

### A simple method to determine mating type in *Neurospora discreta*

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In order to circumvent the problem in assigning the mating types of some *N. discreta* strains by fluffy testers, a PCR based method was developed. Between the two reference *N. discreta* strains (FGSC 3228 A and FGSC 4378 a), we observed the false perithecia to be more abundant in the “a” mating type. When we analyzed the octads from a cross of these two, the “false perithecia” appear to be associated with a particular morphology and mating type.

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*Neurospora discreta* exhibits a stringent reproductive isolation from other heterothallic species of the genus *Neurospora*. (Perkins and Raju 1986 *Exp. Mycol.* **10**: 323-338). The species of a wild isolate is determined on the basis of its fertility in crosses with tester strains (Perkins and Turner 1988 *Exp. Mycol.* **12**: 91-131). The mating type is assigned by spot crosses on lawns of fluffy (*fl*) tester strains (Perkins *et al.* 1989 *Fungal Genet. Newsl.* **36**:64-66). During the course of our studies, we found that some strains of *N. discreta* displayed a poor and delayed reaction on these plates, making it uncertain to designate the mating types. The mating type is specified by the idiomorphs, *mat A* and *mat a* (Metzenberg and Glass 1990 *BioEssays* **12**: 53-59). The *mat A* locus contains three open reading frames. Of these *mat A-1* is the major ORF involved with fertility and vegetative incompatibility (Ferreira *et al.* 1996 *Mol. Gen. Genet.* **250**: 767-774). In order to assign the mating type of a strain, we have looked for the presence or absence of the *mat A-1* ORF by PCR.

**Amplification of the *mat A-1* ORF by PCR:** Conidia from seven-day old cultures grown on glycerol complete medium (Perkins and Raju 1986 *Exp. Mycol.* **10**: 323-338) were used as inoculum for liquid Vogel’s N Medium (Davis and de Serres 1970 *Methods Enzymol.* **A17**: 79-143) supplemented with 1.5 % sucrose as the carbon source. After growth for 24-48 h at 34°C, the mycelium was harvested by passing the culture medium through a filter paper on a Buchner funnel under suction and then lyophilized. DNA was isolated with a minor modification of the method given by Zolan and Pukkila (1986 *Mol. Cell. Biol.* **6**: 195-200). Two hundred nanogram of genomic DNA was used as template in a 25 µl reaction mixture in a 0.5 ml PCR tube (Bangalore Genei Pvt. Ltd., Bangalore). Two primers were designed from the sequence of the *N. discreta mat A-1* gene sequence, intron, and 3’ end (Randall and Metzenberg 1995 *Genetics* **141**(1): 119-136; GenBank accession number L42307). The sequence of the primers is as follows: forward primer (23-mer, 5¢ ATGTCGGGCGTCGACCAAATTGT 3¢) and reverse primer (23-mer, 5¢ ATCTCGACGACATGTCAAATCCG 3¢). The expected product of 1.3 kb would span the complete *mat A-1* ORF, including a 57 bp intron. The reaction conditions for amplification were as follows: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3.0 mM MgCl<sub>2</sub>, 0.2 mM of dNTPs (Amersham Pharmacia Biotech Asia Pacific Pvt. Ltd.), 1.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech Asia Pacific Pvt. Ltd.), and 0.2 µM of each of the primers (Bangalore Genei Pvt. Ltd.). The procedure involved denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 1 min, 1 min annealing at 68°C, and 2 min extension at 72°C in each cycle, and a final extension step for 10 min at 72°C. The amplifications were carried out in a Techne Progene Thermal Cycler set at maximum ramp rate. A portion of the reaction mixture (5 µl) was separated on 1 % agarose gels containing 0.5 µg/ml of ethidium bromide with 1X Tris-acetate (TAE) (pH 8.0) as the buffer, and then visualized by UV illumination. Images were analyzed using the Kodak Digital Science 1D system. Figure 1 shows the product amplified from genomic DNA of different strains. As expected, no product was evident in controls, i.e., “a” strains of either *N. crassa* or *N. discreta*. A product of the expected size (1.3 kb) is apparent in *N. discreta* “A” strains. In case of *N. crassa*, the size of product is 1.4 kb. This was further confirmed by restriction analysis of the amplified products (Figure 2). The *mat A-1* ORF from *N. crassa* (OR23-I V A, FGSC 2489) and *N. discreta* (FGSC 3228 A) have been reported to be highly similar in their sequence (88 %, Randall and Metzenberg 1995 *Genetics* **141**(1): 119-136; 94.1%, Pöggeler 1999 *Curr. Genet.* **36**:222-231). The observation that the primers designed for *N. discreta* sequence amplify the *mat A-1* ORF from *N. crassa* imply that this region is conserved in both the species.

