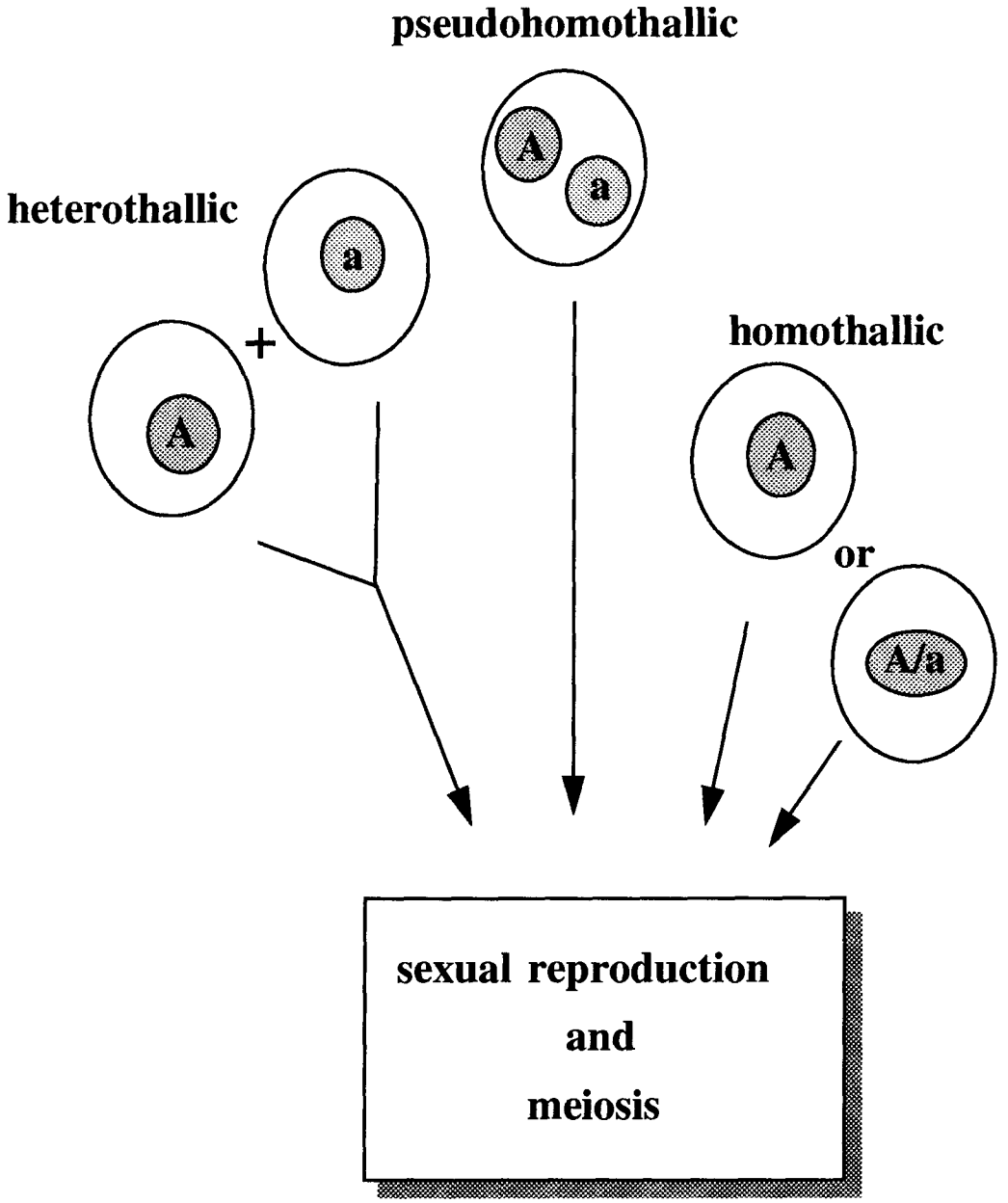

Figure 2 ***Neurospora*** **mating strategies.**
Heterothallic mating requires the union of individuals with opposite mating-types. Pseudohomothallics have multinucleate spores which typically contain nuclei with both mating-types. *N.tetrasperma*, the only known*Neurospora* pseudohomothallic, is self-fertile in >75% of reproductive events. Outcrossing is possible with the estimated 10% to 20% of ascospores that are self-sterile. Constitutive homothallics include those with only A-specific sequences (the majority) and *N.terricola* which contains both mating-type sequences in single nuclei.

opposite mating-types must interact to initiate the sexual cycle. The second and third *Neurospora* reproductive strategies are forms of homothallism or self-fertility. Homothallic individuals are capable of undergoing sexual reproduction and meiosis without a mating event in the traditional sense (76). A vegetative colony can spontaneously develop sexual fruiting bodies that produce fertile meiotic progeny in a manner that is macroscopically indistinguishable from sexual reproduction in heterothallic species (76). At the nuclear and molecular level, however, there is considerable variation in the mating-type constitution of what are generally considered homothallic species.

The pseudohomothallic class, represented in *Neurospora* by the single species *N.tetrasperma*, is functionally homothallic but has a reproductive mechanism that is perhaps more accurately considered heterothallic. Each sexual spore or ascospore normally contains haploid nuclei of both mating-types, A and a, and produces a self-fertile culture. A majority of cultures from single vegetative spores (conidia) are also self-fertile because most conidia are multinucleate and contain nuclei of both mating-types (49,72). The majority of *N.tetrasperma* reproductive events are "homothallic" since the presence of A and a mating-types in single propagules precludes any need for mating. It is presumed that *N.tetrasperma* idiomorphs encode functional mt A-1 and mt a-1 polypeptides and that these polypeptides interact in a manner similar to that outlined for *N.crassa*. Such a process would suggest a heterothallic mating mechanism in an otherwise pseudohomothallic species but no direct evidence exists to support this. Nevertheless, certain aspects of the *N.tetrasperma* life cycle are clearly heterothallic in nature. Approximately 10% of ascospores and 20% of conidia are self-sterile. Cultures derived from these propagules are homokaryotic and cross-fertile with strains of opposite mating-type whether self-sterile themselves or binucleate with both mating-types (72). *N.tetrasperma* is predominantly inbred but a significant proportion of meioses result from these outcrossings involving self-sterile individuals.

The *N.tetrasperma* A and a idiomorphs, while functional during the sexual cycle, do not elicit vegetative heterokaryon incompatibility in *N.tetrasperma* (72). The same

idiomorphs, when transformed into mutant (A^m or a^m mutants that have lost heterokaryon incompatibility) *N.crassa* strains, do cause a vegetative incompatibility response when opposite mating-types are associated in forced heterokaryons. The *N.tetrasperma* idiomorphs are thus capable of eliciting the incompatibility phenotype (in *N.crassa*) yet A and a nuclei are not incompatible when they occupy the same *N.tetrasperma* conidium. A plausible explanation for this fact might be the constitutive expression in *N.tetrasperma* of a *tol*-like factor which suppresses incompatibility.

N.tetrasperma also differs from heterothallic *Neurospora* species by lacking the specialized female hyphae known as trichogynes (72). In heterothallic species, these hyphae are attracted to and grow toward pheromones produced by opposite mating-type conidia (9). Contact between male conidia and female trichogynes, thought to be regulated by mt a-1 and mt a-1, initiates mating and leads ultimately to karyogamy and meiosis (30). *N.tetrasperma* bypasses such a mating event. Presumably the interaction of A and a nuclei, still controlled by mt A-1 and mt a-1, occurs within the self-fertile spore, obviating the need for macroscopic mating structures such as trichogynes.

True *Neurospora* homothallics, and there are five species of this type, have nuclei with only one mating-type configuration. Selfing appears to be obligate in at least one species, *N.terricola* (Beatty, unpublished results); self-sterile ascospores are not observed as they are in *N.tetrasperma* (72). Two homothallic classes have been reported in *Neurospora*, a class in which individuals contain only the A mating-type gene and a class in which both mating-types are present in single nuclei [Table 1] (29). The former class includes four species while the latter class is represented by a single species, *N.terricola*. *N.tetrasperma* and *N.terricola* both typically harbor A and a mating-types in single cells (ascospores in *N.terricola*; ascospores and conidia in *N.tetrasperma*) and both are self-fertile. Only *N.terricola*, however, is an obligate homothallic; the pseudohomothallic *N.tetrasperma* has the ability to outcross.

Table 1. ***Neurospora*** **species and their origins.**
All heterothallic and A-type homothallics have been isolated from temperate climates. *N.terricola* and *N.tetrasperma* are the only *Neurospora* isolates from temperate climates. Each of these species may represent a monophyletic group (see Part Two Discussion).

Heterothallic species	**Climate in which first isolated**
Neurospora crassa	tropical
Neurospora discreta	tropical
Neurospora intermedia	tropical
Neurospora sitophila	tropical
Pseudohomothallic species	
Neurospora tetrasperma	temperate
Homothallic species	
Neurospora africana	tropical
Neurospora dodgei	tropical
Neurospora galapogosensis	tropical
Neurospora lineolata	tropical
Neurospora terricola	temperate

Homothallic species have access to relatively little genetic diversity when compared with outbreeding heterothallic or pseudohomothallic populations but there is no evidence that homothallism is any less successful a means for reproduction (68). Of what use, then, are mating-types to organisms that are self-fertile? What is the nature of the mating-type genes of homothallics? Are they similar to mating-type genes of heterothallic species; are they functional or is their presence residual, reflecting prior evolutionary events? And what degree of functionality, if any, is present in homothallics? Do mt A-1 and mt a-1 have only meiotic functions or might they be active in as yet unidentified mating events? These are some of the questions to which I addressed my research. I chose to examine *N.terricola* in particular detail because of its unique status in the *Neurospora* genus. In addition to molecular identity at the mating-type locus, *N.terricola* is also unique in several morphological and cytogenetic characteristics (32,76). Considerable work has been done to describe the evolution of *N.tetrasperma* (16,49,56,72,90) but comparatively little research has had as its focus the molecular genetics and evolution of obligate homothallics. Recent investigations into the *N.africana* mating-type locus (Glass, unpublished results) have begun to increase understanding of A-type homothallics and my work on A / a -type homothallics, including representatives from genera closely related to *Neurospora*, should lead toward a better understanding of the evolution of homothallism in filamentous ascomycetes.

Neurospora life cycle

Neurospora heterothallics are familiar colonizers of the sites of recent fires. Charred wood is a preferred substrate though there are many reports of growth on decaying vegetation and on food products, particularly bread (14,68,69,83). All known *Neurospora* species grow vegetatively as coenocytic, branched hyphae. These hyphae are clonal and multinucleate and have genetic complements that reflect exactly those of their parents. Vegetative colonies are self-perpetuating when sufficient nutrients are available and hyphae of heterothallic species form two types of vegetative spores, micro- and macro-conidia.

These, upon germination, can initiate new mycelia though microconidia seem to serve primarily as fertilizing agents to initiate sexual reproduction (68). Vegetative reproduction continues until nutrient depletion, particularly nitrogen limitation, induces the beginning of the sexual phase of the life cycle [Figure 3]. Heterothallic *Neurospora* species can grow vegetatively for extended periods of time without forming sexual structures; conidia are produced in generational succession as long as nitrogen, inorganic salts and a carbon source are available (69). In contrast, homothallic species do not form conidia. Instead, hyphal growth which usually occurs in soil rather than on exposed substrates, proceeds only for a few days before the spontaneous formation of proto- sexual structures or protoperithecia (76). These same structures differentiate from heterothallic mycelia but only when nitrogen availability is limited.

In heterothallic species, the sexual phase of the life cycle begins when the female protoperithecium is fertilized, which induces further differentiation into the mature sexual structure or perithecium. Fertilization takes place between a male conidium or hyphal tip and a specialized hypha of protoperithecial origin, the trichogyne. Regardless of the cell types of the fertilizing agents, mating can only occur between individuals with different mating-types. One function of the mating-type locus in *Neurospora* is presumably to regulate the production of pheromones. The female trichogyne detects and grows toward concentrations of these pheromones whereupon contact is made with the male conidium (9). The male nucleus, presumably under its own genetic control (95), migrates down the trichogyne and into the interior of the perithecium. One nucleus then enters the ascogonium and becomes associated with the female nucleus. A series of synchronous nuclear divisions gives rise to a cluster of dikaryotic ascogenous hyphae in which the original male and female nuclei are juxtaposed (73). Karyogamy then takes place in the penultimate cell of one hypha followed immediately by meiosis and a single mitotic division to produce eight haploid sexual progeny, the ascospores. The eight ascospores are arranged linearly in individual asci which fill the interior of the now flask shaped perithecium. A forcible

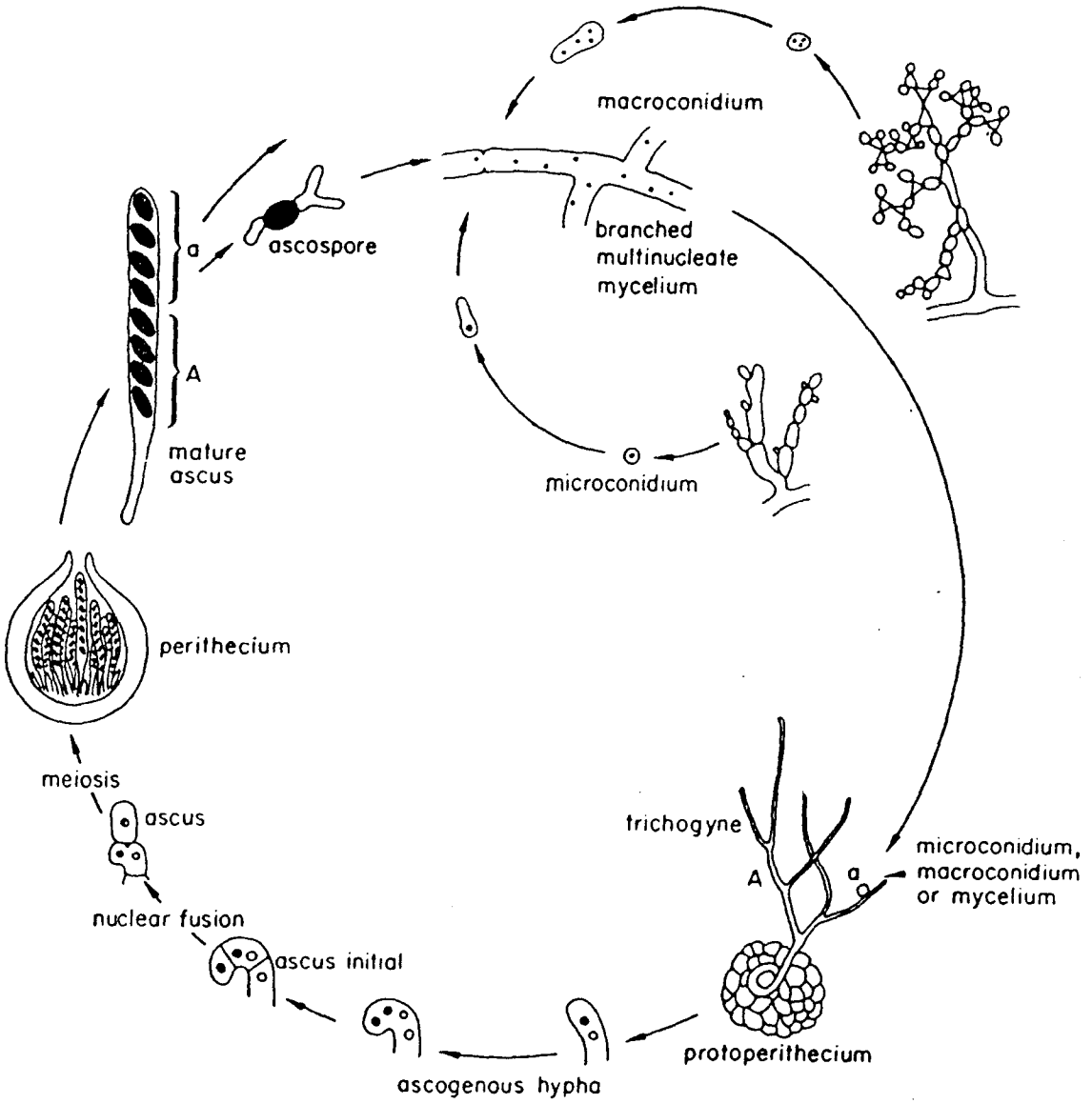

Figure 3 **Life cycle of *Neurospora crassa.***
Figure adapted from Fincham, Day, and Radford (1979) .

discharge of ascospores ensures their dispersal and heat-induced germination gives rise to new vegetative growth to complete the life cycle.

Life cycle differences between hetero- , homo- , and pseudohomothallics

Neurospora homothallics lack micro- and macroconidia and trichogynes (29,36,76). The distinctive orange color observed in heterothallic vegetative colonies is due to an abundance of carotenoids in the conidia; as homothallics lack conidia, their mycelia are relatively colorless. The absence of conidia also means that there is no direct evidence for the action of pheromones in these species, the action of which has been observed directly in *N.crassa* (9). Otherwise, the life cycles of heterothallics and homothallics are indistinguishable macroscopically. Both form identical hyphae, protoperithecia, and perithecia; only ascospore ornamentation differs (3,74). Variations have also been documented in the spindle forming structures and nucleoli of homothallics (76), pseudohomothallics (12), and heterothallics (74,76). Homothallic *Neurospora* species all have spindle-forming structures known as polar caps (described below) that are unknown in heterothallics (76).

The cytological picture for ascogenous hypha and ascus initiation is identical in both homothallic and heterothallic species (76). The pseudohomothallic *N.tetrasperma* differs only in the behavior of its nuclei in the ascus. Two nuclear divisions occur (one meiotic and one mitotic) prior to ascospore delimitation, but only four ascospores are normally delimited (12,72). Each of the four ascospores encloses two nuclei, one of each mating-type. An ensuing division makes *N.tetrasperma* ascospores four-nucleate (12,76). Homothallic and heterothallic *Neurospora* species all have identical nuclear divisions but delimit only eight-spored asci (76).

The chromosome number is seven for all *Neurospora* species (62,76,87). Several characters, however, are unique to *N.terricola*, including:

i) *hemispherical nucleolus*-- These are spherical in all other *Neurospora* species, heterothallic and homothallic (76).

ii) *presence of polar caps*-- most prominent in *N.terricola* though present in other homothallics. These are nucleus-associated structures, distinguishable from nucleoli, which may play a role in spindle formation. Their presence has not been confirmed in heterothallics (76).

iii) *morphology of ascospores*-- *N.terricola* has ovoid ascospores in contrast to the spindle-shaped ascospores of other homothallic species. *N.terricola* ascospores are also unique in having a single germinal pore, the fixed perforation of the epispore of mature ascospores; all other *Neurospora* species have bipolar ascospores (3,32,76).

Designation of species in the *Sordariaceae*

Heterothallic species designations are based primarily on crossing abilities with established reference strains. For the most part, inter-specific mating does not yield fertile progeny. There are exceptions, however, such as the relatively high percentage of viable offspring that result from crosses between *N.crassa* and *N.intermedia* (67). Homothallic species do not lend themselves so easily to systematics since they do not mate. Ascospore morphology and ornamentation is the alternative character upon which species designations are made. Species of the genus *Neurospora* are distinguished from those of closely related genera principally by their longitudinally ribbed ascospores. These are characterized by the presence of ridges or "ribs" alternating with "intercostal veins" (44). Reticulation amongst homothallics varies from the deeply furrowed veins of *N.dodgei* to the shallow rib-vein topography of *N.lineolata*. Size and shape also varies from the relatively large, oblong ascospores of *N.galapogosensis* to the smaller and more ovoid ascospores of *N.terricola* (3) [Figure 4]. A final character is the presence of single versus double germ pores. As described previously, only *N.terricola* has a single germinal pore; all other homothallics have two germinal pores (3,76). These various features and ornamentations, though subtle in some cases, are consistent and have constituted the established basis for delimiting species. Inspections by Austin et.al. (3) of greater than 100 ascospores of each of the

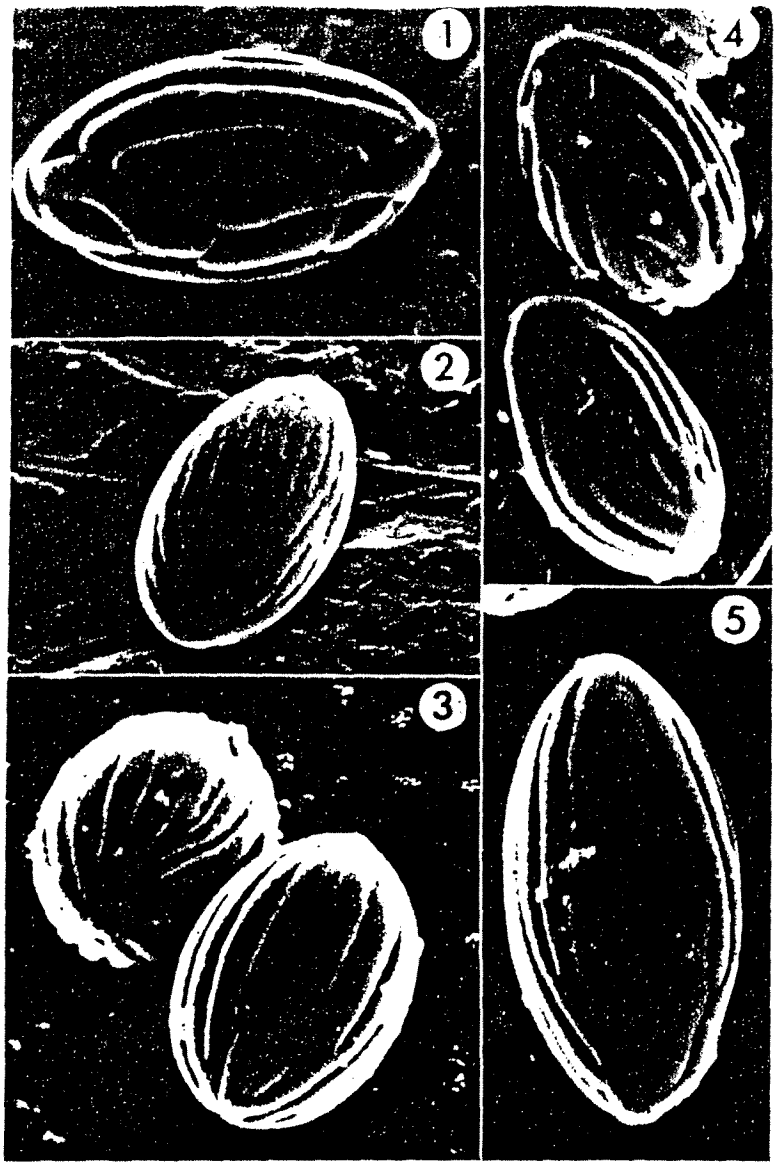

Figure 4 **SEM views of ascospores of five homothallic species of *Neurospora*.** **1**. *N.dodgei*; **2**. *N.lineolata*; **3**. *N.terricola*; **4**. *N.africana*; **5**. *N.galapogosensis*. *N.terricola* ascospores are smaller and more ovoid than those observed in other *Neurospora* homothallics. Figure adapted from Austin et.al. (1974).

