

## RESEARCH ARTICLE

# *Neurospora crassa* *fmf-1* encodes the homologue of the *Schizosaccharomyces pombe* Ste11p regulator of sexual development

SRIVIDHYA V. IYER, MUKUND RAMAKRISHNAN and DURGADAS P. KASBEKAR\*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

### Abstract

The *Neurospora crassa* *fmf-1* mutation exerts an unusual ‘perithecial-dominant’ developmental arrest; *fmf-1* × *fmf-1*<sup>+</sup> cross becomes arrested in perithecial development regardless of whether the mutant participates in the cross as the male or female parent. We localized *fmf-1* to the LG IL genome segment between the centromere-proximal breakpoint of the chromosome segment duplication *Dp(IL)39311* and the centromere. By mapping crossovers with respect to RFLP markers in this region we further localized *fmf-1* to an approximately 34-kb-genome segment. Partial sequencing of this segment revealed a point mutation in the gene *NCU 09387.1*, a homologue of the *Schizosaccharomyces pombe* *ste11*<sup>+</sup> regulator of sexual development. The *fmf-1* mutation did not complement a *NCU 09387.1* deletion mutation, and transformation with wild-type *NCU 09387.1* complemented *fmf-1*. *S. pombe* Ste11 protein (Ste11p) is a transcription factor required for sexual differentiation and for the expression of genes required for mating pheromone signalling in *matP* and *matM* cells. If FMF-1 also plays a corresponding role in mating pheromone signalling in *Neurospora*, then protoperithecia in an *fmf-1* × *fmf-1*<sup>+</sup> cross would be unable to either send or receive sexual differentiation signals and thus become arrested in development.

[Iyer S. V., Ramakrishnan M. and Kasbekar D. P. 2009 *Neurospora crassa* *fmf-1* encodes the homologue of the *Schizosaccharomyces pombe* Ste11p regulator of sexual development. *J. Genet.* **88**, 33–39]

### Introduction

The *female and male fertility-1* (*fmf-1*) mutant of *Neurospora crassa* has an astonishing phenotype; its cross with the wild-type (*fmf-1* × *fmf-1*<sup>+</sup>) becomes arrested in sexual development when the perithecia attain only 40% of their normal diameter, regardless of whether the mutant participates in the cross as the male or female parent (Johnson 1979). That is, *fmf-1* exerts an unusual ‘perithecial-dominant’ developmental arrest. Intriguingly, the mutant is heterokaryon-recessive; *fmf-1* × *fmf-1*<sup>+</sup> crosses are fertile if the *fmf-1* mutant nucleus is derived from an [*fmf-1* + *fmf-1*<sup>+</sup>] heterokaryon. Using the *tolerant* (*tol*) mutation to suppress mating type heterokaryon incompatibility (Newmeyer 1970), Johnson (1979) constructed the heterokaryon [(*tol pan-1 fmf-1 A*) + (*tol trp-4 fmf-1<sup>+</sup> a*)], crossed it with multiply marked *mat a* strains, and mapped *fmf-1* to between *mat* and *cr-1* on

linkage group (LG) I; tentatively, close to *arg-1*. Sequencing of the *N. crassa* genome revealed that *mat* and *cr-1* are separated by > 3.3 Mbp (Galagan *et al.* 2003).

The objective of this study is to map the *fmf-1* mutation to a single gene, in the expectation that identification of the *fmf-1* gene would help us to formulate hypotheses to account for its unique mutant phenotype. This long-mysterious (or, long-neglected) gene has now been found to be the homologue of *Schizosaccharomyces pombe* *ste11*<sup>+</sup>. The Ste11 protein (Ste11p) is a transcription factor that regulates sexual development and is required for mating pheromone signalling in both *matP* and *matM* cells. Homology between *fmf-1*<sup>+</sup> and *ste11*<sup>+</sup> suggests that the FMF-1 protein might play a corresponding role in mating pheromone signalling in *Neurospora*. If that be the case, the *fmf-1* × *fmf-1*<sup>+</sup> perithecial development could be arrested because the protoperithecia are unable to either send or receive mating pheromone signals.

\*For correspondence. E-mail: kas@ccmb.res.in.

We dedicate this paper to our friend Namboori Bhaskara Raju, Stanford University, to recognize his many quiet, yet very significant, contributions to fungal biology. He continues to be such a fine example for the rest of us.

**Keywords.** perithecial development; chromosome segment duplications; mating pheromones; yeast; *Neurospora*.

## Materials and methods

### Gene, phenotype and protein symbols

Gene symbols are italicized, while phenotype symbols are not. Phenotypes are designated with caps (e.g., Hyg<sup>+</sup> or Hyg<sup>R</sup>, and Fmf<sup>+</sup> and Fmf<sup>-</sup>) and no gene number is used. Protein names are all caps and no italics (i.e., FMF-1).