**Restriction analysis of the amplified product:** The Clone (CLONE MANAGER version 3.11) programme was used to construct a hypothetical restriction map, from the reported sequence of the *mat A-1* ORF of *N. discreta* (FGSC 3228 A). We digested the amplified products with different restriction enzymes (*Hind* III, *Kpn* I and *Hha* I) to compare the observed restriction pattern with the expected pattern. A single *Hind* III site is present in *N. crassa* (Glass *et al.* 1990 *Proc. Natl. Acad. Sci. USA* **87**: 4912-4916). This is conserved in the other strains tested. Same is true for the two *Hha* I sites. The Papua New Guinea strains lack one of the two *Kpn* I sites, which is present in the reference strain (FGSC 3228 A) isolated from Kirbyville. This difference might arise from different geographical origins of these strains.

**Association of mating type, morphology and false perithecia:** The Kirbyville strains (FGSC 3228 A/P851 A and FGSC 4378 a/P 8127 a), which are also the species tester reference strains for *N. discreta*, are known to form “false” perithecia on Vogel’s N medium and glycerol complete medium (Perkins and Raju 1986 *Exp. Mycol.* **10**: 323-338). We observed that the false perithecia are more abundant in the “a” mating type. To explore the inheritance of this particular character, we performed an unordered tetrad analysis (Perkins 1974 *Genetics* **77**:459-489) of a cross of these strains. Eight cultures of an octad were classified into two types based on their morphology. Four of them were pinkish and showed conidiation only on the surface. The other four were

yellowish, had good aerial hyphae and conidia formed a “plug” on top of the agar slant. Interestingly, only the second type formed false perithecia. The false perithecia were visible after 8-10 days of growth. This was observed for all the five octads collected. A 1:1 segregation suggests that this character may be determined by of a single nuclear gene. More interesting was the observation that all these false perithecia forming cultures were of the “a” mating type, as determined by testing on fluffy lawns. This was true for all of the five octads tested. The mating types were further confirmed by PCR. For this purpose, the template was prepared from 7 day old conidia of cultures grown on glycerol complete medium, by the quick method of “boiling” (Yeadon and Catcheside 1996 *Fungal Genet. Newsl.* **43**: 71). 2.5 µl of this template was used for a 25 µl reaction. Figure 3 shows the PCR products for one of the octads.

In *N. crassa*, the *mat a* idiomorph has a single ORF (*mat a-1*) (Staben and Yanofsky 1990 *Proc. Natl. Acad. Sci. USA* **87**:4917-4921). This ORF contains a HMG box domain, characteristic feature of a number of regulatory proteins that bind to the sequences with a CTTTG core (Philly and Staben 1994 *Genetics* **137**: 715-722). The *mat a-1* ORF is highly similar (~90%) among all the species of *Neurospora* (Pöggeler 1999 *Curr. Genet.* **36**:222-231). Only in *N. discreta* does the mating type “a” appear to be associated with a particular morphology and false perithecia. False perithecia are also abundant in single-mating-type cultures of *N. tetrasperma* (Perkins and Raju 1986 *Exp. Mycol.* **10**: 323-338). In this case also we found them to be more abundant in one of the mating types. But unlike *N. discreta*, here it was the “A” mating type (FGSC 1270 A) and not the “a” mating type (FGSC 1271 a). Together, these observations suggest that apart from determining mating type, the idiomorphs may regulate many more target genes, and these might be different in different species.