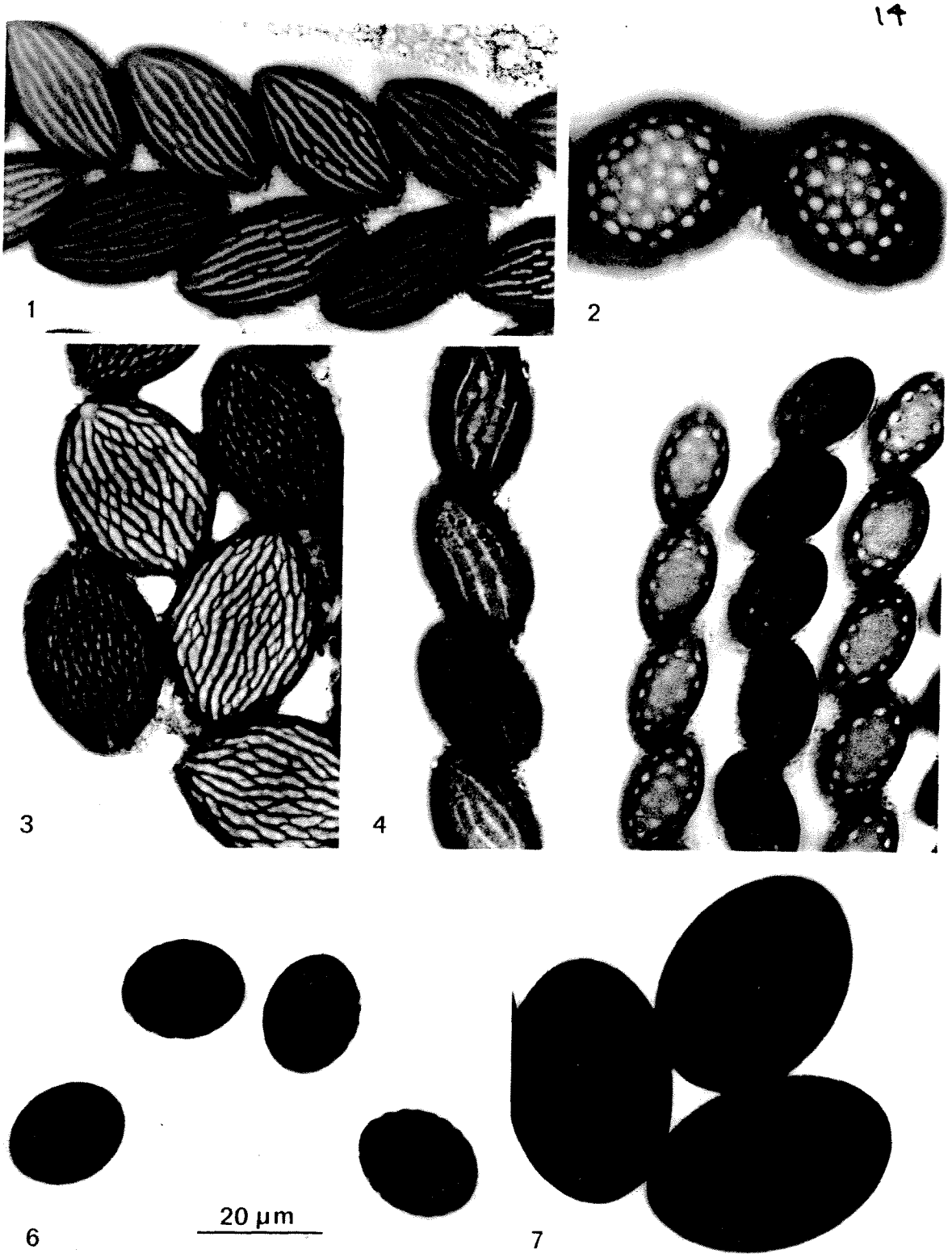

Figure 5 ***Sordariaceae* ascospore ornamentation.** **1**. *N.crassa*: longitudinal veins are unbranched; **2**. *Gelasinospora* species: large ovoid ascospores show prominent round pits. **3**. *A.sublineata*: veins are highly developed, branched, and reticulated. **4**. *Neurospora* species: veins unbranched and less reticulated. **5** and **6**. *Gelasinospora* species: very small ascospores with pits. **7**. *Gelasinospora* species: mature ascospores of this strain are huge and have no visible pits (immature spores show many small pits). From Glass et.al. (1990).

Neurospora homothallics revealed no significant variations from the characters described. Any further confirmation of a *Neurospora* homothallic taxonomy will presumably have to come from an examination of sequence and other molecular data.

Genus and species identifications of other *Sordariaceae* homothallics are based on ascospore and fruiting body morphology. *Gelasinospora* species have pitted ascospores [Figure 5] (23) while *Anixiella* species are characterized by their ascocarps which are spherical as compared to the flask-shaped ascocarps (perithecia) of *Gelasinospora* and *Neurospora*. In addition, the ascocarp of *A.sublineolata* lacks an ostiole, the perithecial opening through which ascospores are forcibly discharged in *Gelasinospora* and *Neurospora* species (23,29).

There are numerous indications that *Gelasinospora* and *Anixiella* are very closely aligned with *Neurospora*. Spindle formation and much of the meiotic progression in *Gelasinospora calospora* (43) is indistinguishable from that in *N.crassa* (74,76). The chromosome number is seven in *G.calospora* (43) as it is in all homothallic species of *Neurospora* (76). At least one *Anixiella* species, *A.sublineolata*, has ribbed ascospores (23) that are nearly indistinguishable from those of certain *Neurospora* species (3) [Figure 5]. Ascospores in the two genera are similar enough to have prompted one worker to reclassify *A.sublineolata* as a *Neurospora* species (97). Finally, as will become clear, there is striking evidence of alignment at the molecular level, namely a conservation in selected *Gelasinospora* and *Anixiella* species of *Neurospora* mating-type idiomorphs and flank sequences.

N.crassa mating-type locus

N.crassa individuals are haploid and contain only one mating-type sequence, A or a, that is present at the mating-type locus. The A and a sequences are entirely dissimilar and do not, therefore, fit the traditional definition of alleles. Their dissimilarity has prompted the term "idiomorphs" by the researchers who cloned them (26,88). As idiomorphs, A and a

are defined as unique 5301 and 3235 bp sequences, respectively, that in opposite mating-types occupy the same locus on linkage group I [Figure 1].

While the mating-type idiomorphs are unique, sequences bordering the idiomorphs (flank sequences) are nearly identical in A and a strains (26,88) [Figure 1] . It is these flank sequences to the left and right of the idiomorphs that really define the mating-type locus *per se*. At least one of the *N.crassa* flanks is conserved in the homothallic *N.africana* (L.Glass, personal communication; N.Beatty, unpublished results) and portions of both right and left flanks may define the mating-type locus in numerous taxa of the *Sordariaceae* (T.Randall, personal communication; N.Beatty, unpublished results). Sequence data from the right flank has revealed a single ORF that contains a DNA motif common to fungal pheromones and the region containing this ORF appears to be species-specific. Other portions of the right and left flanks, however, are conserved in heterothallic and homothallic species of *Neurospora*, *Gelasinospora*, and *Anixiella* (T.Randall, personal communication; N.Beatty, unpublished results).

Functional analyses of the *N.crassa* mating-type locus have associated at least three distinct functions with specific portions of the A and a idiomorphs [Figure 1]. A contains at least two functional segments, one of which acts in both the vegetative and sexual phases of the life-cycle. The A idiomorph contains a single ORF that encodes a polypeptide, mt A-1, of 288 amino acids (26). Similarly, the a idiomorph contains an ORF that encodes a polypeptide, mt a-1, of 382 amino acids (88). These polypeptides control entry into the sexual cycle; mutants in which the A or a ORF has been disrupted cannot mate (26,33). Mt A-1 and mt a-1 also act vegetatively to prevent the formation of mixed mating-type heterokaryons. In a reaction known as heterokaryon incompatibility, juxtaposition of vegetative mycelia with opposite mating-types results in severely limited growth or cell death at the point of contact (27). This reaction is dependent on intact copies of both mtA-1 and mt a-1 and presumably these polypeptides interact differently in vegetative

growth than they do in the sexual cycle. Otherwise, A and a nuclei could not be associated in the ascogenous dikaryon without eliciting an incompatibility reaction.

A and a functions have been determined by deletional (26,88) and mutational (13,28,33,34) analyses. The former has been employed to associate mating and heterokaryon incompatibility functions with specific regions of the mating-type locus. The latter analysis has led to the generation of mating-type mutants which have helped to elucidate the specific activities of mt A-1 and mt a-1 and other functional regions of the idiomorphs (28). Griffiths and DeLange (34) and Griffiths (33) generated a class of mutants, A^m and a^m, with mutations in mt A-1 and mt a-1 respectively. In functionality tests, these mutants were found to be sterile and heterokaryon compatible supporting the idea that mt A-1 and mt a-1 function in mating and vegetative incompatibility. Subsequently, other A mating-type mutants have been generated by repeat induced point (RIP) mutation (28). RIP operates on any duplicated sequence in *Neurospora* by altering both copies and rendering them dysfunctional. Alterations come in the form of G-C to A-T transition mutations and arise only during mitotic divisions of haploid nuclei in ascogenous hyphae (80,81). The result is that RIP can be used to generate mutants simply by introducing an extra copy of the sequence one wishes altered. Both the native and introduced copies are changed and selection for transformants yields functional mutants.

RIP-generated mutations (28) in regions (other than mt A-1) of the A idiomorph have revealed a novel functional region [Figure 1]. Strains with these mutations are morphologically indistinguishable from the wild type in early perithecial development but are greatly diminished in their ability to produce fully developed perithecia and ascospores. This same region, while required for perithecial development and certain meiotic functions, is apparently not necessary for vegetative incompatibility (28).

A region of the a idiomorph with similar function has been assessed by deletional analysis (Staben and Yanofsky 1990). *N.crassa* sterile mutants a^{m1}, a^{m33}, and a^{m30} (33,34) were transformed with various deletional portions of the a idiomorph and assessed

with regard to mating identity, heterokaryon incompatibility and perithecium maturation function. In this manner, mating and incompatibility functions were associated with the mt a-1. A second region of the idiomorph, not including the ORF, was found to function in the maturation of perithecia and the generation of ascospores (88). [Note: The second functional region is now in question; C.Staben, unpublished results; L.Glass, personal communication.] Both the A and a idiomorphs, then, contain ORFs which function in mating and opposite mating-type vegetative incompatibility; both also harbor additional regions that are required for perithecium development and certain meiotic functions.

PART ONE

MAPPING THE HOMOTHALLIC MATING-TYPE LOCI

INTRODUCTION I

Heterothallic *Neurospora* mating-type loci contain a single idiomorph, A or a, and conserved flanking sequences that are identical in both A and a strains. The idiomorphs code for mating, meiotic, and vegetative incompatibility functions and flank sequences have been implicated in the production of sexual pheromones (T.Randall, unpublished results).

Heterothallic sexual reproduction requires a mating event between strains with opposite mating-types. Mt A-1 and mt a-1 maintain control over cellular recognition events during vegetative and sexual growth. The role played by these polypeptides in self-fertilizing or homothallic species, though, is less clear. Especially in A/a-type homothallics such as *N.terricola*, the existence of mating-type genes is difficult to reconcile. Homothallics may require sexual reproduction in order to utilize meiotic repair mechanisms or to produce desiccation resistant ascospores. Mt A-1 and mt a-1 have important meiotic functions in homothallic species and yet the mating and vegetative incompatibility functions associated with mt A-1 and mt a-1 seem extraneous.

A mating-type locus, defined by an idiomorph and flank sequences, is well conserved in heterothallic *Neurospora* species including *N.crassa*, *N.intermedia*, and *N.sitophila* (L.Glass, personal communication). The A-type homothallic *N.africana* contains an A idiomorph that is >90% similar to the *N.crassa* idiomorph (L.Glass, unpublished results). Little is known, however, about the conservation in *N.africana* of *N.crassa* flank sequences. Even less is known about the mating-type loci of other *Neurospora* homothallics. Preliminary work has demonstrated that the *N.crassa* A idiomorph is highly similar in representative heterothallic (27) and homothallic (29) *Neurospora* species. Numerous *Gelasinospora* and *Anixiella* species also contain similar A sequences. A / a -type homothallic species of *Neurospora*, *Gelasinospora* and *Anixiella* contain A **and** a

sequences, both of which have a high degree of homology to the corresponding *N.crassa* idiomorphs (29). It is not clear, however, whether these species harbor both A and a mating-types in their entirety and whether both mating-types occupy a single locus.

The analysis of the mating-type loci of A/a-type *Sordariaceae* homothallics presented here has as its aim the formulation of a tenable evolutionary history of homo- and heterothallism. Arguments can be made for a history in which heterothallism evolved into homothallism. The presence in homothallic species of elaborate mating-type loci is reconcilable if they evolved first in heterothallic species and have been subsequently inherited by homothallics. Alternatively, it is counter-intuitive that homothallic species would develop mating and incompatibility functions they do not require. It is unclear whether A and a sequences function in A / a -type homothallics but a detailed picture of the mating-type locus(i) of selected homothallics may suggest mechanistic models to support the concept that a heterothallic *Neurospora* (or other *Sordariaceae*) species is ancestral to all homothallic species. The loss of mating-type-specific sequences, for example, can supply a directionality to cladistic trees and allow an evolutionary history to be constructed for *Neurospora* species. Coupled with information about the functionality in homothallic species of A and a idiomorphs (part II of this thesis), cladistic trees can then be expanded to address the evolution not only of species but of mating strategies in general.

MATERIALS AND METHODS I

List of strains

The strains employed in the analysis of mating-type locus conservation and linkage are listed in Table 2.

Table 2. Mating-type and ascospore morphology of *Sordariaceae* homothallic isolates. *Neurospora* species, heterothallic and homothallic, are known to have bi-polar ascospores. *N.terricola* is the single exception and has only a single germ pore. *N.terricola* is also unique in being the only *Neurospora* species isolated from a temperate region. FGSC refers to Fungal Genetics Stock Center: Department of Microbiology, University of Kansas Medical Center.

species	mating-type(s)	source	where collected	ascospore morphology
Anixiella sublineolata	A and a	FGSC 5508	Japan	intercostal veins and ribs; two germ pores
Gelasinospora calsopora	A and a	FGSC 6535	French Congo	pitted ascospores
Gelasinospora reticulospora	A and a	FGSC 6537	Colombia	pitted ascospores
Gelasinospora 142-1	A and a	R.L. Metzenberg	Yucatan Peninsula	pitted ascospores
Gelasinospora 143-4	A and a	R.L. Metzenberg	Yucatan Peninsula	pitted ascospores
Gelasinospora S23	A and a	D.D. Perkins	India	pitted ascospores
Neurospora africana	A	FGSC 1740	Nigeria	intercostal veins and ribs; two germ pores
Neurospora crassa A	A	FGSC 987	Louisiana	intercostal veins and ribs; two germ pores
Neurospora crassa a	a	FGSC 988	Louisiana	intercostal veins and ribs; two germ pores
Neurospora terricola	A and a	FGSC 1889	Wisconsin	intercostal veins and ribs; one germ pore

Growth conditions

Homothallic strains were grown in liquid media cultures containing 1x Modified Westergaard's salts [0.1% KNO_3, 0.08% KH_2PO_4 (anhydrous), 0.02% K_2HPO_4(anhydrous), 0.05% $MgSO_4$-$7H_2O$, biotin (25µg/mL), trace elements + NaCl + $CaCl_2$] and 2% fructose.

Cultures were kept stationary at room temperature (~25°C) for seven to ten days or until perithecia were clearly visible. Optimal mycelial yield was obtained after the onset of fruiting but before the forcible discharge of ascospores. The time required for the formation of perithecia varied from species to species and was generally shortest (4-5 days) for *Anixiella* cultures and longest (8-10 days) for *N.africana* and *N.terricola* cultures. Heterothallic strains were cultured in liquid media containing 1x Vogel's salts and 2% sucrose. Mycelia were allowed to grow for six to eight days until conidiation was well established. Both homo- and heterothallic mycelia were harvested by vacuum filtration onto Whatman 1 filter paper disks. Hyphal mats were then washed twice with 0.9% NaCl and transferred to sterile tubes.

Isolation of DNAs

After desiccation, hyphae were pulverized by vortexing with a glass rod. Then the following protocol (modification of the method of Berlin and Yanofsky, 1989) was performed to isolate DNAs of both homo- and heterothallic strains:

1. transfer 350µL pulverized hyphal powder to 1.5µL Eppendorf tube
2. suspend sample in 400µL of salt-detergent solution (4mg/mL sodium deoxycholate, 10mg/mL polyoxyethylene cetyl ether, 2M NaCl)
3. vortex and incubate at room temperature for 20 minutes
4. spin at 12,000rpm for 10 minutes
5. collect supernatant and transfer to fresh tube
6. add 1:4 vol:vol supernatant:TCA/EtOH [TCA/EtOH = 4.5M Na-TCA - in an ice bath, add concentrated NaOH until pH reaches neutrality]
7. mix gently by inversion and incubate on ice for 20 minutes
8. pellet nucleic acids by microfuging for 30 seconds

9. wash pellet with 70% EtOH, dry in speed vac, and re suspend in 300µL 10mg/mL RNAse
10. incubate at 50°C for one hour,
11. add 1:1 sample:phenol/CCl_4, vortex, spin, take supernatant
12. add NH_4OAc to 0.3M; then add 2.5 volumes 95% EtOH
13. mix well by inversion, incubate 10 minutes room temperature, spin, wash dry, resuspend

Digestion, electrophoresis and capillary blotting

N.terricola

N.terricola genomic DNA was digested with one of two groups of restriction enzymes. Group **I** incorporated the following single and double digestions: EcoRV, HindIII, EcoRV/HindIII, EcoRI, BglII, EcoRI/BglII, PstI, BamHI, PstI/BamHI. Group **II** incorporated single digests with more rare cutters: BssHII, KspI, MluI, SacI, ScaI, SmaI, SphI, XbaI. Digestions were performed with 5µg DNA and 20u enzyme for three hours at 37°C.

Other *Sordariaceae* homothallics

Genomic DNAs from the remaining homothallics were digested with restriction endonucleases organized into Groups **III** EcoRV; **IV** PstI; and **V** BamHI, HindIII, SacI, subjected to gel electrophoresis, and transferred to nylon membranes in the manner described for *N.terricola*. Membranes were hybridized to the same *N.crassa* probes utilized in the mapping of the *N.terricola* idiomorphs and flank regions.

Digested DNAs were electrophoresed in 0.8% agarose gels at 35 volts for 14-18 hours. Gels were stained and photographed to visualize the efficiency of digestions. The following denaturations (77) were then performed to facilitate DNA transfer to membranes:

1. acid depurinate in 0.25M HCl for 10 minutes
2. alkali denature in 1.5M NaCl / 0.5M NaOH 2 x 15 minutes
3. neutralize in 1.0M Tris-Cl pH 7.4 / 1.5M NaCl 2 x 15 minutes

Transfer (77) to Nylon membranes (Amersham Corp.) was performed by capillary transfer with 10X SSC. Blotting was effectively complete after 12 hours and gels were transilluminated with UV light to confirm the absence of fluorescent EtBr (indicating a complete transfer). Membranes were baked at 65°C for two hours and stored in plastic bags.

Probes and hybridizations

Idiomorph and flank region probes were constructed from cloned portions of the *N.crassa* A and a mating-type loci. This was done by analyzing consensus maps of *N.crassa* clones and digesting with restriction enzymes to yield the desired fragments. Digests were confirmed by electrophoresis and target bands were purified by excising them from the gel and extracting the DNA (GeneClean; Bio 101 San Diego, CA). Membranes were hybridized to a variety of probes representing idiomorph and flanking regions of the *N.crassa* mating-type locus. A graphic representation of *N.crassa* clones and the probes constructed from them can be found in Figures 6 and 7. Membranes were incubated in pre-hybridization fluid (77) for a minimum of three hours. During this incubation, probes were labeled by the random priming method (T7 Quick Prime kit; Pharmacia Corp.) with [α ^{32}P]dCTP (Amersham Corp.), gravity filtered through a Sephadex G-50 column, and measured in a scintillation counter. The target range for labeled probes was a specific activity of $8x10^6$ to $1.5x10^7$ counts per minute. Probes were denatured by boiling for five minutes and placed on ice. Pre-hybridization fluid was replaced with 15 mL of hybridization fluid (77), labeled probe was added, and membranes were incubated at 65° for 12 to 18 hours. Membranes were then subjected to the following washes: 1% SDS, 0.1x SSC 15' RT; 0.1%SDS, 0.1x SSC 30' 60° (2 times). After three to seven day exposures to Kodak X-OMAT film, restriction fragment size patterns were compared to each other and analyzed with respect to band sizes predicted from established maps of *N.crassa*.

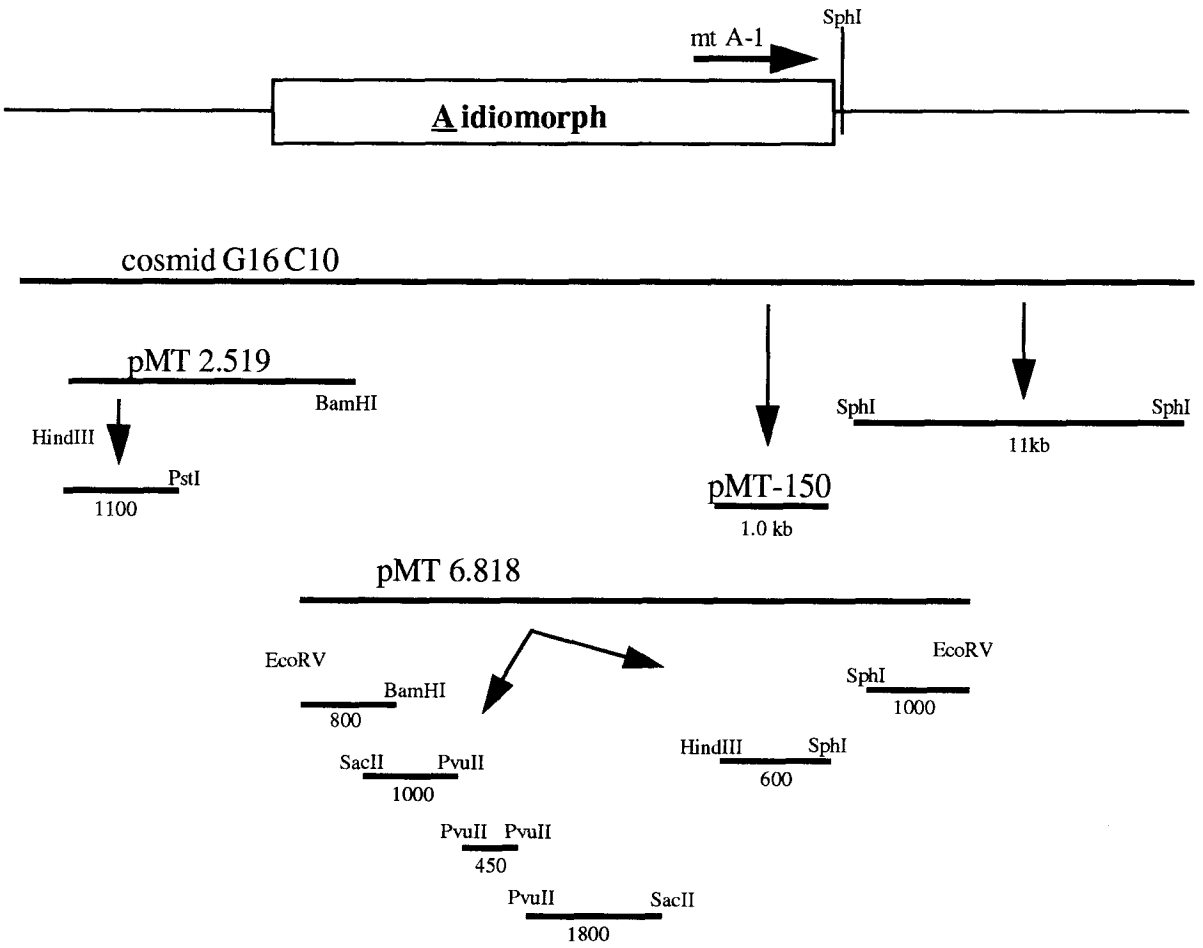

Figure 6 ***N.crassa*** **A clones and probes derived from them.**
The G16 C10 cosmid, pMT 6.818, pMT -150, and pMT 2.519 clones were provided by N.L.Glass. Consensus *N.crassa* A sequences (27) were used to choose restriction sites and to construct probes based on digestion at those sites. Probes are drawn so that they lie beneath the approximate region in *N.crassa* from which they were derived.