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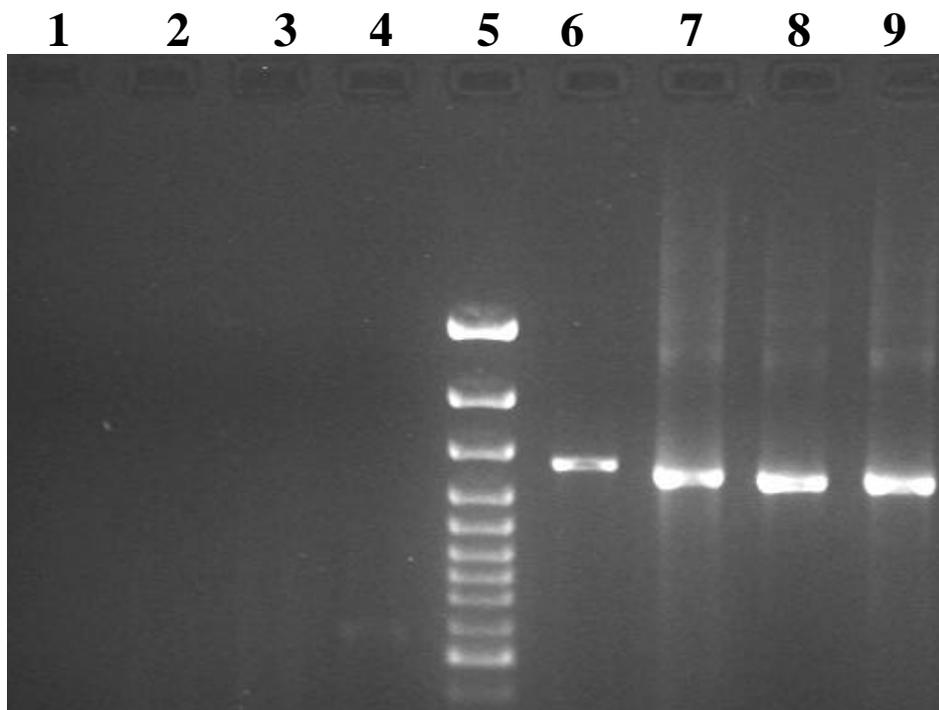


Figure 1: Products of PCR amplification of *mat A-1* ORF from different species of *Neurospora*. Lane 1: control reaction in the absence of DNA, lane 2: *N. crassa* ORS-6 a (FGSC 4200), lane 3: *N. discreta* P8127 a (FGSC 4378), lane 4: *N. discreta* Bandipur a (FGSC 6789), lane 6: *N. crassa* OR23-I V A (FGSC 2489), lane 7: *N. discreta* P851 A (FGSC 3228), lane 8: *N. discreta* P1913 A (collected from Papua New Guinea), lane 9: *N. discreta* P1859 A (collected from Papua New Guinea). Lane 5: Standard marker DNA, GeneRuler™ 100 bp Ladder Plus (MBI Fermentas), size of fragments 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, 3000 bp.

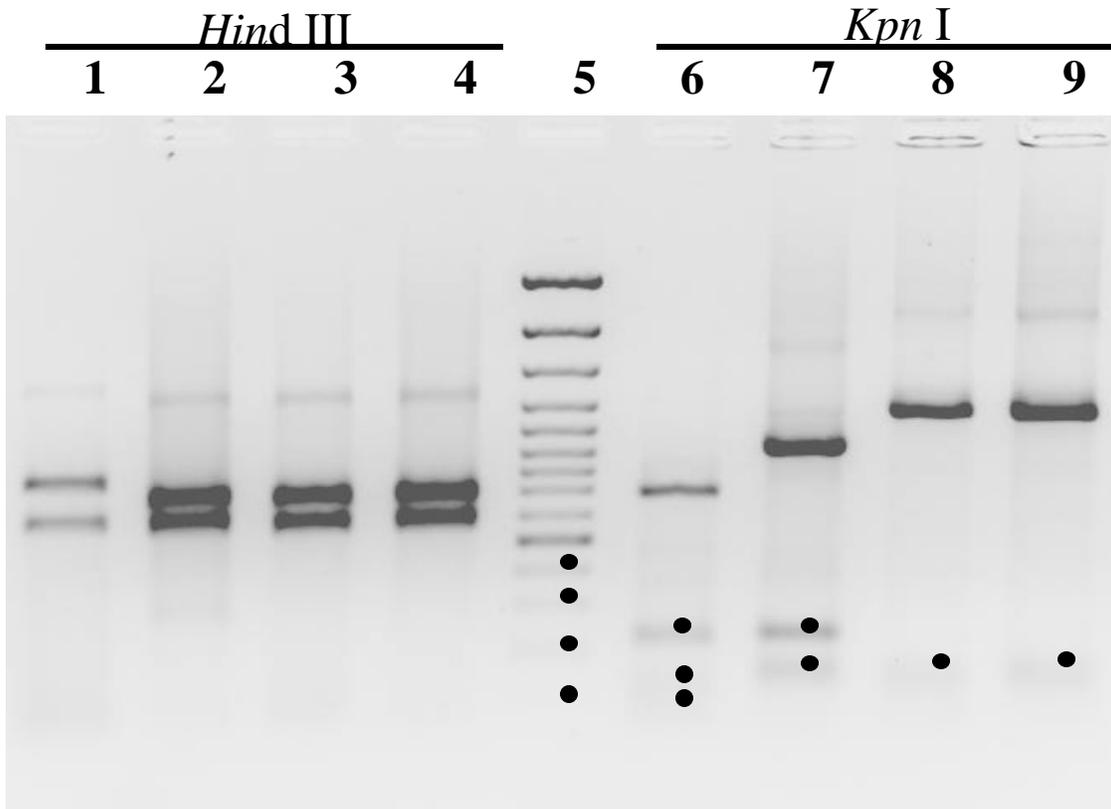


Figure 2: Restriction digestion pattern of the amplified products. Lane 1 and 6: *N. crassa* OR23-I V A (FGSC 2489), lane 2 and 7: *N. discreta* P851 A (FGSC 3228), lane 3 and 8: *N. discreta* P1913 A (collected from Papua New Guinea), lane 4 and 9: *N. discreta* P1859 A (collected from Papua New Guinea). Lane 5: Marker DNA, size of fragments 100,200,300,400,500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, 3000 bp. The restriction fragments add up to give the total size of the amplified product. The position of faint bands is highlighted by dots.

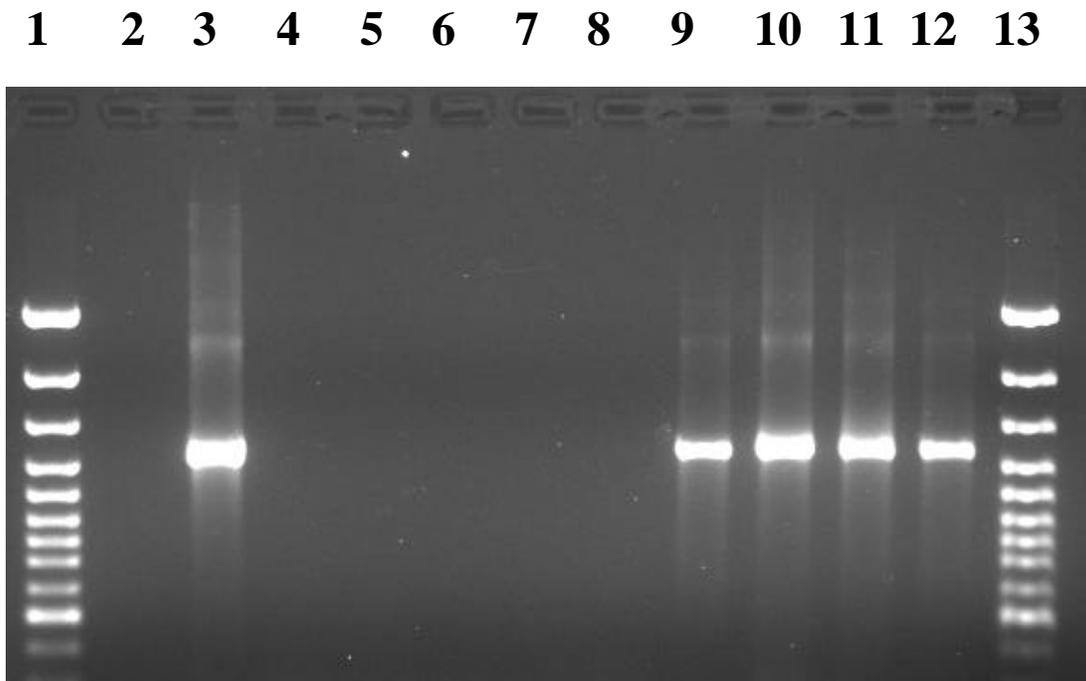


Figure 3: Products of PCR amplification of *mat A-1* ORF from all the eight cultures of a single octad of a cross of Kirbyville strains. Lane 2: reaction performed in the absence of DNA, lane 3 and 4: parents. Lane 3: *N. discreta* P851 A (FGSC 3228), lane 4: *N. discreta* P8127 a (FGSC 4378). Lane 5-12: 8 eight cultures of a single octad. Lane 5-8: mating type a, lane 9-12: mating type A. Lane 1 and 13: Marker, size of fragments 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, 3000 bp.