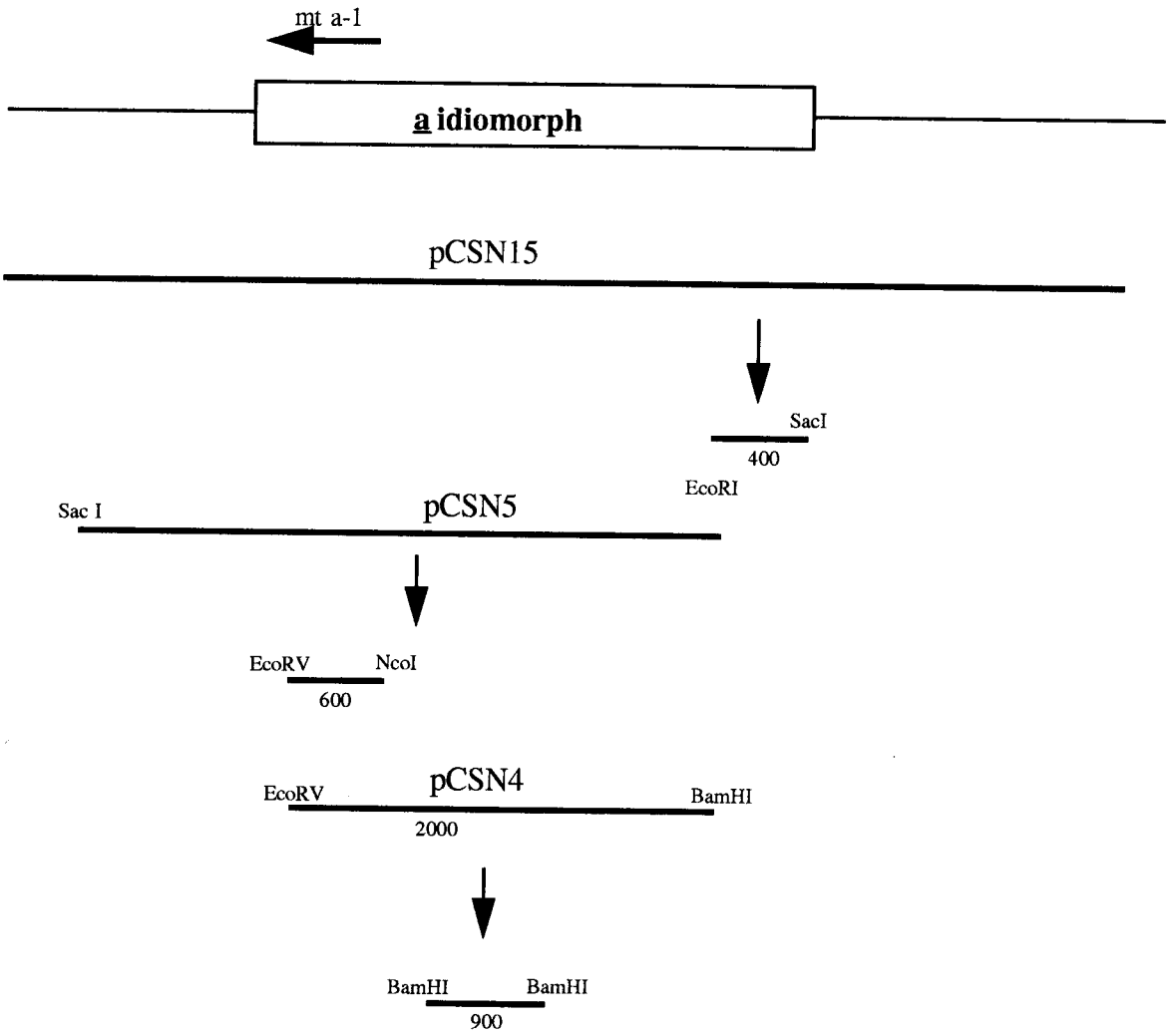

Figure 7 ***N.crassa*** **a clones and probes derived from them.**
The pCSN15, pCSN5, and pCSN4 clones were provided by N.L.Glass. *N.crassa* consensus a sequences (88) were used to choose restriction sites and to construct probes based on digestion at those sites. The pCSN4 plasmid was used in its entirety as a probe. Probes are drawn so that they lie beneath the approximate region in *N.crassa* from which they were derived.

RESULTS I

Determining conservation of homothallic mating-type loci

A idiomorph

DNA from each of the homothallic isolates was digested with a Group **IV** enzyme (PstI) and hybridized to selected *N.crassa* A probes. A HindIII/SphI probe representing the *N.crassa* mt A-1 and two central idiomorph probes, PvuII/SacII and PvuII/PvuII [Figure 6], hybridized strongly to *N.terricola*, *Gelasinospora*, and *Anixiella* DNA. Based on these strong hybridizations between *N.crassa* and homothallic DNAs, it is clear that the majority of the *N.crassa* A idiomorph is conserved in the homothallic species examined [Figures 8,12,14].

The distal portion of the idiomorph, opposite mt A-1, is more variable. Though most of the homothallic species tested contain the *N.crassa* A idiomorph in its entirety, at least one homothallic species is missing a significant segment of the idiomorph. A BamH1/EcoRV 800 bp probe [Figure 6], derived from the end of the idiomorph opposite mt A-1, hybridized strongly to all homothallic species except *N.terricola* [Figure 14]. Strong hybridization was apparent in all lanes except that with *N.terricola* DNA [Figure 14, lane g], indicating that a portion of the A idiomorph at least as large as that spanned by the 800 bp probe is missing from the *N.terricola* genome. A probe, PvuII/PvuII [Figure 6], generated from a sequence slightly internal to the BamHI/EcoRV probe, hybridized well to *N.terricola* DNA, indicating that the *N.crassa* region spanned by this probe is present in the *N.terricola* genome. It is clear, then, that the border in *N.terricola* between A-specific sequence and "flank"sequence lies in the 400bp intervening region between the BamHI/EcoRV and PvuII/PvuII probes. Thus, *N.terricola* lacks approximately 1.0 kb of the *N.crassa* A idiomorph; the *N.crassa* idiomorph is 5301 bp while the *N.terricola* idiomorph is approximately 4300 bp. All other homothallic species tested have A idiomorphs that are similar in size to that of *N.crassa*.

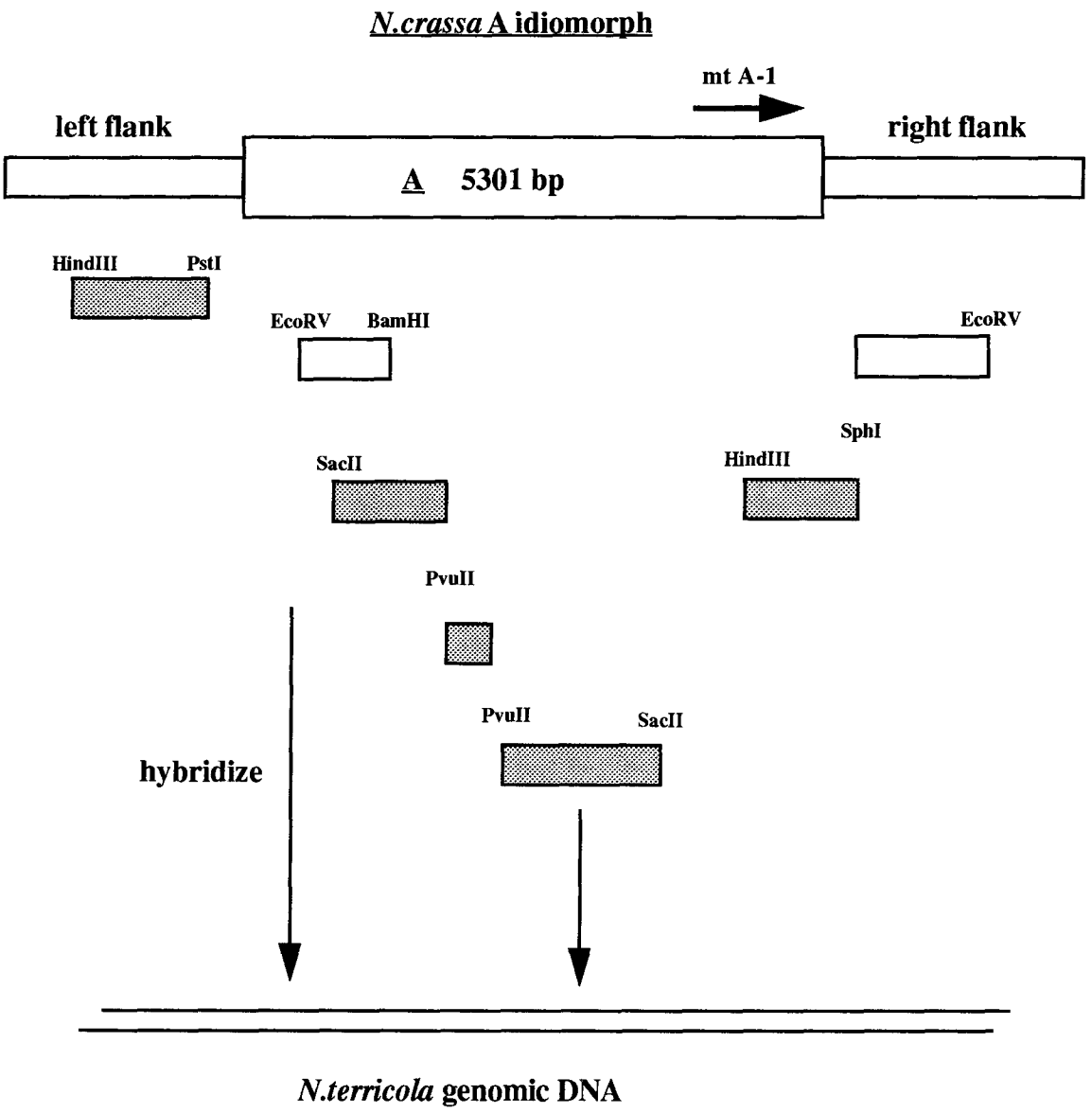

Figure 8 **Results of *N.terricola* genomic DNA hybridizations to *N.crassa* A probes.** Probes generated from *N.crassa* clones were hybridized to *N.terricola* genomic DNA. Shaded boxes represent those probes that hybridized. The unshaded boxes represent probes that did not hybridize to *N.terricola* though it did hybridize strongly to *Gelasinospora* and *Anixiella* DNAs. The HindIII/PstI left flank fragment is included as a reference. In *N.terricola*, this flank sequence is contiguous with the a idiomorph. (See Figure 9). In *G.reticulospora* and *G.S23*, the same flank sequence may be contiguous with the a idiomorph, the A idiomorph or both.

a idiomorph

The *N.crassa* a idiomorph was found to be largely conserved in each homothallic species examined. Three *N.crassa* a idiomorph probes, pCSN4 (entire), pCSN4 EcoRV/NcoI and pCSN15 EcoRI/SacII [Figure 7] were hybridized to homothallic DNAs digested with the Group **IV** enzyme (PstI). A pCSN4 probe, which represents the majority of the *N.crassa* a idiomorph, hybridized strongly to all homothallic DNAs [Figure 15: **1**]. A pCSN4 EcoRV/NcoI probe, corresponding to mt a-1, also hybridized well (data not presented as a photograph) to homothallic DNAs. The pCSN15 EcoRI/SacII probe, from the end of the idiomorph opposite the ORF, did not hybridize to any homothallic DNAs [Figure 15: **2**]. The weak hybridization in lanes c, d, and i of Figure 15 (probe **2**) can be attributed to a small amount of probe contamination, possibly with A idiomorph sequences. The difference in intensity between the a conrol DNA hybridization (lane j) and the weak hybridizations in neighboring lanes, however, makes clear the fact that the *N.crassa* right flank sequence is absent or highly diverged in *Sordariaceae* homothallics.

The pCSN15 EcoRI/SacII probe represents a portion of the *N.crassa* a idiomorph, extending from the EcoRI site to the right flank border, that is absent from the genomes of all the homothallic species examined. The missing portion includes the EcoRI/SacII fragment itself as well as the remaining 200bp that separate the SacII site from the border of the idiomorph [Figures 7, 9]. The a idiomorphs of the homothallic species tested are missing approximately 700 bp of the *N.crassa* a idiomorph. The homothallic a idiomorphs measure approximately 2500 bp in length while the *N.crassa* idiomorph is 3235 bp in length.

To clarify the *N.terricola* a idiomorph border, Group **I** digested genomic DNA was hybridized to a third *N.crassa* a probe, pCSN4 BamHI/BamHI, representing a region internal to the EcoRI/SacII fragment which is not present in *N.terricola* [Figures 7,9]. Given the absence in *N.terricola* of sequences represented by the pCSN15 EcoRI/SacII probe, the intent was to determine the approximate region where conservation of the

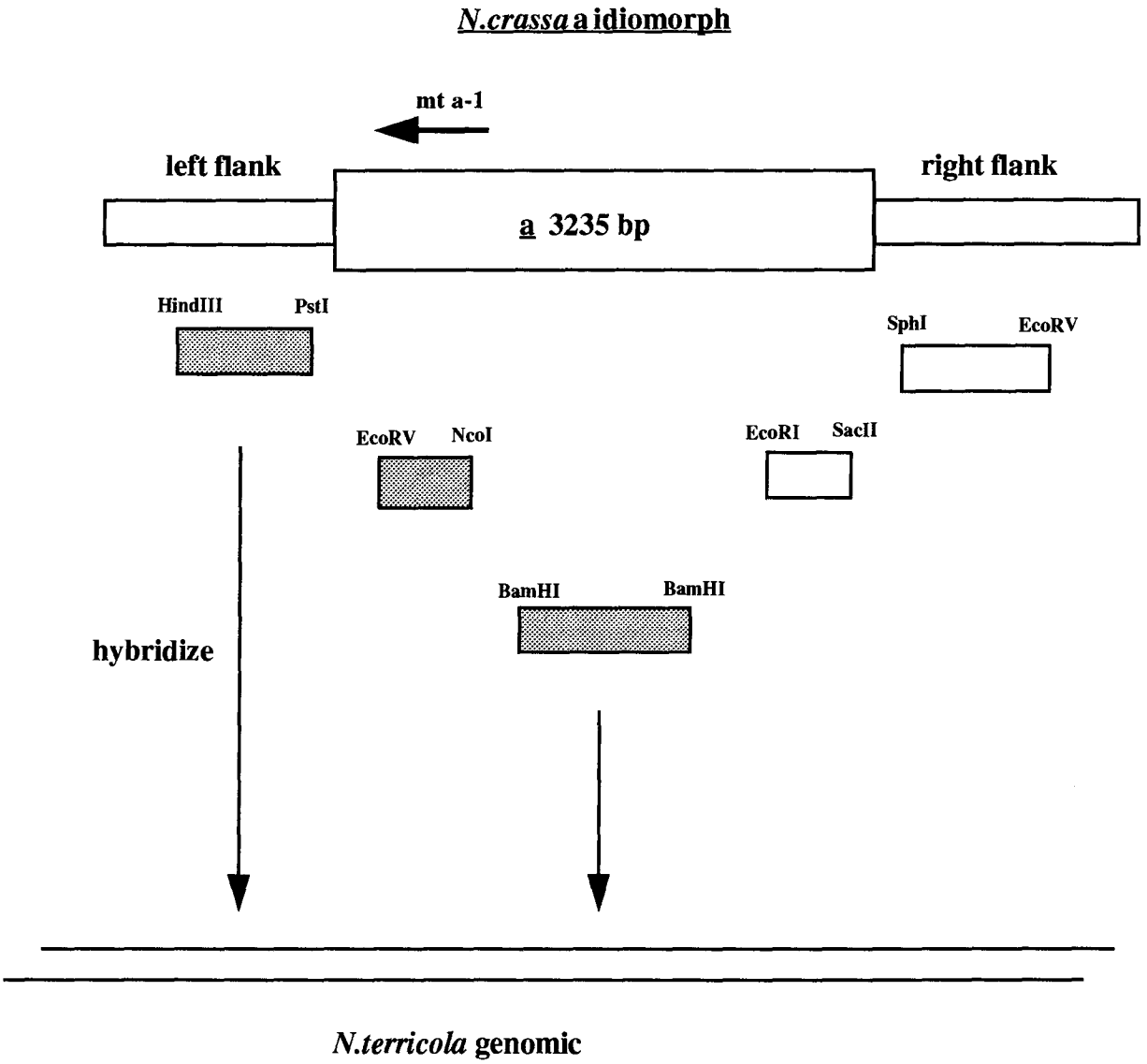

Figure 9 **Results of *N.terricola* genomic DNA hybridizations to *N.crassa* a probes.** Probes generated from N.crassa clones were hybridized to *N.terricola* genomic DNA. Shaded boxes represent those probes that hybridized and unshaded boxes those that did not hybridize. Flank probes are identical to those in the Figure 8. In most homothallic species tested, left flank sequences are contiguous with the a idiomorph [Figure 10]. Right flank sequences are absent in all cases.

N.crassa a idiomorph resumes. Hybridization yielded a characteristic a pattern similar to that observed from hybridization to the pCSN4 probe [see Figure 12: **2**]. Sequences represented by the BamHI/BamHI (900bp) probe, then, are contiguous with the a ORF and part of the *N.terricola* a idiomorph. The *N.terricola* a idiomorph border must lie between the BamHI site and the EcoRI site.

Summarizing a idiomorph data, approximately 700bp of the *N.crassa* a idiomorph is missing from the genomes of all *Sordariaceae* homothallics tested. In the remainder of the homothallic isolates, the a idiomorph is well conserved and, based on the intensity of hybridizations, appears to be highly similar to the *N.crassa* a idiomorph.

Analysis of A and a linkage in homothallic *Sordariaceae*

A / a linkage in *N.terricola*

Neurospora terricola genomic DNA, when digested with the Group **I** restriction enzymes, yielded fragments ranging from 0.5 to 12 kb in length. These fragments were subjected to gel electrophoresis, transferred to nylon membranes, and membranes were hybridized to *N.crassa* A idiomorph, a idiomorph, and flank region probes. The RFLP pattern [Figure 12: **1**] observed after *N.terricola* hybridization to a *N.crassa* HindIII/SphI mt A-1 probe [Figure 6] was entirely dissimilar to that [Figure 12: **2**] generated from a *N.crassa* NcoI/EcoRV mt a-1 probe [Figure 7]. The fact that *N.terricola* hybridizations to *N.crassa* mt A-1 and mt a-1 probes each produced a unique pattern indicates that the location of each *N.terricola* A and a idiomorph is also unique. (The ORF is considered integral to the idiomorph so that the ORF locus also describes the idiomorph locus). Based on analysis of band sizes, no single *N.terricola* fragment hybridized to both A and a *N.crassa* probes. The A and a idiomorphs in *N.terricola* must be separated by a region at least as sizable as the largest unique band observable from the autoradiographs, in this case some 10kb.

To elucidate further the linkage between *N.terricola* A and a idiomorphs, Group **II** enzymes were employed. The genomic fragments (3-20 kb) produced by these less frequent cutters were larger on average than those (0.4- 10kb) produced by the Group **I** enzymes. Of eight Group **II** enzymes employed, three gave similarly sized bands in hybridizations to both A and a *N.crassa* idiomorphs. BssHII, MluI and SmaI digests each yielded bands of similar size in A and a hybridizations [Figure 13: lanes a, c, f]. Two interpretations of these data are possible. The *N.terricola* A and a idiomorphs may be separated by an intervening sequence greater than 10kb but smaller than 18kb in length; the similar size bands may represent the same fragment and indicate linkage between the A and a idiomorphs. Alternatively, the similar sizes of MluI and SmaI bands in A and a hybridizations, respectively, may be random and insignificant. In either case, size differences of large bands are difficult to resolve in low percentage (0.8%) agarose gels. Pulsed field gel electrophoresis (CHEF) could likely resolve the linkage between A and a and the association of homothallic mating-type idiomorphs with specific linkage groups.

A / a linkage in other *Sordariaceae* homothallics

Gelasinospora and *Anixiella* DNAs were digested with Group **V** enzymes, subjected to gel electrophoresis, and transferred to nylon membranes. Membranes were hybridized to pMT -150 (G-2) and pCSN4 *N.crassa* probes [Figures 6, 7] representing mt A-1 and mt a-1 sequences, respectively. Autoradiograph band patterns [Figure 18: **2** versus **3**] that resulted from hybridizations to these probes were entirely dissimilar. As with *N.terricola*, unique band patterns were equated with unique positions for the A and a idiomorphs. The two idiomorphs must be separated by a distance at least as great as the largest dissimilar band, in this case 12kb.

Conservation in *N.terricola* of *N.crassa* left and right flank sequences

When a HindIII/PstI *N.crassa* left flank probe [Figure 6] was hybridized to *N.terricola* genomic DNA , a RFLP pattern [Figure 17: **1**] was observed that exactly

matches the pattern [Figure 17: **2**] generated when hybridization was made to the *N.crassa* pCSN4 [Figure 7] probe. Such a matching pattern could only be produced if the left flank and a idiomorph sequences are contiguous in *N.terricola*. It is not clear, however, how the *N.terricola* a idiomorph is oriented and whether the conserved *N.crassa* flank sequence borders to the left or the right. No bands were observed [Figure 19: **1**] when a SphI/EcoRV *N.crassa* right flank sequence [Figure 6] was used as a probe indicating that this sequence is unique and not conserved in *N.terricola*.

Additional hybridizations were performed to investigate the possibility that some portion of the *N.crassa* right flank might be conserved in *N.terricola*. *N.terricola* genomic DNA digested with Group **I** enzymes was hybridized to a large (12kb) SphI/SphI fragment [Figure 11] of the *N.crassa* cosmid G16 C10. This fragment encompasses the 1.0kb SphI/EcoRV right flank probe that did not hybridize to *N.terricola*. The probe also includes an additional 11kb of *N.crassa* flank sequence that extends further out from the (A or a) idiomorph. The intent was to hybridize this large flank probe to *N.terricola* genomic DNA and to look for a RFLP pattern that matches the pattern produced by hybridizations to A or a idiomorph probes. Such a match would indicate some conservation of *N.crassa* right flank sequences in *N.terricola* and the linkage of these sequences to an idiomorph. The result obtained [Figure 20] was a strong hybridization but a RFLP pattern dissimilar to both A and a idiomorph patterns. The presence of *N.crassa* right flank sequences was confirmed but no linkage was established. CHEF gel analysis could likely resolve whether the right flank sequences conserved in *N.terricola* are linked to the A or a idiomorph (or not linked to either idiomorph).

Conservation in *Anixiella* and *Gelasinospora* of *N.crassa* left and right flank sequences

Homothallic DNAs digested with Group **III** and Group **IV** enzymes were hybridized to a pMT 2.519 HindIII/PstI probe [Figure 6] that represents the *N.crassa* left flank region. Each of the homothallic DNAs hybridized strongly to this probe [Figure 14: **2**]. Thus a

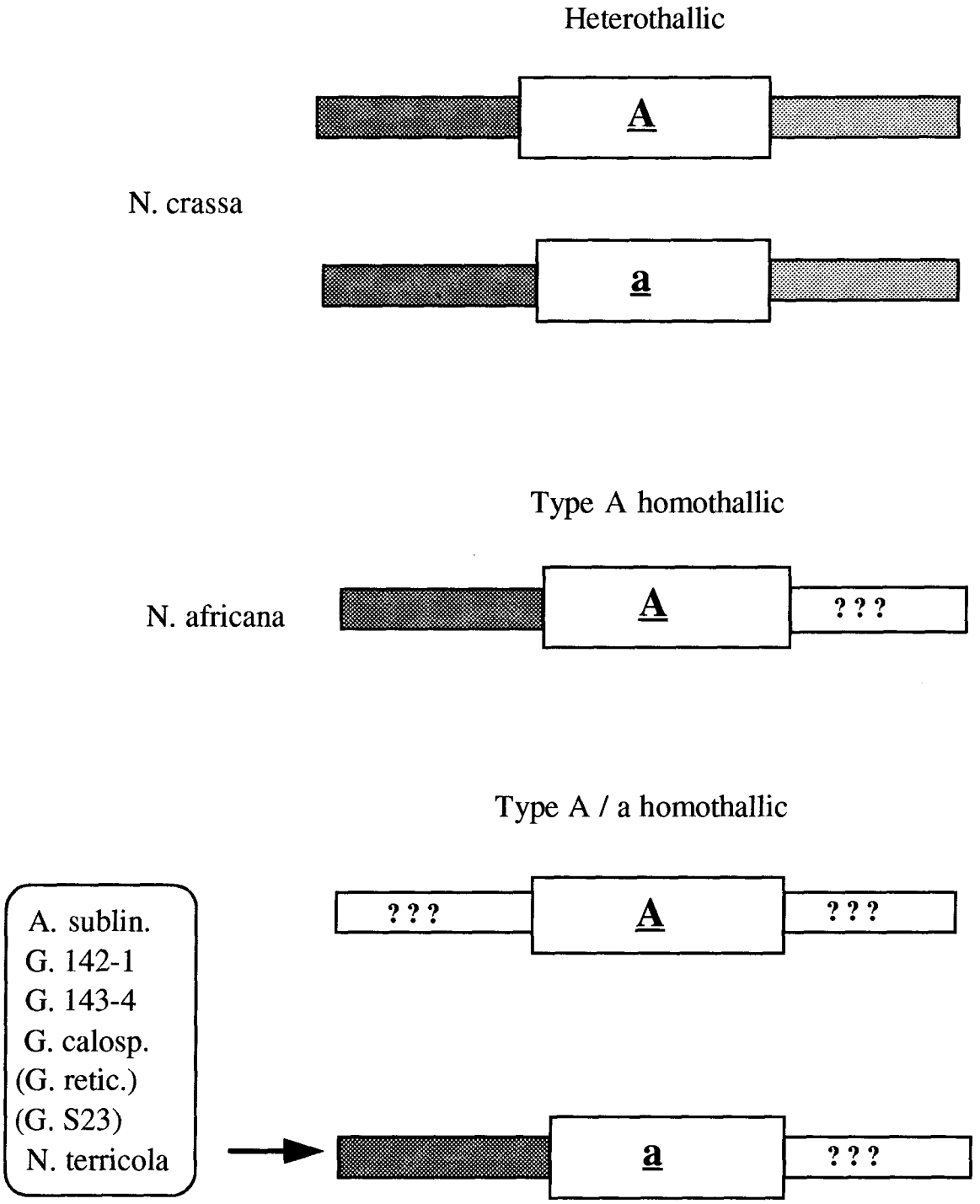

Figure 10 **Summary of linkage between flank sequences and idiomorphs in *Sordariaceae* homothallics.** The *N.crassa* left flank region is similar in *Sordariaceae* hetero- and homothallics.The left flank is contiguous with the A idiomorph of *N.africana*. The same flank is contiguous with the a idiomorph in 6 of 8 A/a-type homothallics tested. Idiomorph/left flank linkage has not been conclusively determined for *G.reticulospora* and *G.S23*. The *N.crassa* right flank is absent or diverged in all homothallics examined.

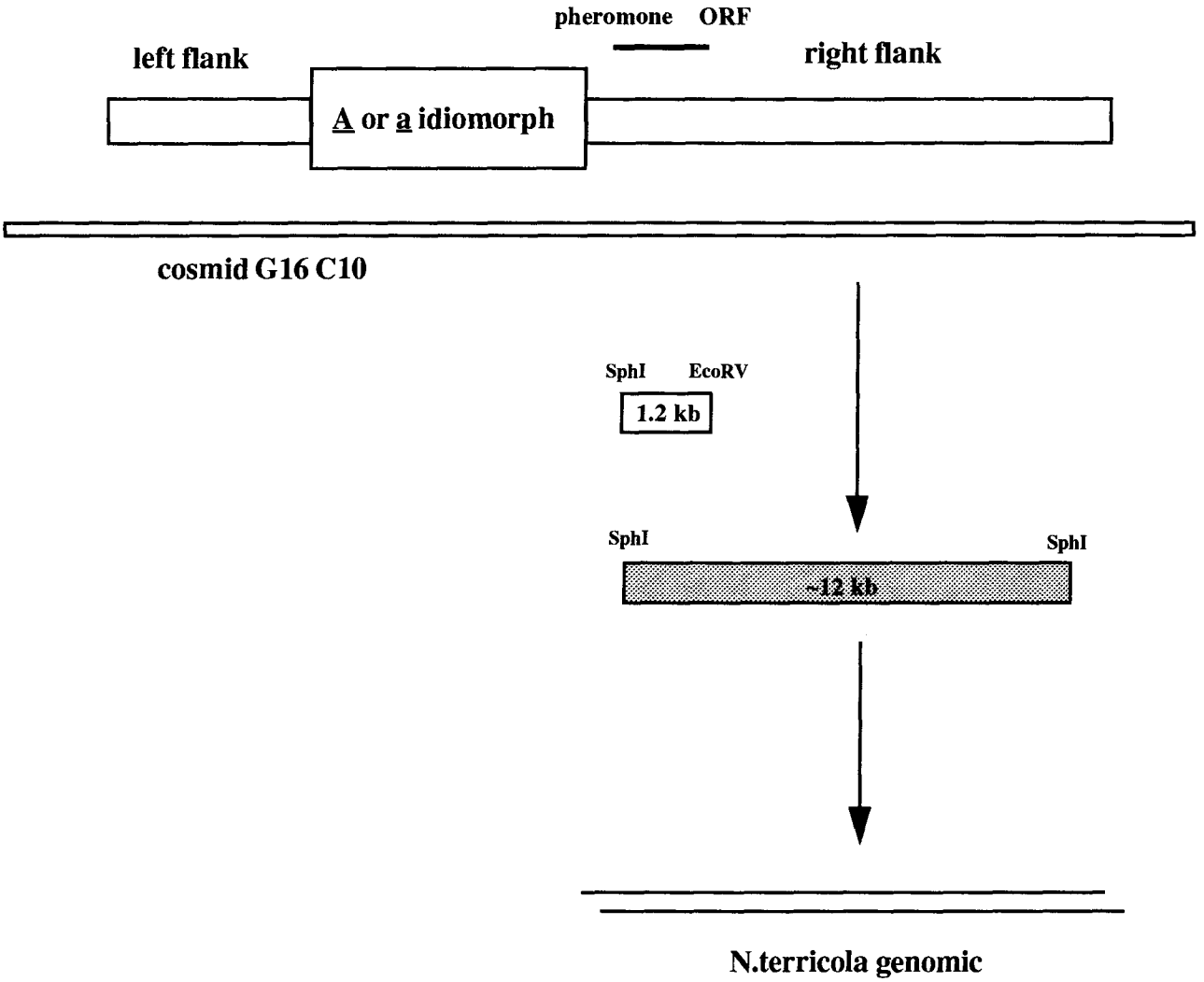

Figure 11 **Detail of *N.crassa* right flank region and its conservation in *N.terricola*.** Probes generated from an *N.crassa* cosmid were hybridized to *N.terricola* genomic DNA. The shaded box represents a probe that hybridized strongly, the unshaded box a probe thatdid not hybridize. The region containing the pheromone ORF is absent or diverged in *N.terricola*, *Gelasinospora*, and *Anixiella* species. This region may be species-specific in the *Sordariaceae*. Distal to this region, *N.crassa* sequences are again present in *N.terricola*, suggesting that the mating-type region as a whole may be conserved. Figure is not to scale.

significant portion of the *N.crassa* left flank region is present in the genomes of each of the homothallic species tested. The single digests from groups **III** and **IV**, however, yielded insufficient data to resolve the question of whether the homothallic left flank sequence is linked to the A or a idiomorph.

To resolve this linkage question, *A.sublineata, G.calospora, G.reticulospora, G.142-1, G.143-4,* and *G.S23* DNAs were digested with Group **V** enzymes. Membranes were hybridized to pMT -150(G-2), pMT 2.519 PstI/HindIII [Figure 6] and pCSN4 [Figure 7] *N.crassa* probes, representing mt A-1, left flank, and mt a-1 sequences respectively. *A.sublineolata*, *G.calospora*, and *G.143-4* each yielded similar RFLP patterns when probed with the *N.crassa* left flank and mt a-1 probes [Figure 18: **1** versus **2**]. This fact suggests that the left flank sequence is contiguous with the a idiomorph in these species. Linkage is less clear for the remaining *Sordariaceae* homothallics. 1 of 3 *G.reticulospora* bands is the same in A idiomorph and left flank hybridizations while 0 of 3 match in a idiomorph and left flank hybridizations. Precisely the same situation was observed in *G.S23* hybridizations. Presumably due to insufficient DNA, *G.142-1* hybridizations were too weak [Figure 18: lanes j-l] to make any conclusions about linkage. Summarizing these data, the left flank region of *N.crassa* is present in all *Sordariaceae* homothallics examined. This flank is contiguous with the a idiomorph in *A.sublineolata*, *G.calospora*, and *G.143-4*. The same flank region may be contiguous with the A idiomorph in *G.reticulospora* and *G.S23* but such a conclusion is equivocal.

Similar analyses were made of the right flank region. An *N.crassa* pMT 6.818 SphI/EcoRV right flank probe [Figures 6] was hybridized to homothallic DNAs digested with Group **III** and Group **IV** enzymes. No hybridization was observed to any of the eight [Table 2] homothallic DNAs; six repeat hybridizations [Figure 19: **1**] with DNA from each strain yielded no observable bands. Thus all *Sordariaceae* homothallics tested, including *N.terricola*, lack the *N.crassa* right flank sequence encompassed by the SphI/EcoRV right flank probe. Most of the *Anixiella* and *Gelasinospora* species tested do harbor a distal right

flank sequence from *N.crassa*, however. Hybridizations of homothallic DNAs to a G16 C10 SphI/SphI probe [Figure 11] showed the presence of this sequence in all species tested [Figure 19: **2**] with the exception of *G.142-1* and *G.S23* [lanes d and f].

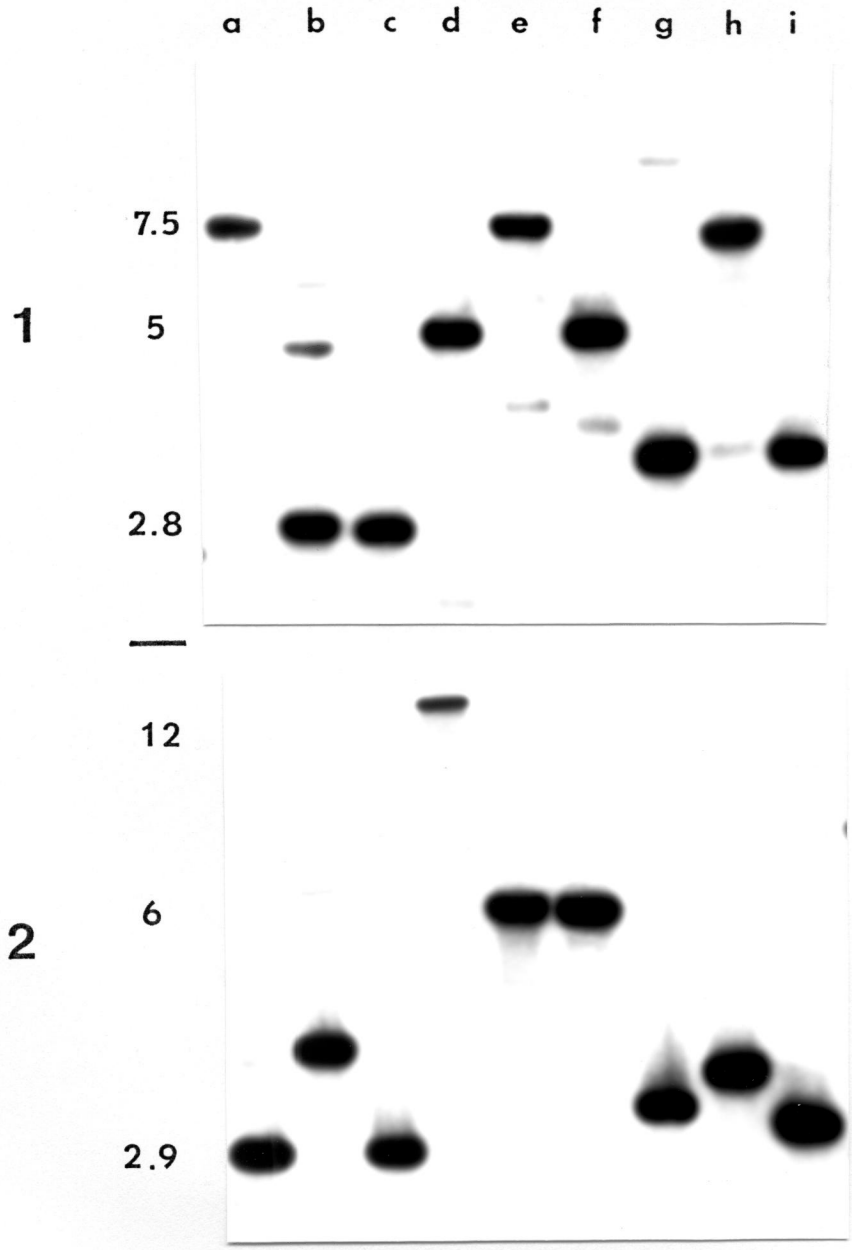

Figure 12 ***N.terricola*** **A and a idiomorphs are not closely linked.**

N.terricola genomic DNA was probed with **1**. pMT 6.818 (entire) and **2**. pCSN4. [See figures 7 and 8]. Lanes are digests with a)EcoRV, b)HindIII, c)EcoRV/HindIII, d)EcoRI, e)BglII, f)EcoRI/BglII, g)PstI, h)BamHI, i)PstI/BamHI. Band patterns are entirely dissimilar indicating in *N.terricola* that A- and a-specific sequences are separated (unlinked) by at least 12kb (lane d).

Table 3. **Results of hybridizations of homothallic genomic DNA to *N.crassa* idiomorph and flank region probes.** Hybridizations, when positive, were strong in all cases. Failed hybridizations (*N.terricola* genomic to BamH1/EcoRV fragment and all isolate genomic DNAs to SphI/EcoRV right flank fragment) were repeated three or more times to confirm the absence of those sequences from genomes. See Figures 6-10 for graphic representation of probes and hybridizations.

***N.crassa* probe**	**size**	**mating-type**	**region of idiomorph (locus)**	**hybridization to *N.terricola***	**hybridization to *Anixiella*, and *Gelasinospora***
HindIII/SphI	600	A	mt A-1	yes	yes
PvuII/SacII	1800	A	middle	yes	yes
PvuII/PvuII	450	A	middle	yes	yes
PvuII/SacII	1000	A	middle	yes	yes
BamHI/EcoRV	800	A	opposite mt A-1	no	yes
EcoRV/NcoI	600	a	mt a-1	yes	yes
BamHI/BamHI	900	a	middle	yes	yes
pCSN4	2000	a	middle plus mt a-1	yes	yes
EcoRI/SacII	400	a	opposite mt a-1	no	no
HindIII/PstI	1100	----	left flank	yes	yes
SphI/EcoRV	1000	----	right flank	no	no
G16 C10 SphI/SphI	12kb	----	right flank	yes	yes *

* with exception of *G.142-1* and *G.S23*

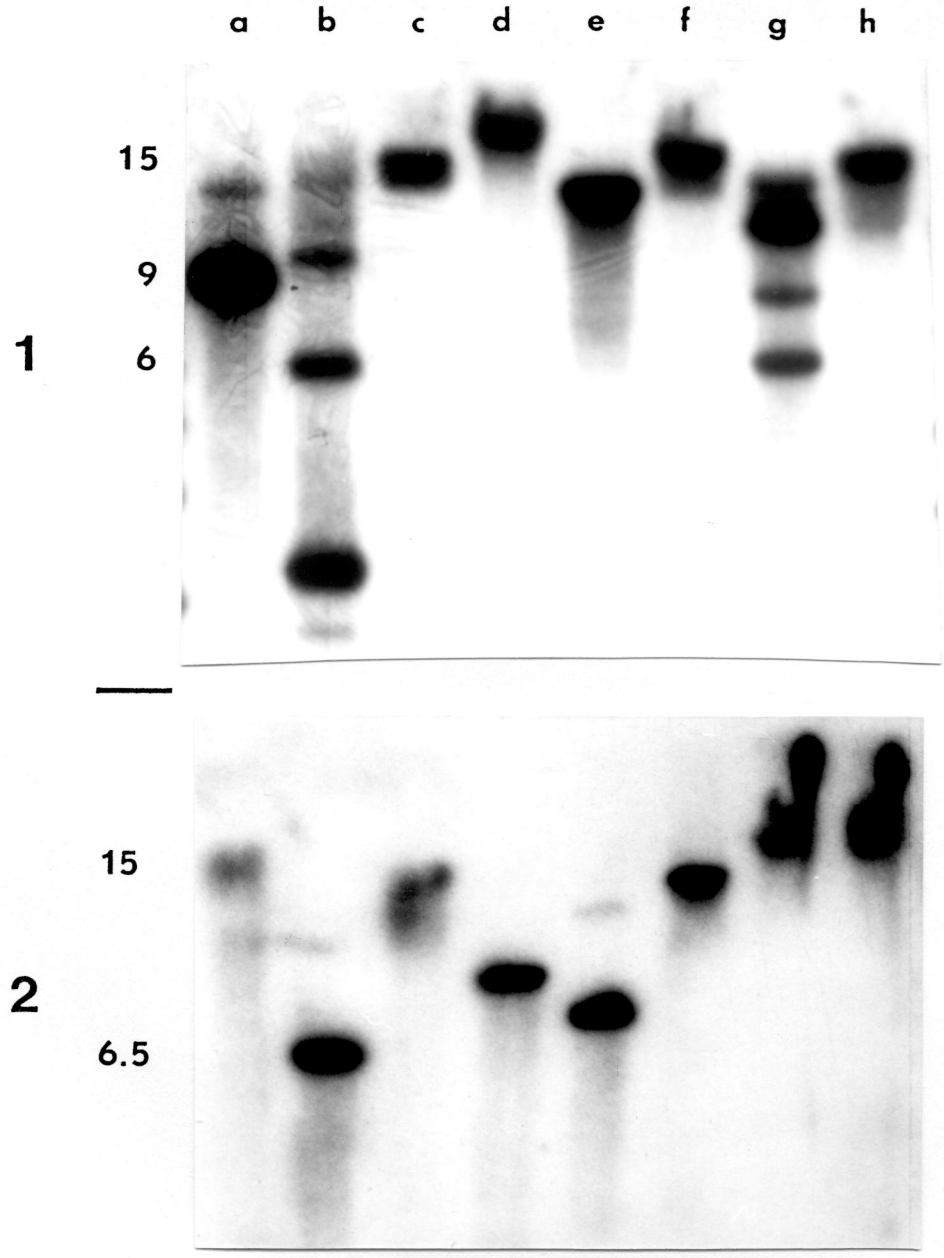

Figure 13 ***N.terricola*** **A and a idiomorphs are not closely linked.**
N.terricola genomic DNA was digested with rare cutters and probed with **1**. pMT 6.818 (entire) and **2**. pCSN4. [See figures 6 and 7]. Lanes are digests with a)BssHII, b)KspI, c)MluI, d)SacII, e)ScaI, f)SmaI, g)SphI, h)XbaI. Band patterns are largely dissimilar indicating in *N.terricola* that A- and a-specific sequences are separated (unlinked) by at least 16kb (lane d). Lanes a, c and f have bands that are similar in size in digests **1** and **2**, a result which may or may not be significant in terms of linkage (see Materials and Methods I: Analysis of A/a linkage in *N.terricola*).

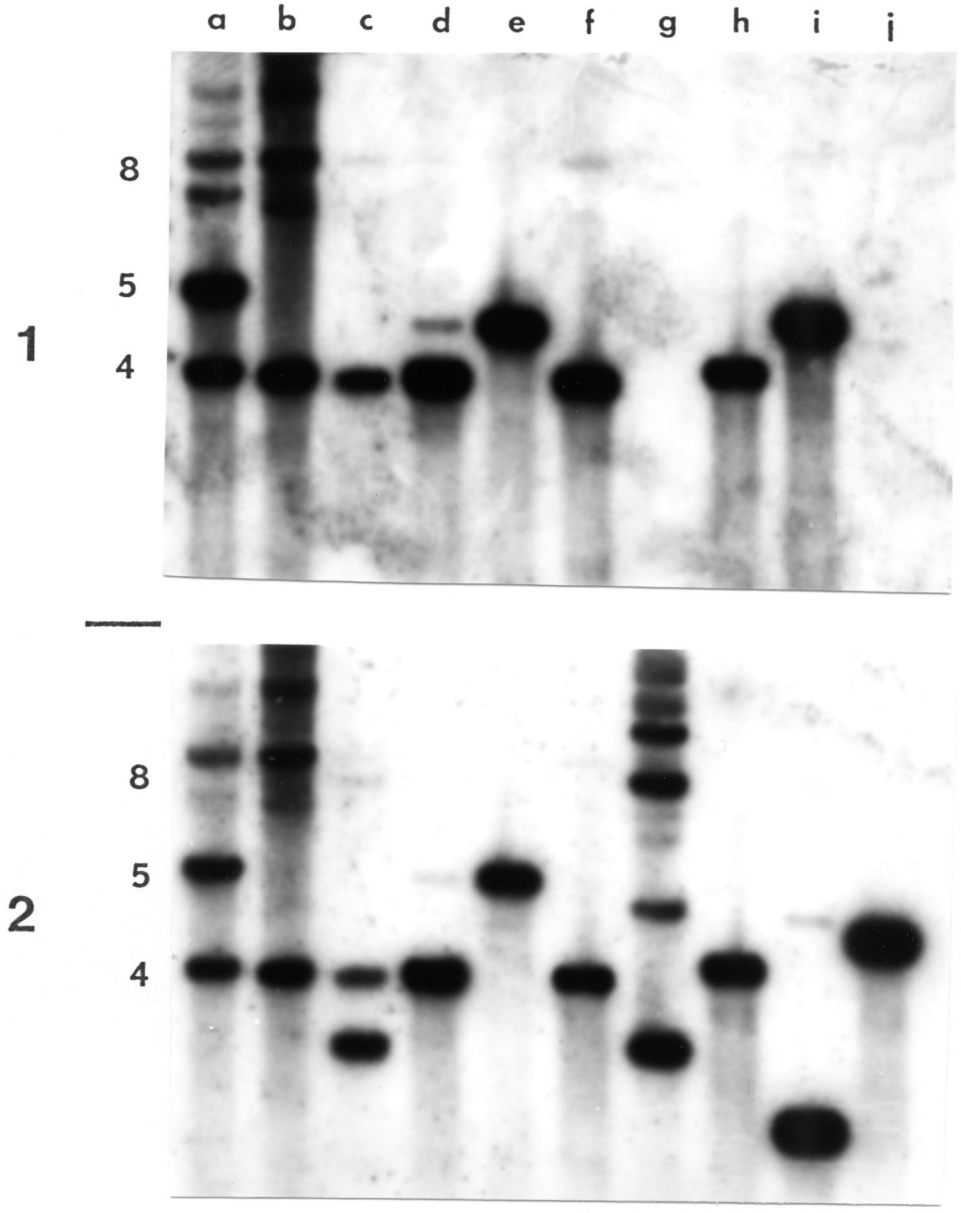

Figure 14 ***Sordariaceae*** **homothallics, with the exception of *N.terricola*, contain the entire *N.crassa* A idiomorph; all contain a left flank sequence.** Homothallic DNA was digested with PstI and probed with **1**. pMT6.818 EcoRV / BamHI (800bp) and **2**. pMT2.519 HindIII / PstI (1100bp). [Figures 6 and 8]. Probe **1** represents an internal A-specific sequence that is absent (lane g) in *N.terricola*. Probe **2** represents the *N.crassa* left flank region which is present in all species tested. Similar band patterns in **1** and **2** suggest that the left flank is linked to the A idiomorph in a number of the homothallic species but such a conclusion is based on the comparison of a single band in most cases. Lanes a, b, and g are partial digests and show multiple bands (unreliable for linkage analysis) [see Figure 18]. Lanes are as follows: a)*A.sublineata*, b)*G.reticulospora*, c)*G.143-4*, d)*G.142-1*, e)*G.calospora*, f)*G.S-23*, g)*N.terricola*, h)*N.africana*, i)*N.crassa* A, j)*N.crassa* a.

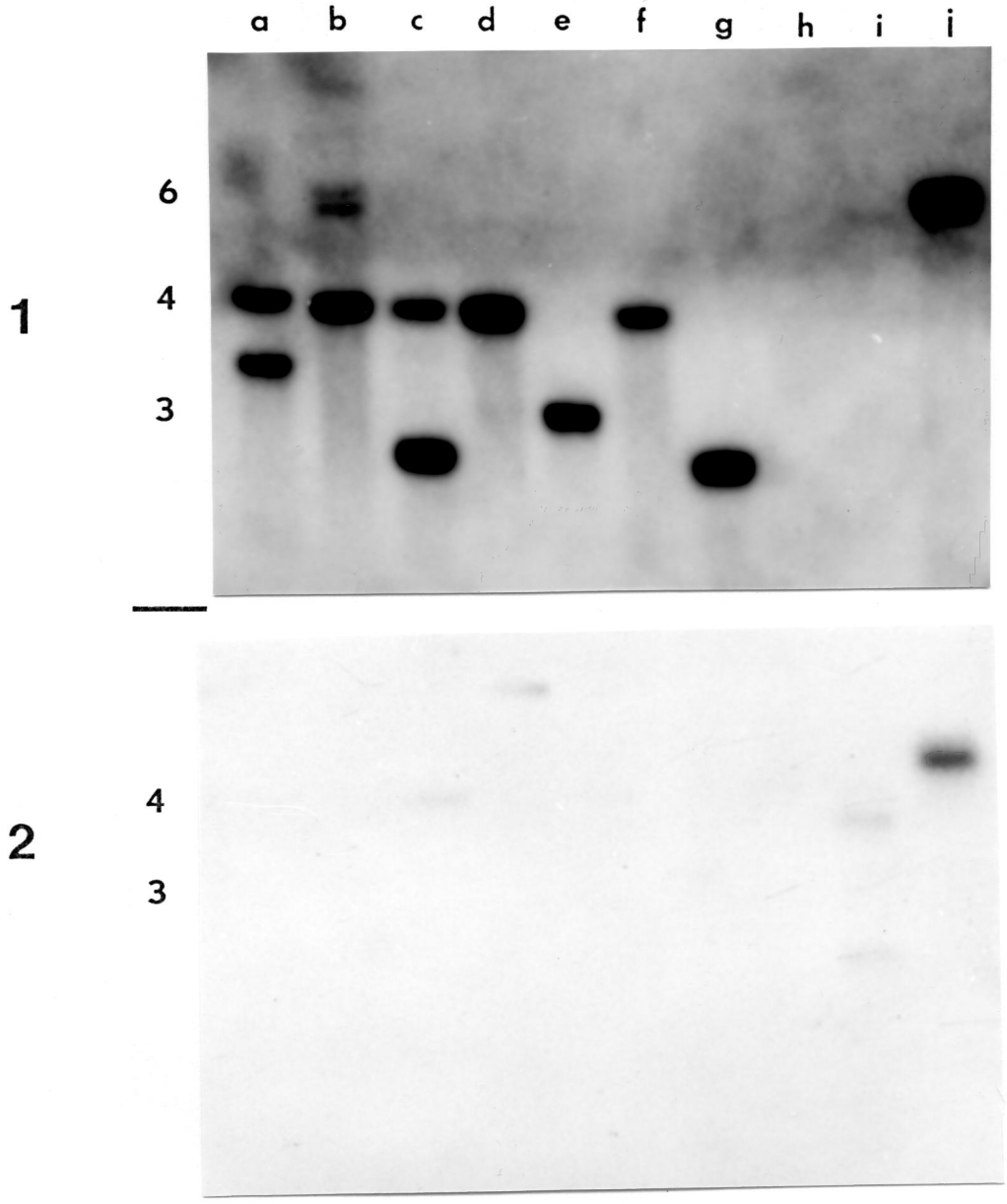

Figure 15 ***Sordariaceae* homothallics lack a portion of the *N.crassa* a idiomorph.** Homothallic DNA was digested with PstI and probed with **1**. pCSN4 and **2**. pCSN15 EcoRI / SacII (400bp) [Figures 7 and 19]. Probe **1** represents the majority of the a idiomorph including mt a-1. Probe **2** represents a segment of the a idiomorph from the end opposite mt a-1. This region is diverged or absent in all homothallics tested. Faint bands visible in **2** are probably due to slight probe contamination, probably with A idiomorph sequences. Lanes are as follows: a)*A.sublineata*, b)*G.reticulospora*, c)*G.143-4*, d)*G.142-1*, e)*G.calospora*, f)*G.S-23*, g)*N.terricola*, h)*N.africana*, i)*N.crassa* A, j)*N.crassa* a.

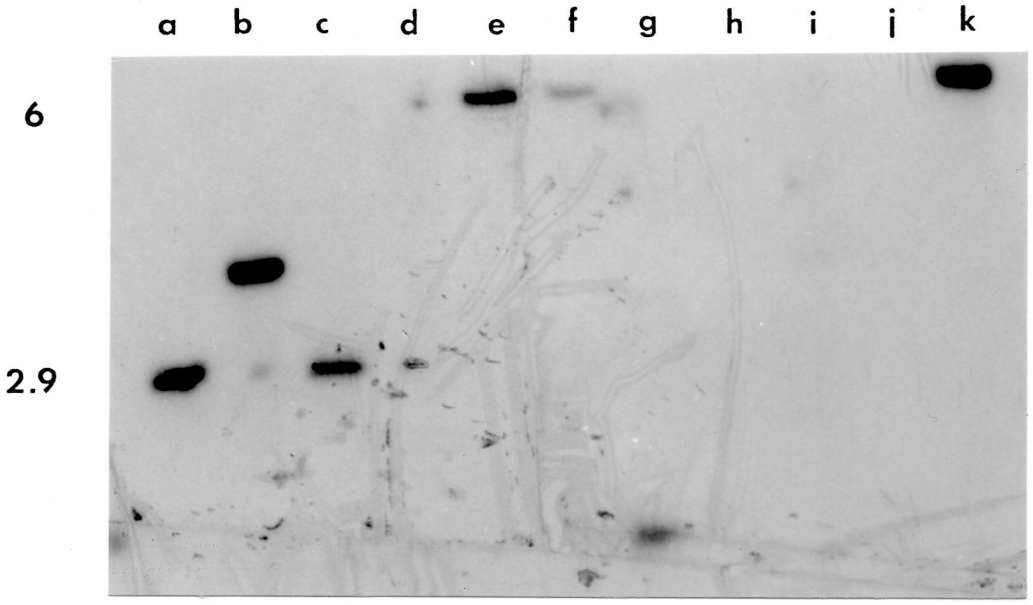

Figure 16 **Clarifying the border of the *N.terricola* a idiomorph.**
N.terricola genomic DNA was digested with Group **I** enzymes and probed with a BamHI/BamHI (900bp) fragment representing a central portion of the *N.crassa* a idiomorph [see Figures 7 and 19]. Hybridization yielded a characteristic a pattern similar to that observed from hybridization to an ORF a (pCSN4) probe [see Figure 12: **2**]. Sequences represented by the BamHI/BamHI (900bp) probe, then, are contiguous with the a ORF and part of the *N.terricola* a idiomorph. The *N.terricola* a idiomorph border must lie between the BamHI site and the EcoRI site [Figures 7,9,15] so that at least 600bp of the *N.crassa* a idiomorph are missing from the *N.terricola* genome. Lanes are digests with a)EcoRV, b)HindIII, c)EcoRV/HindIII, d)EcoRI, e)BglII, f)EcoRI/BglII, g)PstI, h)BamHI, i)PstI/BamHI, j)*N.crassa* A, k)*N.crassa* a. Lanes h and i contain insufficient DNA for visible hybridization.

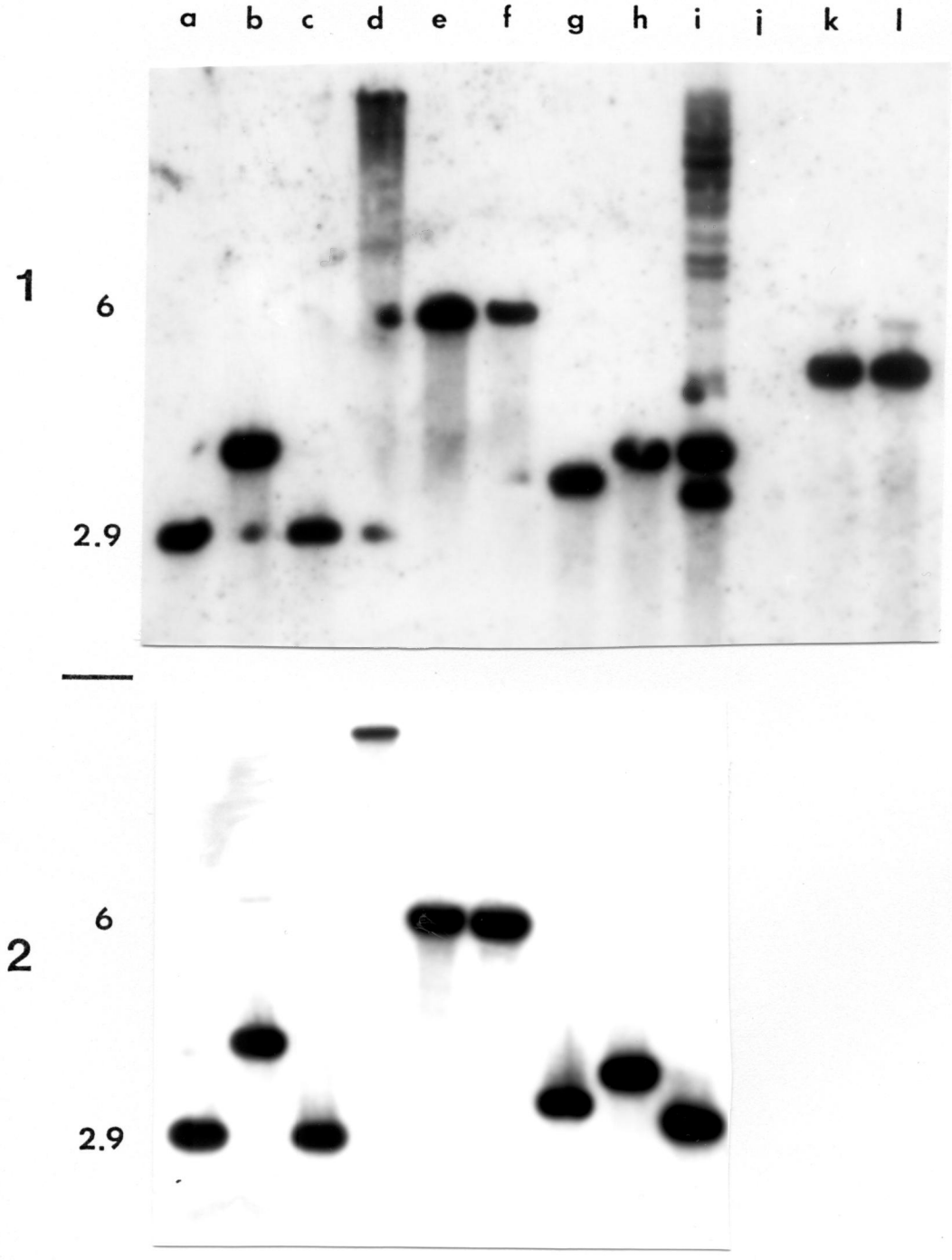

Figure 17 ***N.crassa* left flank sequences are contiguous with the a idiomorph in *N.terricola*.** *N.terricola* genomic DNA was probed with **1.** pMT2.519 PstI / HindIII (1100bp) and **2.** pCSN4 [Figures 6 and 8]. The band patterns match exactly indicating that the idiomorph and flank sequences are contiguous in *N.terricola*. Lanes were digests with a)EcoRV, b)HindIII, c)EcoRV/HindIII, d)EcoRI, e)BglII, f)EcoRI/BglII, g)PstI, h)BamHI, i)PstI/BamHI, j)*N.africana*, k)*N.crassa* a, l)*N.crassa*A.

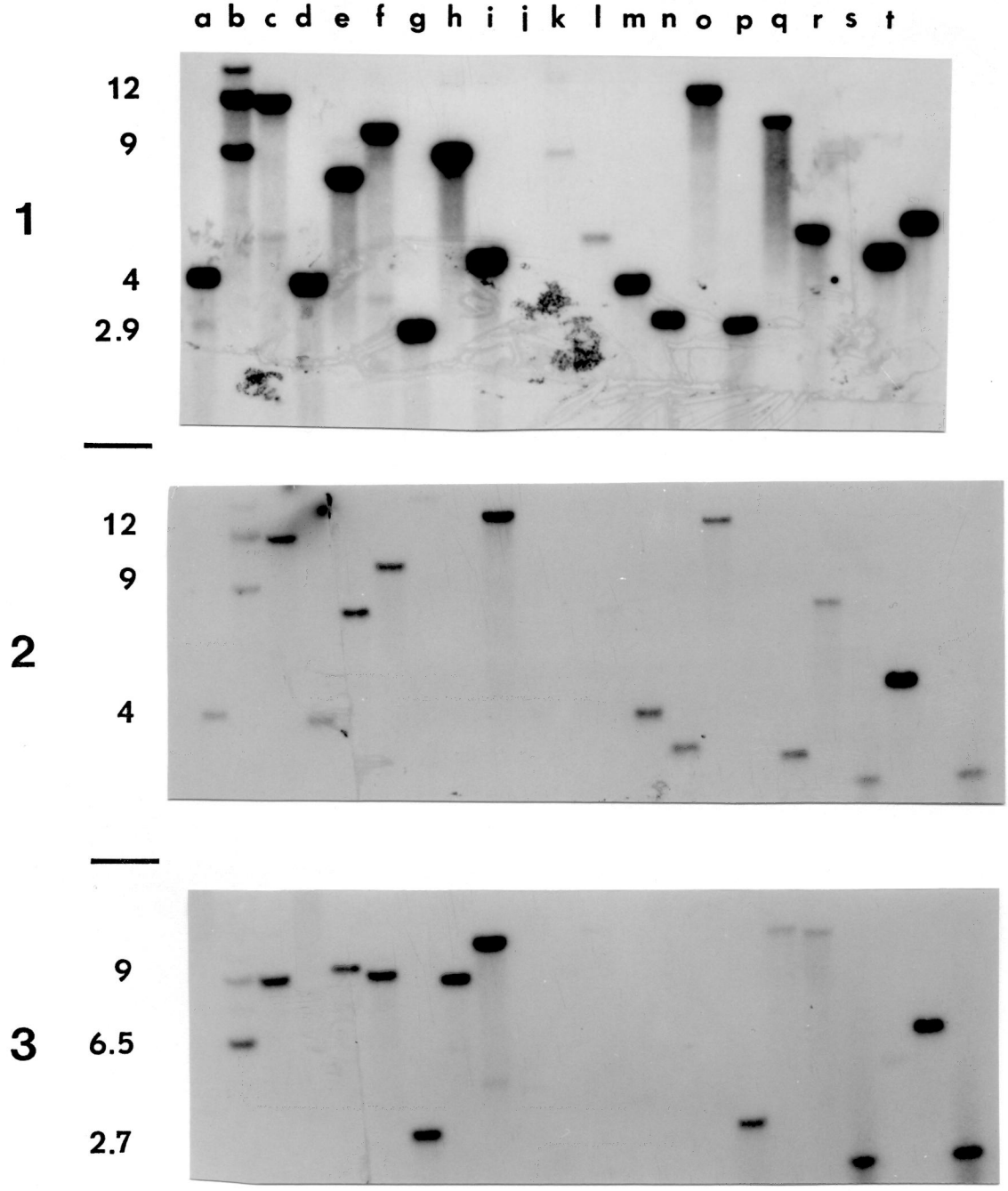

Figure 18 **Idiomorph and left flank linkage in *Sordariaceae* homothallics.**
Homothallic genomic DNA, digested with Group **V** enzymes, was probed with **1**. pMT2.519 HindIII/PstI (1100bp), **2**. pCSN4, and **3**. pMT-150(G-2) [Figures 6-9]. Three digests (consecutive lanes) were performed with each species. Enzymes were BamHI, HindIII, SacI. Lanes were: a-c)*A.sublineata*, d-f)*G.calospora*, g-i)*G.reticulospora*, j-l)*G.142-1*, m-o)*G.143-4*, p-r)*G.S-23*, s)kb ladder, t)*N.crassa* a, u)*N.crassa* A. Band patterns are similar with probes **1** and **2** for all species with exception of *G.reticulospora G.S23*. *G.142-1* is inconclusive. Similar band patterns indicate contiguousness between flank sequence and idiomorph. Lanes without bands probably have insufficient DNA.

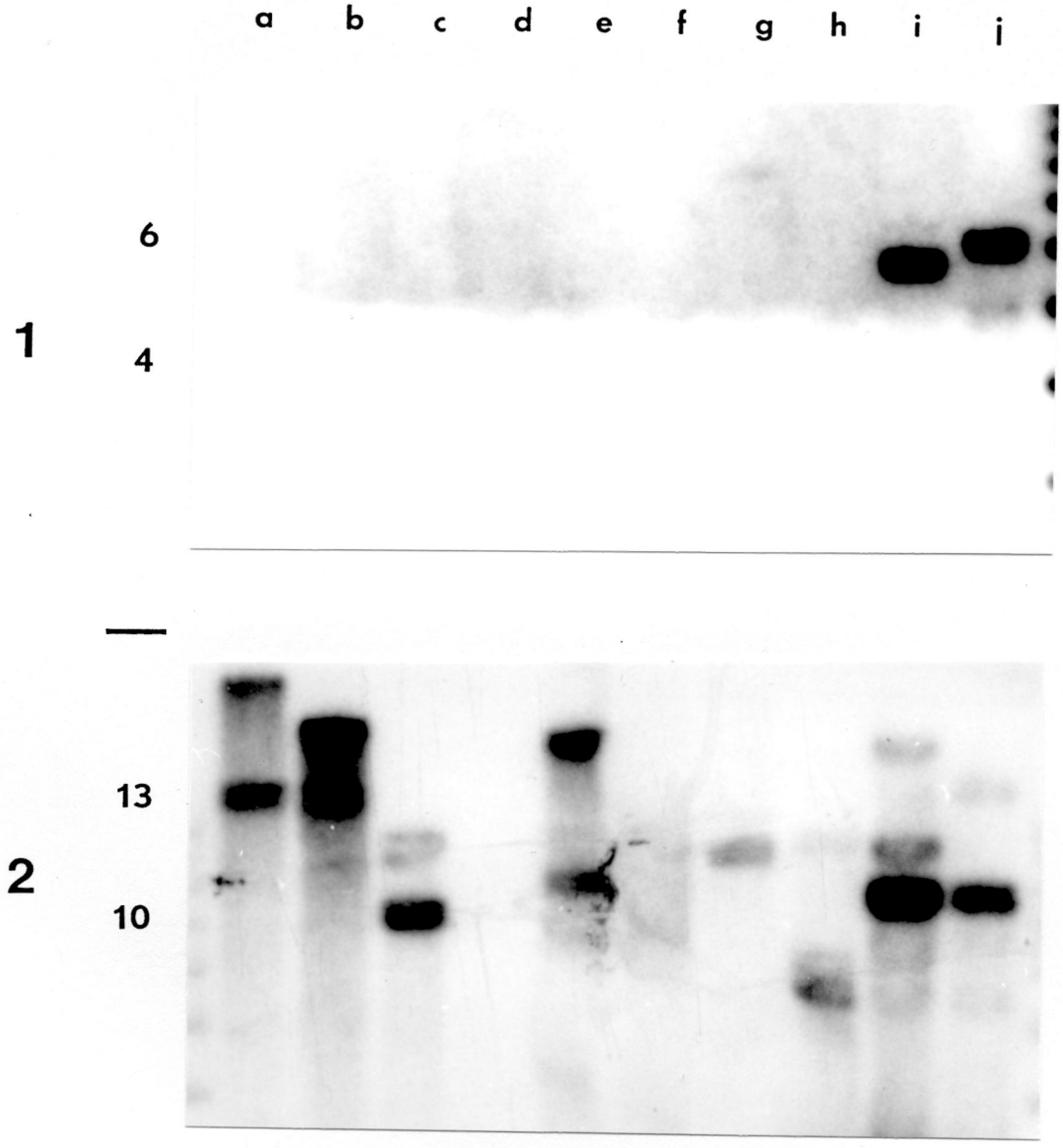

Figure 19 **Idiomorph linked *N.crassa* right flank sequences are not present in homothallic *Sordariaceae*. More distal right flank sequences are, however, conserved.** Homothallic DNAs were digested with PstI and probed with **1.** pMT 6.818 SphI/EcoRV (1.0kb) and **2.** G16 C10 SphI/SphI (12kb). Lanes are as follows: a)*A.sublineata*, b)*G.reticulospora*, c)*G.143-4*, d)*G.142-1*, e)*G.calospora*, f)*G.S-23*, g)*N.terricola*, h)*N.africana*, i)*N.crassa* A, j)*N.crassa* a. The right flank sequence that is contiguous with an idiomorph in *N.crassa* is absent in homothallic species. The more distal *N.crassa* flank sequence is present in most *Sordariaceae* homothallics but not clearly linked to an idiomorph. Exceptions are lanes d and f which represent species that appear to lack the *N.crassa* distal right sequence.

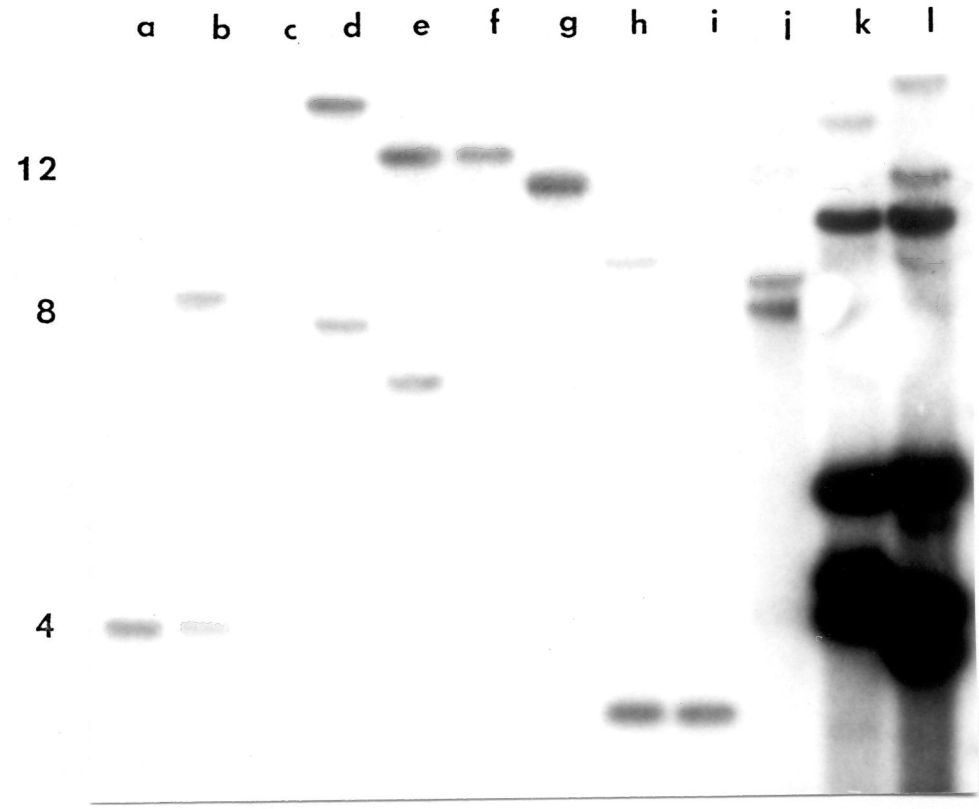

Figure 20 **Distal right flank sequences are present in *N.terricola* but not clearly linked to an idiomorph.** *N.terricola* genomic DNA was digested with Group **I** enzymes and probed with the *N.crassa* G16C10 SphI/SphI probe (Figure 11) representing a distal portion of the right flank region of *N.crassa*. Lanes are digests with a)EcoRV, b)HindIII, c)EcoRV/HindIII, d)EcoRI, e)BglII, f)EcoRI/BglII, g)PstI, h)BamHI, i)PstI/BamHI, j)*N.africana*/ EcoRV, k)*N.crassa* a, l)*N.crassa* A. The band pattern does not match that produced when *N.crassa* A or a internal sequences were used as probes. Rather, the pattern seen here is novel and indicates only that the *N.crassa* sequence is present in the *N.terricola* genome; Nothing can be determined with regard to the location of the sequence or its linkage to an idiomorph. The large number of bands in control (genomic) DNA lanes can be attributed to the size of the probe (~12kb) and its hybridization to sequences other than mating-type.

DISCUSSION I

The well described mating-type locus of *N.crassa* is comprised of idiomorphs, A or a, and sequences that flank the idiomorph to the left and right. The idiomorphs are dissimilar while the flank sequences are identical between mating-types. A *N.crassa* haploid nucleus invariably has single copies of idiomorph and flank sequences, occupying the same locus in A and a individuals.

It was this researcher's prediction that characteristics of the tightly packaged mating-type locus of *N.crassa* might be conserved in *Sordariaceae* homothallics, enabling the creation of mechanistic models to explain the evolution of one or more forms of homothallism. Though the A and a idiomorphs differ in *N.crassa*, the similarity between flank sequences helps define a mating-type *locus* as such.

Left flank region

The *N.crassa* left flank region is well conserved in the *Sordariaceae*. Every species examined, hetero- and homothallic, has a sequence highly similar to the left flank region of *N.crassa*. An idiomorph, A or a, and a conserved left flank sequence support the concept of a single mating-type locus in both heterothallic and homothallic species. *N.crassa* A and a idiomorphs are both bordered by an identical left flank sequence. The same flank sequence is contiguous with the a idiomorph in four of the six homothallic species examined. The remaining two homothallics (*G.reticulospora* and *G.S23*) each contain a sequence similar to the *N.crassa* left flank sequence but linkage is yet undetermined.

It is interesting that the *N.crassa* left flank region is contiguous with the a idiomorph in most of the homothallic species tested. Such a result suggests that the two sequences may have acted as a unit in the evolution of a homothallic mating-type locus. It is also possible that A/a-type homothallics have resulted from a single mechanistic event in which a heterothallic ancestor underwent an unequal crossover at the mating-type locus. A crossover in the left flank regions of A and a individuals might have occurred so as to isolate the A idiomorph from its left flank while leaving a left flank sequence connected with

the a idiomorph. The fact that a present day heterothallic (*N.crassa*) contains the same left flank sequence in both A and a strains suggests a functional requirement for that region. The left flank present in A/a-type homothallics (and contiguous with the a idiomorph) may be present as an artifact while in heterothallics the same sequence has been retained for function in both A and a strains.

Right flank region

The *N.crassa* right flank region, represented by the 1.0 kb SphI/EcoRV probe [Figures 6, 7, 10] did not hybridize to genomic DNAs of any *Sordariaceae* homothallic examined. This same *N.crassa* right flank sequence is not conserved in other heterothallic *Neurosporas* either (N.L.Glass, personal communication). This *N.crassa* flank region may be absent in other species or substantially diverged but appears, in any case, to be highly variable in the *Sordariaceae.* This interpretation is supported by the recent work of Tom Randall (unpublished results) who has sequenced a portion of the right flank region from *N.crassa.* He has identified an ORF that contains a motif common to fungal pheromones. Furthermore, he has attempted hybridizations between various *Sordariaceae* homothallic genomic DNAs and the *N.crassa* right flank region that contains the pheromone ORF. In no instance did he observe hybridization between these DNAs indicating that this region is highly divergent between species. Hybridizations were also performed between the *N.crassa* right flank region and genomic DNAs of heterothallic *Neurospora* species. Heterothallic DNAs also failed to hybridize to portions of the *N.crassa* right flank, suggesting an extensive variability for this region in the *Sordariaceae.*

Given these results, it is tempting to speculate that the right flank region is species-specific in the *Sordariaceae* and codes for distinct pheromones unique to individual taxa. Alternatively, heterothallics alone may have pheromone ORFs and functional pheromones. Homothallic species may simply have variable right flank sequences that are present residually and reflect prior evolutionary events. There is no direct evidence (isolation of

actual pheromone, for instance) that any homothallic species possess a pheromone ORF and there is certainly no plausible reason why homothallics should have any need of pheromones since they have no conidia, no trichogynes, and no observable mating mechanisms.

N.terricola A / a linkage data suggest a novel homothallic mating-type locus

While the conservation of left flanking sequences in heterothallic species of *Neurospora* suggests a single heterothallic mating-type locus, the idea of a well defined homothallic mating-type locus is not strongly supported by the data that describes the linkage between *N.terricola* A and a idiomorphs. The A and a idiomorphs are not closely linked in *N.terricola.* Yet the similar sizes (18kb) of *N.terricola* MluI and SmaI bands in hybridizations to *N.crassa* A and a idiomorph probes suggest that the *N.terricola* idiomorphs may not be completely un- linked. Idiomorphs separated by 18 kb cannot strictly be said to occupy the same locus. Indeed, preliminary CHEF gel analysis (Myron Smith, unpublished results) of whole *N.terricola* chromosomes indicate that A and a idiomorphs may be resident on different linkage groups altogether.

The *Neurospora* homothallic mating-type locus clearly differs from heterothallic loci in the constitution of the right flank region. This is true for both A-type (*N.africana*) and A/a-type (*N.terricola*) homothallics. The (lack of close) linkage between the idiomorphs of *N.terricola* cannot, however, be interpreted as reasonable grounds on which to distinguish the homothallic from the heterothallic mating-type locus. No other *Neurospora* species can be described in terms of idiomorph linkage since *N.terricola* alone contains both idiomorphs. A *Sordariaceae* A/a-type homothallic mating-type locus is, however, a reasonable concept. *N.terricola* is similar to selected *Gelasinospora* and *Anixiella* A/a-type homothallics in harboring idiomorphs that appear to be unlinked. Homothallics in these genera are also alike in the constitution of their left flank regions. Four of six *Gelasinospora* and *Anixiella* species examined, like *N.terricola*, contain *N.crassa* left flank

sequences that are contiguous with the a idiomorph. These data strongly suggest a conserved mating-type locus in *Sordariaceae* A/a-type homothallic species and a requirement of linkage between the left flank and the a idiomorph for functional homothallism. CHEF gel analysis of the linkage between A and a idiomorphs must be completed, however, before the question of a homothallic mating-type locus and its evolution can be resolved.

Evolution of homothallic mating-types

Idiomorph conservation and linkage data support the hypothesis that *N.terricola* and *N.africana* (a representative A-type homothallic) each evolved, monophyletically, from a heterothallic ancestor distinct from the ancestor that gave rise to *N.crassa*. Such distinct evolutions could account for the lack of hybridization between *N.crassa* right flank sequences and *N.terricola* and *N.africana* genomic DNAs. The conservation throughout the *Neurospora* genus of a single mating-type locus defined by left and right flank sequences would suggest a close evolutionary relatedness between *N.africana* and *N.terricola*. Such a close relationship, however, is not supported by other data, namely the linkage between left flank and idiomorph sequences in these species. Left flank sequences are contiguous with the a idiomorph in *N.terricola* and the A idiomorph in *N.africana*. A linear relationship between *N.terricola* and *N.africana*, one in which one species evolved directly into the other, is not likely since such an event would require the left flank sequence to be separated from one idiomorph and re-linked to the opposite idiomorph. On the other hand, distinct evolutions from a common ancestor could easily associate a left flank sequence with either idiomorph. The fact that *N.terricola* lacks some 1.0 kb of the *N.crassa* A idiomorph also distinguishes *N.terricola* from *N.africana* which contains the A idiomorph in its entirety. It is unlikely that *N.africana* would have evolved from *N.terricola* to regain the lost 1.0 kb fragment. *N.africana* must have preceded *N.terricola* evolutionarily if both species are

monophyletic. Alternatively, each species itself may be monophyletic in which case no conclusions can be drawn about when the two species evolved.

A mechanism for the evolution of homothallic mating-type loci

Given that *N.terricola* does contain some *N.crassa* right flank sequences, it is likely that *N.terricola* and *N.crassa* descended from a species ancestral to all heterothallic *Neurospora* species. A mechanism can be proposed to account for the presence of both A and a idiomorphs in single nuclei of *N.terricola*. The hypothesis posits the nuclear juxtaposition of A and a idiomorphs as a result of meiotic non-disjunction. In a mutant meiotic event, haploid genomes containing A and a could have in participated in cross-over events but failed to dissociate. Instead of eight haploid meiotic progeny (the ascospores) resulting from normal disjunction, the result of such a mutant meiosis would be four ascospores, each harboring A and a idiomorphs. These idiomorphs could be resident in the same nucleus or separate nuclei depending on the precise nature of the aberrant meiosis. In the preferred model [Figure 21], the idiomorphs are contiguous with no intervening sequences between them. Interestingly, the tetrasporic product of this mutant meiosis is an individual much like *N.tetrasperma*, a species proposed as an evolutionary intermediate in the General Discussion of this thesis. Raju (1978) has proposed that a single mutation could result in the conversion of a tetrasporic state (*N.tetrasperma*) to an octasporic state (*N.terricola* et.al.) in *Neurospora*. Finally, the *N.terricola* A and a idiomorphs could have been separated (even to different linkage groups) by translocation events. The *N.terricola* left flank sequence could have been translocated as a unit with the a idiomorph, leaving the A idiomorph with a novel (undescribed) left flanking region. Figure 21 summarizes some of these mechanistic events.

N.africana and the other A-type *Neurospora* homothallics can be reconciled easily as species in which the a idiomorph was lost altogether. The A idiomorph and contiguous left flank region remain at the mating-type locus. Distal portions of the right flank may or may

not be present in these species and they may or may not have regions encoding pheromone ORFs. As comparatively little work has been done to describe *Gelasinospora* and *Anixiella* heterothallic loci, it is not reasonable to extend the *N.terricola* model to A/a-type homothallics in these genera without reservation. Nevertheless, given the degree of conservation between the left flank and idiomorph regions of these groups and those of *N.crassa*, application of the *N.terricola* model is not altogether untenable.

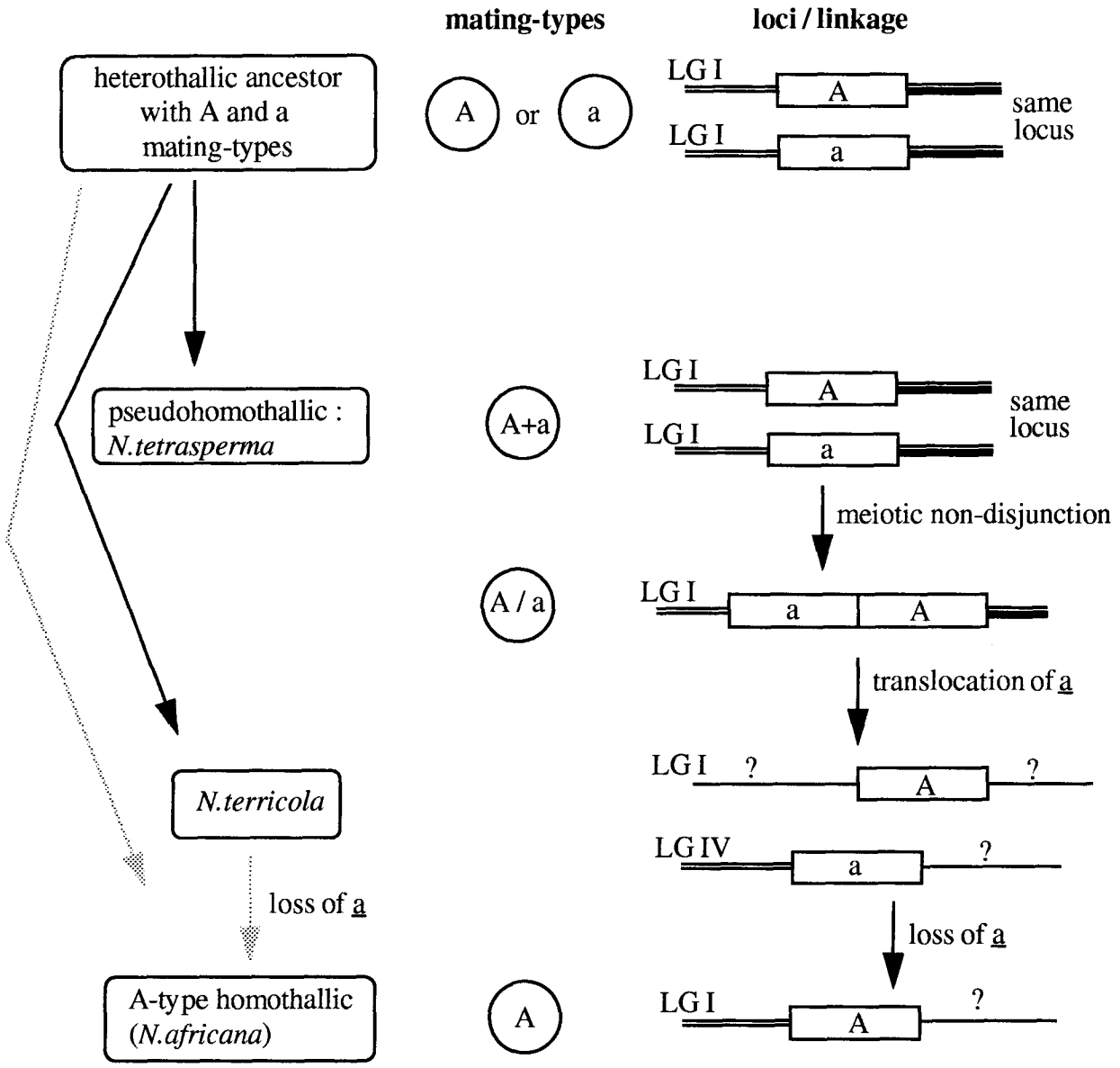

Figure 21 **Mechanistic model for the evolution of homothallic *Neurospora* species.** The mating-type locus of an ancestral heterothallic is essentially unchanged in existing heterothallics such as *N.crassa*. Cytogenetic events bring A and a nuclei together into single ascospores or conidia and give rise to *N.tetrasperma*. Evolution to pseudohomothallism is monophyletic. Meiotic non-disjunction brings A and a idiomorphs (and some flank sequences) together at the mating-type locus, yielding A/a-type homothallism such as that in *N.terricola*. Alternatively, non-homologous integration could put a on a different linkage group (IV). A-type homothallics, represented by *N.africana*, arise after the loss of a-specific sequences. These homothallics may diverge (grey arrows) from *N.terricola* or directly from the ancestral heterothallic.

PART TWO

SEQUENCE AND FUNCTIONALITY ANALYSIS OF *N.TERRICOLA* ORF A

INTRODUCTION II

The presence of mating-type sequences in homothallic genomes is puzzling since self-fertile species have no mechanism for mating. It is likely that evolution in the *Sordariaceae* proceeded from heterothallism to homothallism. If this is true, homothallic species may contain mating-type sequences that are residual and no longer functional. Alternatively, homothallics may employ some but not all the functions associated with heterothallic *Neurospora* mating-type loci.

N.crassa mating-type idiomorphs are known to be required for meiotic functions as well as mating events. Homothallic *Neurosporas* may well require functional A and a idiomorphs for meiosis and the production of viable ascospores. Yet *N.africana* contains only A-specific sequences and is capable of a robust sexual cycle. The a idiomorph may be entirely absent so that the A idiomorph alone is sufficient for sexual reproduction. Alternatively, *N.africana* may contain an undescribed polypeptide (a diverged mt a-1 or a novel protein) that performs functions similar to those furnished by the a idiomorph; this putative polypeptide may not be similar enough to *N.crassa* a to be detected by DNA hybridizations. *N.africana* A is functional when transformed into *N.crassa* sterile mutants and a functional copy is required for the formation of perithecia (N.L.Glass, personal communication). A similar result is expected for *N.terricola* A. Based on DNA hybridizations, both the A and a idiomorphs are highly conserved from *N.crassa* and A is likely to be functional throughout the genus. Idiomorph and flank region similarity suggests a close evolutionary relationship between *Neurospora* hetero- and homothallics.

While it is relatively easy to hypothesize molecular genetic mechanisms that could account for an evolution from heterothallic to homothallic species, mating-type idiomorph functionality is harder to interpret. If, for instance, the *N.terricola* a idiomorph is functional

and necessary for sexual reproduction, then this species is likely to be monophyletic and not linearly related to A-type homothallics. Other *Neurospora* homothallics are unlikely to have quickly gained reproductive independence from an ancestral species in which the a idiomorph was functional. Alternatively, if the *N.terricola* a idiomorph is *non*-functional, a A-type homothallic such as *N.africana* could easily have lost a sequences and descended from *N.terricola* in a single mutational event. A-type and a-type homothallics could be linearly related and represent members of a single phylogenetic line from a heterothallic ancestral species.

Evolutionary questions such as these can be made more tenable with specific sequence and functionality data. Part two of this thesis examines the sequence and functionality of A in *N.terricola*. Sequence data from the predicted ORF region of the idiomorph is compared with similar data from *N.crassa* (26,88) and *N.africana* (N.L.Glass, unpublished results). Comparisons of mt A-1 sequences are made between *N.crassa*, *N.africana*, and *N.terricola*. These comparisons are used in conjunction with data from Part I of this thesis to speculate on the evolution of hetero- and homothallism in *Neurospora*. A preferred evolutionary model is proposed which positions heterothallic species ancestral to homothallics and pseudohomothallic species as intermediates. (Arguments will be presented to support an evolutionarily transitional status for pseudohomothallism). A final analysis speculates on evolutionary relationships in the whole *Sordariaceae* family.

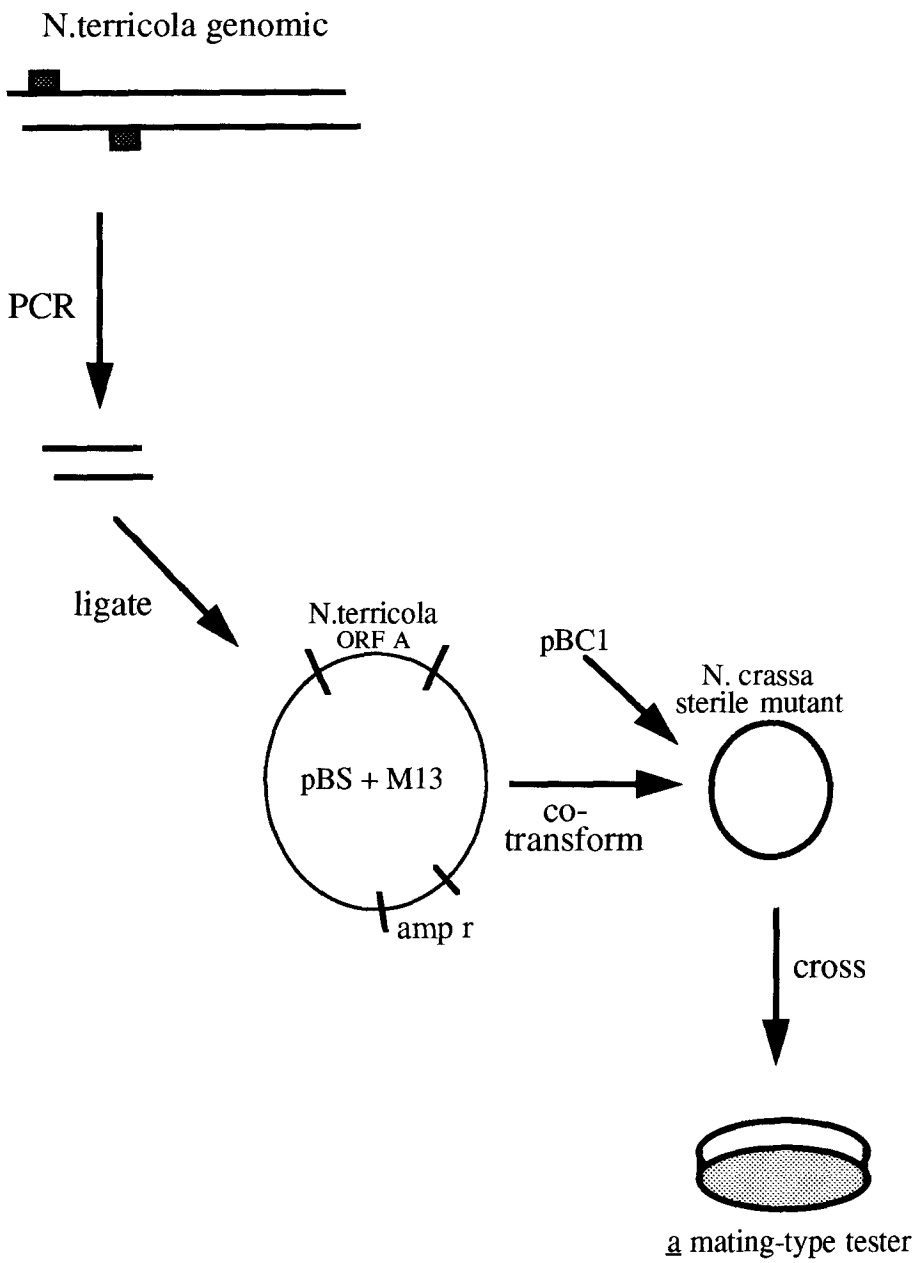

Figure 22 **Strategy to test functionality of *N.terricola* ORF A .**
N.crassa oligonucleotide primers were used to amplify the *N.terricola* A ORF. Blunt ligation into a Bluescribe vector yielded an A clone which was cotransformed with a benomyl resistance gene into sterile mutant spheroplasts. Cotransformants were crossed with an opposite mating-type (a) reference strain and functionality was assessed by observing for the development of perithecia.

MATERIALS AND METHODS II

PCR

A strategy for testing the functionality of *N.terricola* ORF A is presented schematically in Figure 22. Oligonucleotide primers were chosen from consensus *N.crassa* A and a sequences (26,88) to span the entire ORFs of mt A-1 and mt a-1. 24 mer and 25mer A primers were synthesized (Oligonucleotide Synthesis Laboratory, UBC) while 19mer and 24mer a primers were generously donated by C. Staben.

primer	sequence	mating-type
1778	5' CCACCTTCACCCAAACTTCCCACC 3'	A
3194	5' GGGTTACTGGAAGATGAGGTACCAT 3'	A
Y694	5' GAGGTGATATCCTTGGTGACCGGG 3'	a
Y759	5' GAATGCTATTCAGGGCCGG 3'	a

The following reaction conditions were employed:

	A	a	
Template DNA	100ng	100ng	
dNTPs	10mM	10mM	
$MgCl_2$	2.0mM	2.0mM	
Primers	50µM	50µM	
Taq buffer*	1x	1x	*(Perkin Elmer-Cetus)
Taq polymerase*	5u	5u	
---	---	---	
ddH_20	to 100µL	to 100µL	

PCR template DNAs were *N.terricola* and *N.crassa* genomic, the latter used as a control. DNAs were isolated in the same manner described in Materials and Methods of Part I of this thesis. Samples underwent the following cycle in a Perkin Elmer (model 480) thermocycler:

1.	95°	90s	
2.	55°	80s	
3.	72°	180s	
4.	repeat steps 1-3		5 times
5.	95°	90s	
6.	55°	120s	
7.	72°	120s	
8.	repeat step 5-7		20 times
9.	95°	120s	
10.	72°	480s	

Amplification was confirmed by subjecting a fraction of the reaction mix to electrophoresis and visualizing the appropriately sized band. In this manner, the predicted 1.1 and 1.6 kb bands were seen in the A and a amplifications respectively. A second confirmation was performed by Southern hybridization analysis. A and a PCR products were subjected to gel electrophoresis, transferred to Nylon membranes (Amersham Corp.), and hybridized to *N.crassa* A (pMT -150 (G-2)) and a (pCSN4) probes [Figures 6, 7] according to the method described in Materials and Methods I of this thesis. Strong hybridizations were observed in both cases indicating that *N.terricola* mating-type sequences had been amplified.

Cloning *N.terricola* ORF A

1. Purification of PCR fragments

N.terricola A and a PCR products were electrophoresed and re-confirmed by visualizing the appropriate sized bands. Slits were cut into the gel just distal to the PCR bands and the bands electroeluted into DE-81 (Stratagene Inc.) paper inserted into the slits. The paper was removed and centrifuged to spin off excess buffer. DNA, which binds to the DE-81 paper, was then extracted by the following method:

1. place DE-81 paper in 0.8mL eppendorf tube nested in 1.5mL tube, the former pierced at the bottom to allow flow through

2. extract DNA by adding 20µL of 1.0M NaCl-saturated urea to DE-81 paper
3. incubate at 60° for 3 minutes
4. spin at 12,000g for 3' to collect eluent
5. repeat extraction two additional times
6. add combined eluents to prepared 1.0 mL syringe Sephadex G-50 column
7. spin 6000g for 10' to collect eluent (only DNA passes through; urea binds to the column)

2. Blunt end ligation of A

Purified A PCR DNA was given blunt ends by the following reaction:

PCR fragment	100ng	
dNTPs	20mM	
T4 buffer*	1x	*(Pharmacia)
T4 polymerase*	15u	
---	---	
ddH_2O	to 20µL	

Blunt-ended DNA was then purified again by adding the entire T4 reaction mix to a 1.0mL syringe Sephadex G-50 column and spinning at 6000g for 10 minutes.

Bluescribe M13 (Stratagene) was chosen as the cloning vector and linearized with HincII. Vector DNA was then extracted once with 2:1:1 DNA(aq.):phenol:$CHCl_3$ and once with 1:1 DNA(aq.):$CHCl_3$. Vector DNA was precipitated and re-suspended in TE. The following ligation reaction was then performed:

A DNA from T4 reaction	100ng	
vector DNA	500ng	
ligation buffer*	1x	*(Stratagene)
DNA ligase*	10u	
---	---	
ddH_20	to 25µL	
incubate overnight at 16°C		

3. Transformation

DH5-α *E.coli* cells were transformed by adding the entire 25μL ligation reaction to 100μL of cells and placing on ice for one hour. Cells were then placed at 42° for three minutes, iced for three additional minutes, and added to 0.9mL of LB broth. After incubation at 37° for one hour, 100μL of cells were plated (LB + 100μg/mL ampicillin) with 20μL of X-gal and incubated overnight at 37°.

4. Selection

Putative transformants were visualized as white colonies in a background of blue colonies after a 12-18 hour incubation. The bluescript vector contains a *lac-Z* gene for the production of β-galactosidase which, in the presence of X-gal, constitutively produces a blue pigment when transcribed. The *lac-Z* promoter and *lac-Z* gene are separated by a multiple cloning site which ordinarily does not interfere with transcription. When a fragment is ligated into the multiple cloning site, however, transcription is disrupted, no blue pigment is produced, and white colonies are formed. White colonies, then, represent putative recipients of the insert or clones.

In this manner, potential *N.terricola* A clones were screened. White colonies were picked and cultured overnight in LB + ampicillin and DNA was extracted by alkaline lysis [a modification of the method of Vollmer and Yanofsky (95) by R.Subramaniam]. Enzymes were chosen from those that cut in the multiple cloning site of the vector. By digesting putative clones with restriction enzymes that cleave on both ends of the polylinker, the insert could be cut out of the vector. This fragment and the vector could then be visualized as distinct bands on a gel, confirming success in producing a clone. The A clone was named "pNTA" (for "*N.terricola* A").

Cloning *N.terricola* ORF a

Blunt cloning was not successful with the PCR-amplified *N.terricola* ORF a, even after repeated attempts.

Sequencing pNT A

Sequencing work of the *N.terricola* A ORF (from pNT A) was performed by Rajgopal Subramaniam and N. Louise Glass.

Testing functionality of *N.terricola* ORF A

The strategy for testing functionality involved cotransformation of *N.crassa* sterile-mutant strains with a plasmid containing an antibiotic resistance gene pβC1 (for selection) and the *N.terricola* A ORF [Figure 22]. The pβC1 plasmid contains an altered *Neurospora* β-tubulin gene that confers resistance to the antibiotic benomyl. Sterile mutant cells (see below) cotransformed with pβC1 and an *N.crassa* mt A-1 were employed as positive controls. Cells that receive both genes (cotransformants) form colonies that will mate with tester strains of opposite mating-type. These colonies are not fertile and do not produce ascospores; they do, however, form reproductive structures (perithecia) and in this way signal the action of a functional gene. Cotransformations with pβC1 and *N.terricola* DNA are expected to produce colonies that will mate and fruit in a similar manner if the *N.terricola* DNA includes a functional ORF.

The following materials were used for cotransformations:

	A
N.terricola DNA	pNTA
antibiotic resistance gene	pβC1
recipient strain	A^{m56}

Transformants were selected by growth on media containing 0.5μg/mL benomyl. Recipient cells were *N.crassa* spheroplasts (prepared by N.L.Glass) of A^{m56} strains (33) that harbor a single frame shift mutation in mt A-1, effectively rendering them sterile. Growth on media with benomyl ensures that all transformant colonies have received the benomyl resistance gene but there is no direct evidence that spheroplasts have also received

N.terricola mating-type DNA. Rather, cotransformation (the receipt of both DNAs) is *predicted* to occur if the ratio of DNA copies (pβC1:pNTA) is in the range of 1:2 to 1:2.5. Confirmation of the receipt of pNTA can be confirmed if it is unequivocally shown to confer mating ability to the sterile A^{m56} cells. Alternatively, receipt of mating-type DNA can be confirmed by isolating DNA from the cotransformant colony and hybridizing the DNA to *N.terricola* mating-type DNA.

Cotransformations were performed according to the following protocol (96):

1. aliquot 50μL spheroplasts into eppendorf tubes on ice.
2. add 1μg pβC1 DNA + 2.5μg pNTA DNA; incubate 30' on ice.
3. add 1.0mL 40% PEG / 500mM MOPS pH 8.0 / 50mM CaCl2
4. incubate 30' RT
5. add cells to prepared 8.0mL aliquots of regeneration top agar
6. add entire mixture to prepared plates (bottom agar + 0.5μg/mL benomyl)
7. incubate at RT until cotransformant colonies become visible (3-5 days)

Putative pNTA / pβC1 cotransformant colonies were allowed to grow until vegetative tissue breached the surface of the agar. Sterile disks of Whatman 3 filter paper were applied to the surface of the agar so as to cover all visible colonies and colonies were allowed to grow into and inoculate the disks (approximately 24 hours). The filter paper was then removed and transferred to prepared plates with established growth of an opposite mating-type tester strain, in this case a fluffy (fl) mutant of A (FGSC strain 4347). The fl mutant is an aconidial female receptor strain with enhanced fertility; fl strains have the phenotype of colonial growth in dense mats that facilitates use in mating-type test crosses. Inoculated filter paper disks were allowed to fertilize a fl A plate whereupon reproductive structures (perithecia) would become visible in two to five days.

Control cotransformations were performed concurrently with *N.terricola* mating-type cotransformations. All procedures were identical to those described for *N.terricola* but with transforming DNA substitutions or omissions. Positive controls were cotransformations with *N.crassa* PMT-150(G-2) mt A-1. Negative controls were

transformations only with pβC1 DNA; transformation without mating-type DNA was expected to result in transformant colonies but no perithecial development upon crossing to an opposite mating-type strain.

Testing "escape" from homothallism in *N.terricola*

One hundred individual ascospores were picked, heat shocked (60°C, 30 minutes), and allowed to germinate on minimal media (modified Westergaard's salts + 2% fructose). Individual slants were observed first for germination and mycelial growth and then for the development of perithecia. Perithecia were in turn observed to see if they shot ascospores. Escapes were considered to be those slants that demonstrated vegetative growth but no development of perithecia.

RESULTS II

Sequence analysis of *N.terricola*, *N.africana*, and *N.crassa* A ORFs

Results from nucleotide sequence comparisons of *N.crassa*, *N.africana*, and *N.terricola* A ORFs are presented in Figure 23. The carboxy and amino terminal regions of all three sequences are quite similar: 91% similarity in the amino terminal region and 86-87% similarity in the carboxy terminal region for all three species. Intron sequences are considerably more variable. The *N.terricola* intron is 86% similar to the *N.crassa* intron while the *N.africana* intron is only 77% similar to the *N.crassa* intron. The *N.terricola* and *N.africana* introns are 90% similar to one another.

Functionality of *N.terricola* ORF A

Results of functionality tests are presented in Table 4 and Figure 24. *N.terricola* ORF A was considered functional if cotransformation into *N.crassa* spheroplasts resulted in the growth of colonies that could mate with an opposite mating-type tester strain (see Materials and Methods II). Positive mating was assayed by observing for the development of brown or black perithecia at the sites of cotransformant colonies. Negative controls were transformations with pβC1 [selection] DNA and no mating-type DNA. Positive controls were cotransformations with pMT-150(G-2) [*N.crassa* A] or pCSN4 [*N.crassa* a] and pβ C1 DNA. Six replicate negative control transformations with pβC1 only yielded a total of 14 colonies, none of which mated with A or a mating-type tester strains. Six replicate positive control cotransformations with pβC1 and pMT-150(G-2) yielded a total of 78 colonies, 48 of which mated with a tester strains. Nine replicate cotransformations with pNTA [*N.terricola* A] and pβC1 DNA yielded a total of 128 colonies, 40 of which mated with a tester strains [Table 4]. No difference was visually discernible between perithecia from positive control and *N.terricola* cotransformations [Figure 24]; black perithecia were observed for both control and experimental cotransformations but ascospores were not

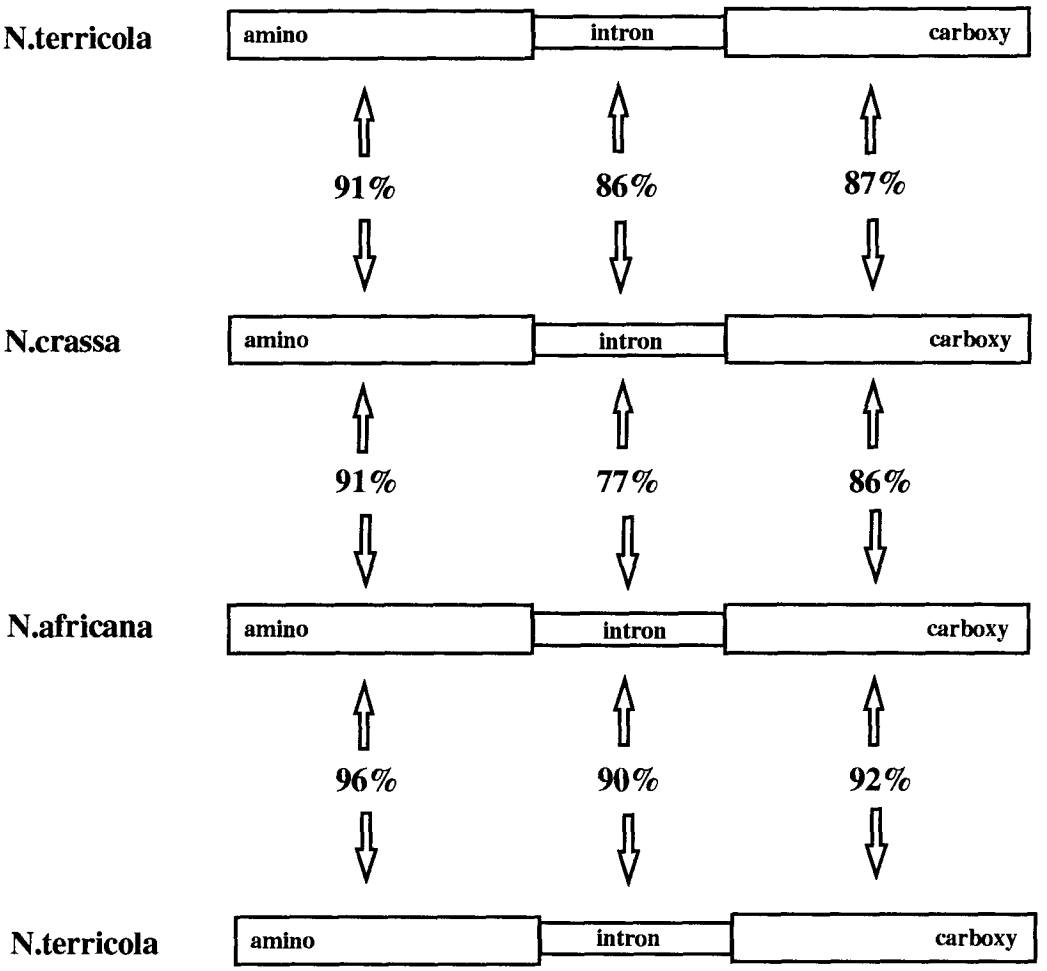

Figure 23 **Graphic representation of DNA sequence similarity between the A ORFs of *N.crassa*, *N.terricola*, and *N.africana*.**
Percentages are percent similarities between DNA sequences in the regions indicated.

Table 4 **Results of cotransformations of A^{m56} spheroplasts with pβC1 and *N.terricola* A mating-type DNAs.**

transforming DNA(s)	replicates	(co)transformant colonies	colonies that mated	MT tester strain
pβC1	6	14	0	fl a
pβC1 pMT-150(G-2)	6	78	48	fl a
pβC1 pNTA	9	128	40	fl a

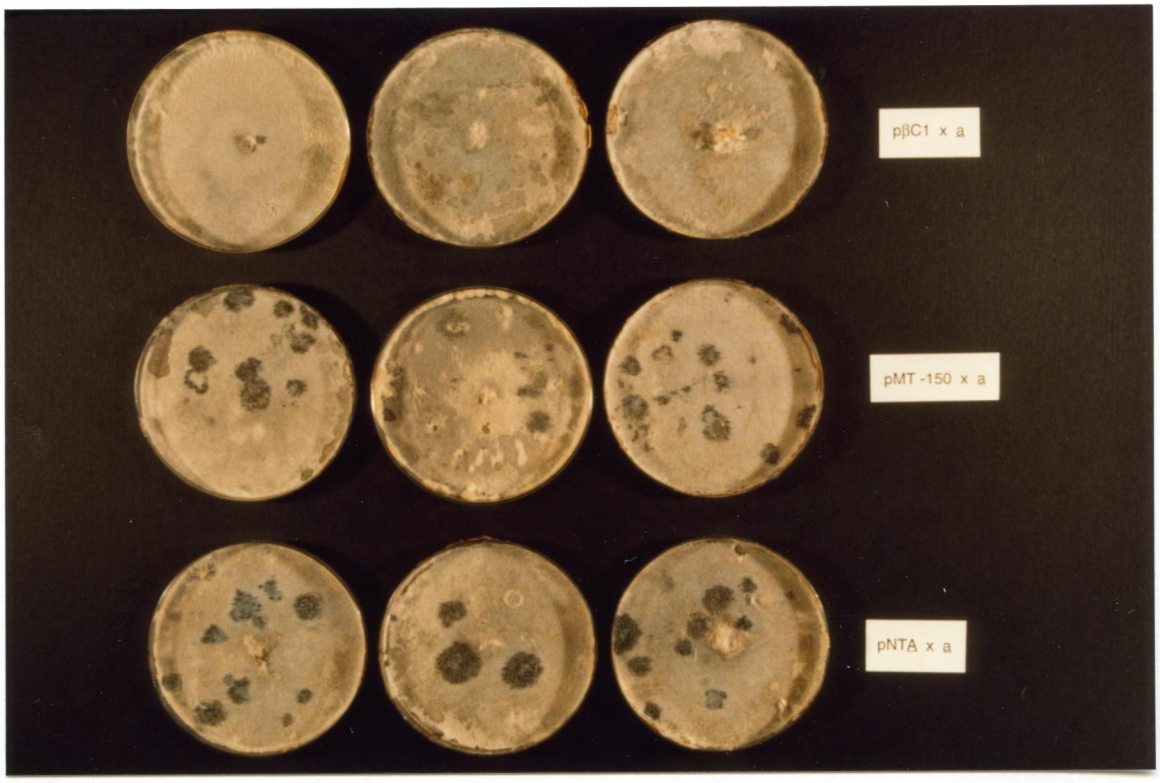

Figure 24 **Results from cotransformations of A^{m56} spheroplasts with pβC1 (selection) and mating-type DNAs.** **1.** Transformation with no DNA **2.** Transformation with pβC1 only **3.** Cotransformation with pCSN4 (*N.crassa* mt A-1) and pβC1 **4.** Cotransformation with pNTA (*N.terricola* ORF A) and pβC1. In each case, (co)transformant colonies were crossed with a tester strain of the opposite mating-type (fl a). The development of prominent black perithecia was interpreted as a positive mating reaction. No difference is observable between the perithecia resulting from positive control (pCSN4) and experimental (pNTA) cotransformations. The *N.terricola* ORF A, therefore, confers full functionality (mating ability) on the recipient sterile spheroplasts.

produced in either case. Ascospore formation was not expected since *N.crassa* sterile mutants have been characterized by the irrecoverable loss of certain meiotic functions including the ability to generate ascospores. Receipt of transforming mating-type DNA does not restore the ability of A^{m56} mutants to form ascospores (N.L. Glass, personal communication). Ascospore formation aside, mating was interpreted to be equally vigorous in control and experimental cotransformations and the *N.terricola* A ORF was confirmed to confer full functionality to the recipient mutant spheroplasts. Though the A ORF has not been proved to function in *N.terricola*, cotransformation results suggest that A *could* function in *N.terricola.*

As a negative control, a second set of matings were attempted between cotransformant colonies and fl strains of the same (A) mating-type. In this case, the same 128 putative cotransformant colonies (described previously) did not mate or yield perithecia. The *N.terricola* A ORF, then, acts authentically as a mating-type gene and interacts only with strains of opposite mating-type.

"Escape" from homothallism in *N.terricola*

A test was devised to determine whether homothallism is stable in *N.terricola.* Experimentally, cultures of *N.terricola* are invariably homothallic; mycelial growth of a single culture always leads spontaneously to the formation of perithecia and the forcible discharge of viable ascospores. Convenience dictates that cultures be grown from multiple inocula, ascospores or vegetative hyphae. The possibility exists that cultures initiated in this manner may be the result of crossing between propagules that contain an A or a idiomorph but not both. It is unclear whether *N.terricola* can lose a mating-type and become functionally heterothallic. Even if the *N.terricola* mating-type idiomorphs are not functional, the question of homothallic stability remains. Is *N.terricola* constitutively homothallic or can individuals lose the ability to self?

An examination of colonies grown from single propagules suggests that *N.terricola* mating could rarely if ever be occurring as the result of crossing between functionally heterothallic individuals. 100 individual *N.terricola* ascospores were picked and heat shocked to induce germination. Of these, 87 germinated and grew into normal mycelial colonies. After 4-7 days of growth, all 87 colonies formed visible brown or black perithecia. Each of these 87 went on to shoot ascospores. As a result, it was concluded that *N.terricola* is constitutively homothallic; in no instance was functional heterothallism observed.

DISCUSSION II

The strong hybridizations between *N.crassa* and *N.terricola* mating-type DNAs suggest a high degree of similarity. This fact was utilized in formulating a strategy to ascertain whether the A ORF of *N.terricola* can confer mating activity to sterile mutants of *N.crassa*. A PCR amplification of *N.terricola* A and a ORFs was predicted to be possible with the use of *N.crassa* oligonucleotide primers. Homology between the genomes of *N.crassa* and *N.terricola* means that *N.crassa* primers could effectively bind *N.terricola* genomic templates and allow a high yield PCR amplification. The successful amplification by PCR of the *N.terricola* ORFs A and a supports the predicted similarity between *N.terricola* and *N.crassa* and provides a tool with which to address the question of idiomorph functionality in *N.terricola*. (A discussion of functionality results will be presented later). Two approaches to the functionality problem were possible with *N.terricola* PCR products; the ORF fragments could be cloned and sequenced or sequenced directly. Subsequently, PCR product or whole ORF containing plasmids could be transformed into *N.crassa* sterile mutants.

Cloning was chosen as the preferable option but came to fruition only with the *N.terricola* A ORF . Blunt cloning is difficult even under ideal conditions and perhaps the rapid cloning of A was effected in part by luck. Repeated attempts to do a similar ligation of a into the same vector were unsuccessful despite countless strategies and technical modifications. Failure to clone a, while frustrating, cannot be construed as biologically significant as ORF a appears to have just as much similarity as ORF A to the corresponding *N.crassa* sequence. Nevertheless, cloning difficulties did not preclude sequencing and functionality tests with the a PCR product.

Monophylogeny Versus Polyphylogeny in *N.crassa*, *N.africana*, and *N.terricola*

Sequence analyses [Figure 23] of *N.crassa*, *N.africana*, and *N.terricola* A ORFs demonstrate that the intron DNA sequence of *N.terricola* is quite similar (86%) to that of

N.crassa. The *N.africana* intron is less similar (77%) to the *N.crassa* intron but the two homothallic introns (*N.terricola* and *N.africana*) are very similar (90%). In the following analyses, it is assumed that intron sequence similarity is a direct measure of temporal evolution, dissimilar sequences having diverged earlier than similar sequences. With that assumption, the A intron sequence data immediately suggest several possible relationships between the species. *N.terricola* and *N.africana* are closely aligned and more similar to each other than either is to *N.crassa*. Comparing *N.terricola* and *N.africana*, the former is evolutionarily closer to (its intron sequence is more similar to) *N.crassa*. If heterothallism is ancestral, *N.africana*, *N.terricola,* and *N.crassa* may be members of one phyletic group in which a heterothallic type, as typified by *N.crassa*, arose first, followed by the juxtaposition of A and a idiomorphs in a single nucleus and the evolution of *N.terricola*. Finally, the loss of a sequences marked the emergence of *N.africana*.

Monophylogeny could also be described by evolutionary events in which homothallism arose twice. In such a history, a heterothallic ancestor (different from *N.crassa*) evolved into *N.terricola* first, maintaining the presence of both A and a idiomorphs in a single individual. The separation of idiomorphs into distinct individuals could have given rise to *N.crassa* followed by cytogenetic events that allowed the a individual to become self-fertile (*N.africana*). Though mechanistically feasible, this double evolution of homothallism is counter-intuitive. It is unlikely that the heterothallic ability to conidiate would be lost in the evolution of *N.terricola* only to be gained again in *N.crassa* and lost a second time in *N.africana*. Such an oscillation between conidial heterothallism and aconidial homothallism is difficult to reconcile unless selective pressures were particularly strong. There is, however, no evidence in *Neurospora* to suggest that one mating strategy is significantly more successful than the other (68).

A case against polyphylogeny

A second interpretation of the A intron sequence data would be that *N.africana* and *N.terricola* each represent a monophyletic evolution from a heterothallic *Neurospora* ancestor as typified by *N.crassa*. [It is assumed that *N.crassa* has evolved directly, and with relatively few changes, from the heterothallic ancestor]. In this history, *N.africana* and *N.terricola* represent individual divergences from a continuous heterothallic lineage and each has membership in a distinct homothallic lineage. Such a history is strongly contradicted by the intron sequence data, however. The high degree of similarity (90%) between the introns of *N.terricola* and *N.africana* strongly refute the hypothesis that these species diverged long ago. Rather, intron similarity between them suggests that they are members of a single phyletic group and have diverged only recently.

All these assignations to phyletic groups and evolutionary models must be considered with the caveats that a) the supporting or refuting data come from a single locus and b) sequence data were derived from single isolates of each species. Only single isolates are available for *N.terricola* and *N.africana*. In positing hypotheses for the evolution of *Neurospora* (and other *Sordariaceae*) mating strategies, we are likely merely scratching the surface of what is a multi-faceted phenomenon. Realistically, homo- and heterothallism may have evolved numerous times in *Neurospora*. Many transitional species must now be lost and we may never be able to describe the entire evolutionary history of *Neurospora*. At the same time, in the analysis of present day hetero- and homothallic species, we may have a window on one-time evolutionary events that reflect the only instances in which homothallism has evolved in *Neurospora*.

Evolution in terms of functionality at the mating-type locus

The evolution of *Neurospora* homothallism can be considered in the context of functionality at the mating-type locus. Presumably, the A and a idiomorphs of an ancestral heterothallic had both mating and meiotic functions as they do in the present day *N.crassa*.

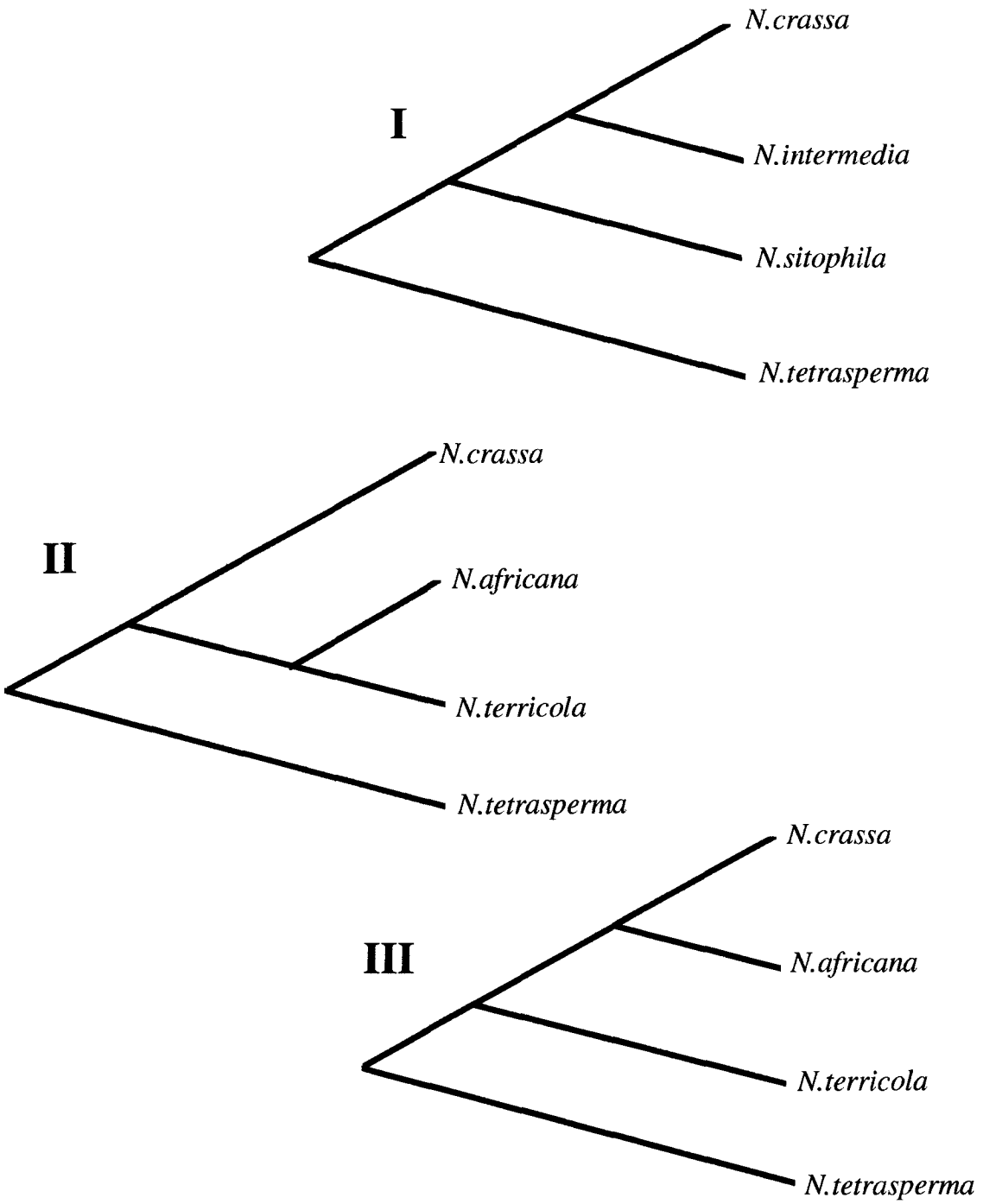

Figure 25

Three cladistic trees for evolution in the genus *Neurospora*. All trees are unrooted. Tree I is adapted fromTaylor and Natvig (1989) and reflects nuclear and mtDNA RFLP data that show *N.tetrasperma* and *N.sitophila* to be monophyletic. *N.tetrasperma* RFLP patterns are the most dissimilar to *N.crassa*, indicating an early divergence. Trees II and III are possible histories for the evolution of homothallic species, based primarily on *N.crassa*, *N.africana*, and *N.terricola* sequence data. Greater sequence similarity is interpreted to indicate a more recent divergence. Tree III is more feasible than tree II since *N.africana* mating-type sequences are closer to *N.crassa* than are those of *N.terricola*.

The fact that the *N.terricola* A ORF confers functionality in *N.crassa* sterile mutants is to be expected if *N.terricola* inherited essentially intact (and functional) idiomorphs directly from a heterothallic ancestor as typified by *N.crassa.* The *N.terricola* A ORF, however, confers mating ability to *N.crassa* sterile mutants but does not confer meiotic ability. One might expect the reverse situation where a homothallic ORF would confer meiotic functions (useful in producing resistant ascospores for propagation) but not the irrelevant mating functions. It is necessary to point out here that *N.crassa* mating-type DNA (pMT -150 G-2), transformed into the same sterile mutants, also confers only mating functions. The inability of transformed A^{m56} mutants to produce ascospores probably reflects unique properties of the mutants themselves and not the wild type functionality of the *N.terricola* and *N.crassa* A ORFs. Nonetheless, the fact that the *N.terricola* A ORF is largely conserved from *N.crassa* and can indeed function in *N.crassa* mutants suggests that evolution has proceeded from heterothallism to homothallism. Why would a homothallic organism that does not mate develop a polypeptide that can function in elaborate mating processes? A more plausible history describes the presence of functional mating-types in *N.terricola* as residual, the polypeptide retained for its meiotic functions.

An obvious question is whether A (and a) function in *N.terricola* and whether their function is meiotic, mating-related, or both. [This question could be answered by the generation of *N.terricola* sterile mutants and their transformation with *N.terricola* wild-type A and a ORFs]. An even more intriguing question is whether *N.terricola* sterile mutants, transformed with A and a ORFs, could be taken through a cross (A transformant **x** a transformant) and produce fertile meiotic progeny. If so, the argument could be made that *Neurospora* homothallism is not fundamentally different from heterothallism at least at the level of mechanisms and molecular interactions at the mating-type locus. *N.terricola* homothallism may retain all the molecular requirements for heterothallism (two mating-types that can function) but not *require* a heterothallic mating strategy. Rather the loss (or repression) of certain genes (conidiation genes that contribute to chemotropic/ pheromonal

mating interactions, for instance) may have coincided with the evolution of species that are self-reproductive. Finally, such an evolution may have reflected selective pressures whereupon environmental conditions were unfavorable for vigorous vegetative growth and conidiation and mates were hard to come by. The predominance of homothallic *Sordariaceae* in soil suggests the occupation of a niche where fitness is related to the ability to self (independence from mating), slower growth, and the production of hardy ascospores.

Evolution in *Gelasinospora* and *Anixiella*

The degree to which the A and a idiomorphs of *Neurospora* are conserved in *Gelasinospora* and *Anixiella* suggests an evolutionary relatedness between these genera. Though no idiomorph and flank sequence data are available for species of *Gelasinospora* and *Anixiella*, it is compelling nonetheless to consider the evolution of homothallism in the whole *Sordariaceae* family and the possibility that the root heterothallic species we have considered may be ancestral not only to *Neurospora* homothallics but to *Gelasinospora* and *Anixiella* homothallics as well. An ancestral *Sordariaceae* heterothallic may have developed different ascospore ornamentations independently from the evolution of mating-types which have remained largely conserved in *Neurospora*, *Gelasinospora* and *Anixiella*. In *Gelasinospora* and *Anixiella*, A/a-types constitute the majority of homothallics while in *Neurospora*, A-types are more common. This fact suggests that homothallism has evolved separately in *Neurospora* and the other two genera though possibly from the same (or a similar) ancestor. It is possible that A/a-type and A-type homothallism are significantly different forms of a reproductive strategy that in the former requires two functional idiomorphs and in the latter only one. This interpretation contrasts that in which the presence of an a idiomorph in A/a-type homothallics is residual (reflecting prior evolutionary events) and the a idiomorph is not functional. In either case, the conservation of A and a idiomorphs and (left) flank sequences seems to be more important than the arrangement of these elements at the mating-type locus(i). The fact that the left flank region

is conserved in all the A/a-type homothallics examined suggests a functional role for this sequence. At the same time, the variability in that sequence's linkage to an idiomorph (linked to a in *G.calospora* et.al.; inconclusive in *G.reticulospora* or *G.142-1*, for instance) suggests that an A/a-type homothallic locus may have evolved more than once, even within a genus.

GENERAL DISCUSSION

Evolution of homo- and heterothallism

Innumerable models can be formulated to explain the evolution of homothallism and heterothallism. A key question is whether heterothallism preceded homothallism or the reverse. This writer favors the hypothesis that heterothallism evolved first followed by one or several events giving rise to homothallic populations. Intuitively, it seems improbable that self-fertile populations would undergo the relatively expensive evolution of mating-types and reproductive structures without strong selective pressure. Clearly there are selective advantages conferred by aspects of sexual reproduction in *Neurospora* but are these advantageous enough to envision the evolution of heterothallism from homothallic populations? Alternatively and more probably, heterothallism arose first in order to meet the need for what are now considered the numerous advantages to sexual reproduction. These advantages might include: i) ability to outcross --use of meiosis for genetic recombination and creation of new and potentially advantageous gene combinations ii) use of meiosis for repair of DNA copied during cell division iii) production of ascospores which are resistant to desiccation and therefore effective as propagules over time and distance (68,69). If heterothallism evolved to meet one or more of these needs, it is easy to see how the same advantages could be useful to homothallic descendants. Advantages conferred by outcrossing are irrelevant in self-fertilizing homothallics but meiosis for repair or the production of hardy ascospores could be valuable to homothallics.

Several approaches have been taken in the attempt to develop an evolutionary history for *Neurospora*. Some workers have used mitochondrial DNA (mtDNA) length mutations and random fragment hybridization analysis of nuclear DNA to describe the relationships between heterothallic species in the genus (56,90,91). Though no such analyses have included constitutively homothallic species, data from homothallic species can nevertheless be merged with heterothallic cladistic trees.

Random fragment length polymorphisms in nuclear DNA have been analyzed in heterothallic (56) and homothallic (29) groupings of *Neurospora* species. Natvig et.al. (56) have detected the existence of distinct clusters of heterothallic isolates that have common RFLP patterns when digested with the same restriction enzyme(s). Analysis of these clusters lends credence to the hypothesis that *N.tetrasperma* and *N.sitophila* are each monophyletic. Conversely, *N.crassa* and *N.intermedia* cannot form monophyletic groups based on comparison of RFLP patterns. Instead they constitute a phyletic branch to themselves. In support of these hypotheses, these same data support established species concepts based on mating. *N.tetrasperma* and *N.sitophila* are not capable of crossing with other *Neurospora* species. *N.crassa* and *N.intermedia*, however, produce a significant percentage of fertile ascospores when crossed (67).

N.tetrasperma is believed to have diverged first from an ancestral *Neurospora* heterothallic because its nuclear RFLP patterns are the most dissimilar to patterns from other *Neurospora* species. *N.sitophila* is believed to represent the second oldest divergence while the remaining *Neurospora* heterothallics form a third phylogenetic group that diverged more recently. [This temporal analysis makes no accommodation for the evolution of true homothallic species]. This hypothesis has since been affirmed by mtDNA length mutation data from the same species (91). The clusters that emerge from mtDNA analyses are consistent with those obtained from nuclear RFLP analyses. Cladistic trees for heterothallics, though, are unrooted and not based on specific knowledge of ancestral *Neurospora* species. For this reason, it is impossible to determine which divergences

occurred earliest. Relationships are translated to histories on the basis of characters and their similarities between species. Groups possessing the most character diversity are thought to represent the oldest divisions. Indeed, the greatest RFLP variability is seen among isolates of *N.crassa* and *N.intermedia*. *N.tetrasperma* is intermediate and *N.sitophila* has the least intra-specific diversity.

Similar data are available for the analysis of evolutionary relationships between homothallic species of *Neurospora*. Glass et.al. (29) compared RFLP patterns of *Neurospora* and other *Sordariaceae* isolates. Digests of genomic DNAs were hybridized to random *N.crassa* cosmid probes and to *N.crassa* mating-type probes. Several groupings emerged from the data. Within the *Neurospora* genus, all the A-type homothallics clustered in a single group; their RFLP patterns were very similar. *N.terricola* yielded unique patterns when probed with random and mating-type probes. This suggests that A-type and A/a-types represent distinct monophyletic groups. The lack of variability within the A-type cluster also engenders the possibility that members of this grouping are actually a single species (29).

Evolutionary models

Nuclear RFLP (56) and mtDNA length mutation (90,91) data for heterothallic species can be considered in the context of homothallic RFLP (29) data and the sequence data presented in this thesis. A cladistic tree can then be proposed that considers the evolution of both homothallic and heterothallic *Neurospora* species [Figure 25]. In the trees presented, *N.tetrasperma*, *N.terricola*, and A-type homothallics may each represent monophyletic groups and mark separate divergences into functional homothallism. Alternatively, there may have been fewer than three distinct evolutions to functional homothallism; at least two groups of homothallics may be linearly related, one representing an intermediate form ancestral to the other. This latter scenario is not likely because of cytogenetic and molecular differences between groups. *N.tetrasperma* is thought to be

monophyletic because its RFLP patterns cluster and because it is the only *Neurospora* species that is four spored. Similar arguments can be made for the monophylogeny of *N.terricola*. *N.terricola* is unique in possessing both mating-types in a single nucleus. RFLP patterns are dissimilar to those of any other homothallic grouping (29) and only *N.terricola* has ascospores with a single germ pore (3,32). Finally, *N.africana* RFLP patterns cluster with those of the other A-type homothallics; no similarity is observed to patterns from any other cluster.

Evolutionary histories based on RFLP analyses may seem counter-intuitive when more variability is observed within a species (*N.crassa*) than within entire groups (A-type homothallics) (56). In this case, however, differences between heterothallic and homothallic reproduction must be considered. The fact that homothallic populations are almost entirely inbreeding means that there is little opportunity for genetic variability to arise. Because of differences in genetic input, homothallic and heterothallic speciations cannot be temporally grouped on the basis of RFLP patterns.

Nauta and Hoekstra (57,58) have applied a population genetic model to weigh the conditions under which heterothallism might have evolved into homothallism and vice versa. The model is applied to theoretical populations in which there are varying extents of selfing and variably deleterious effects realized by selfing. In all the scenarios, the conditions for homothallism to invade heterothallic populations are much more easily realized than conditions for heterothallism to invade homothallic populations. The fact that most homothallic *Sordariaceae* do not form conidia is significant. This means that outcrossing can occur only by occasional mycelial contact and only then if there is no conflict in vegetative incompatibility types. Selfing for homothallics then approaches 100%. Severe inbreeding depression could drive the evolution of heterothallism from homothallic populations if outcrossing conferred a substantial fitness advantage over selfing. In the case of haploid organisms such as *Neurospora*, however, selfing is not especially deleterious. *Neurospora* inbreeding is actually a form of intragametophytic selfing. Such selfing does

not imply any recombination and is effectively equivalent to asexual reproduction. The disadvantages to inbreeding common to diploids reflect the combination of deleterious recessive alleles and these disadvantages do not apply to haploid, intragametophytic selfers. Indeed, populations from nature show homothallism to be no less successful a reproductive strategy than heterothallism (68).

The Nauta and Hoekstra model also predicts that when evolution from hetero- to homothallism (or the reverse) occurs, one should also expect to find polymorphic populations in which both systems exist in the same species. Interestingly, most of the known polymorphic species also produce conidia. Notable examples include *Nectria haematococca*, *Gibberella zeae*, and *Glomerella cingulata* (58,68). Perhaps these species are transitional and represent intermediates in the evolution from hetero- to homothallism. The fact that *Sordariaceae* homothallics are neither polymorphic nor conidial may mean that an evolutionary event is complete and that intermediate species, now extinct or not yet isolated, had lesser fitness. Nauta and Hoekstra's model considers the question of intermediates. Since intragametophytic selfing is not significantly deleterious for haploids, homothallism is expected to eclipse heterothallism in natural populations. In their words, "the only explanation for the existence of heterothallic *Sordariaceae* seems to be that in some cases the fitness threshold for intermediate stages is too high" (58). The evolution from hetero- to homothallism is not likely to occur suddenly and in a single mutational event. Rather, intermediate polymorphic species are predicted. In many cases these polymorphic strains are stable (*G. zeae*, *N. haematococca*, *G.cingulata*) but significant numbers are not stable and presumably remain homothallic.

Conidial homothallics are not observed in the *Sordariaceae*. Nevertheless, the lack of conidia in *Sordariaceae* homothallics does not preclude the possibility that lost intermediate species were both conidial and homothallic. Indeed, the pseudohomothallic *N.tetrasperma* does form conidia (72) suggesting an intermediate status for this species in the evolution from hetero- to homothallism. [*N.tetrasperma*'s evolution was previously considered only

with respect to heterothallism]. It is easy to identify *N.tetrasperma* characters that fit the idea of intermediacy. Sexual reproduction can occur by inbreeding or by outcrossing (72). Both mating-types are present in single ascospores and conidia, bestowing functional homothallism. At the same time, the fact that A and a mating-types occupy separate nuclei implies a close alignment with heterothallic mating mechanisms.

If indeed conidia and microconidia serve in *Neurospora* for fertilization in addition to propagation, then the loss of conidiation in true homothallics is not surprising. *N.terricola* and the A-type species can be thought of as constitutive homothallics because they have lost the ability to outcross; they have no need for conidia as fertilizing agents. At the same time, conidia themselves are very successful as propagules. Perhaps the evolution of homothallism is coincidental with the loss of ability to conidiate. Homothallics would then be disadvantaged as compared to conidiating heterothallics but the ability to self and the independence from mating could compensate. Theoretically, it should still be possible for *N.terricola* to evolve to heterothallism by the unlikely restoration of conidiation function. A similar evolution in *N.africana* is even more unlikely because it would also require the evolution of a functional a idiomorph. Alternatively, *N.africana* may already harbor a second idiomorph that functions like a but is not detectable by hybridization to *N.crassa* a.

Future Work

The mating-type locus of *N.crassa* is largely conserved in homothallic members of the *Sordariaceae*. The majority of the *N.crassa* A and a idiomorphs and the left flanking sequence are present in the genomes of A/a-type homothallic species of *Neurospora*, *Gelasinospora*, and *Anixiella*. The idiomorphs are not closely linked in any of these genera though the a idiomorph is linked to the left flank sequence in most cases. The right flank sequence is diverged or absent in the same species. While the conservation of the idiomorphs themselves is well established, their linkage is not well described. Reasonable mechanistic models for the evolution of a homothallic mating-type locus will require the

mapping of A and a idiomorphs to a specific locus(i). This result could be obtained by CHEF gel analysis and the localization of idiomorphs to linkage groups. Presumably, the association of A and a idiomorphs in single nuclei could then be attributed to crossover (idiomorphs on same linkage group) or translocation (idiomorphs on different linkage groups) events.

The *N.terricola* A ORF confers functionality when transformed into *N.crassa* sterile mutants. A similar result is expected for the *N.terricola* a ORF but awaits confirmation. Of even more interest would be the functionality in *N.terricola* of these ORFs. It is easy to conceive of an evolution in which the idiomorphs of a heterothallic ancestor are present residually in descendant homothallic species; their functionality upon transformation into *N.crassa* sterile mutants could also be residual. A key question is whether an *N.terricola* individual with a mutation in the ORF of A or a (or both) can still enter the sexual cycle and generate viable meiotic progeny. If sexual functions were lost, would the transformation of this mutant with an *N.terricola* mating-type ORF(s) restore functionality and/or ascospore viability? The answers to these questions would hinge on the development of an *N.terricola* sterile mutant, the generation of competent spheroplasts of that mutant, and its successful transformation by *Neurospora* DNA.

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