### *Neurospora crassa* strains

*Neurospora* genetic analysis was essentially as described by Davis and De Serres (1970). Unless otherwise indicated, all the *N. crassa* strains used were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas, USA. They included the standard Oak Ridge strains 74-OR23-1 A (FGSC 987) and OR8-1 a (FGSC 988), the auxotrophic mutant strains *arg-3 a* (FGSC 1069) and *his-1 A* (FGSC 401), and the *fmf-1* mutant strains *fmf-1; pyr-3 A* (FGSC 3108) and *fmf-1; tol pan-1 A* (FGSC 3109). The *pyr-3* and *pan-1* mutations are unlinked to *fmf-1* and confer auxotrophy for uracil and pantothenate (Perkins et al. 2001). The *tol* (*tolerant*) mutation suppresses mating type heterokaryon incompatibility (Newmeyer 1970), and was used to recover *Dp(IL) 39311* strains (see below) which contain both *mat A* and *mat a* idiomorphs. The semi-dominant *Sad-1* (*Suppressor of ascus dominance-1*) mutation suppresses meiotic silencing and, thereby, enhances the productivity of *Dp* × euploid crosses (Shiu et al. 2001). The *Sad-1 A* (FGSC 8740) and *Sad-1 a* (FGSC 8741) strains were kindly provided by the late Robert L. Metzberg.

The *helper-1* strain (FGSC 4564; *a<sup>m1</sup> ad-3B cyh-1*) contains *fmf-1<sup>+</sup>*, and the inactive *a<sup>m1</sup>* allele of *mat a* that does not trigger mating type heterokaryon incompatibility but makes it a passive partner when the heterokaryon is used as a parent in a cross. Only the nuclei of the active partner participate in karyogamy and the production of sexual progeny (Perkins et al. 2001). The *ad-3B* mutation confers adenine auxotrophy. Heterokaryons between the *fmf-1* mutant strains and the *helper-1* strain were isolated by selection for complementation of the auxotrophic markers, and they will henceforth be referred to as [*fmf-1; pyr-3 A*] and [*fmf-1; tol pan-1 A*], respectively. An *arg-3 fmf-1 a* segregant was obtained from the cross *arg-3 a* × [*fmf-1; pyr-3 A*] (out of 315 segregants examined, including 150 *arg<sup>-</sup>*) and used to make the heterokaryon with *helper-1*. This heterokaryon will be referred to as [*arg-3 fmf-1 a*].

The translocation strains *T(IR;VR;IR > VII)In(VL;VR)AR173 a* (FGSC 2469), *T(IR > VII)P7442 mo a* (FGSC 3209) and *T(IL > IIR)39311 a* (FGSC 1246) are described by Perkins (1997) and will be referred to as *T(AR173) a*, *T(P7442) a* and *T(39311) a*, respectively. The wild-isolated (*W*) *mat A* strains Chemax (P4212) Colonia Paraiso (P4212), Mauriceville-1c (FGSC 2225) and Mughalsarai (P0736) were used for RFLP mapping.

### Generation of RIP-induced *his-2* mutants

The oligonucleotides 5' AAGCTGGTGCCAAT-GTTCTTGC 3' and 5' AGGCAGATCACCGTTCAAATCG 3' were custom purchased from Bioserve (Hyderabad, India) and used as primers to amplify a 1463 bp segment of the *his-2* gene by PCR, and the amplified DNA was cloned into the *EcoRV* site of the plasmid vector pCSN44 (Staben et al. 1989). The resulting plasmid (pSIhis2) was transformed by electroporation into conidia of the *his-3 A* strain (FGSC# 6103), and transformants were selected on hygromycin-medium. The PCR reaction conditions, other molecular methods, and transformation protocols were as described by Bhat et al. (2004). The primary transformants were crossed with OR *a*, and among their progeny we obtained several that were hygromycin-resistant, prototrophic and *mat a*, consistent with the hypothesis that the transgene, designated *Dp(his-2)*, was unlinked to *his-3* (and *mat*). The *Dp(his-2) a* strains were crossed with *fmf-1; pyr-3A* and with the wild-isolated *mat A* strains Colonia Paraiso (P4212) and Mauriceville-1c (FGSC 2225). *Dp(his-2) A* progeny from these crosses were then crossed with OR *a* to target RIP to the *his-2<sup>+</sup>* locus on the LG I derived from the *fmf-1* or the wild-isolate parent (Galagan and Selker 2004). Eight RIP-induced *his-2* alleles were obtained on LG I's bearing *fmf-1*; the *fmf-1 his-2 A* segregant #45 was used in the crosses that established that *fmf-1* is not distal to *his-2* (see Results). Three and 21 *his-2* mutants, respectively, were obtained on the LG I's derived from the Colonia Paraiso and Mauriceville-1c strains, and they were used in the localization of *fmf-1* via RFLP mapping.

### Inverse PCR

Genomic DNA (2 µg) of the translocation strain was digested with the restriction enzyme in a 20 µl reaction for 4–5 h. One µg of the digested DNA (10 µl) was ligated using 12 Weiss units NEB ligase for 16 h at 16°C in a 500 µl reaction volume. The circularized DNA was then ethanol precipitated, resuspended in TE and 0.1 µg was used as template for PCR using primers INV1 5' AGCACGTGGGAGAGAATCCTT 3' and INV2 5' TGGATCTGGAAGGTGTCACCT 3'.

### Molecular markers to localize *fmf-1*

PCR based RFLPs were identified to distinguish between the genomes of OR and the wild-isolated strains. The oligonucleotide primers and restriction enzymes used to obtain these RFLPs are listed in tables 1 and 2 of electronic supplementary material at <http://www.ias.ac.in/jgenet/>.

## Results

### *fmf-1* localizes to between the proximal breakpoints of *Dp(IL) 39311* and *Dp(IR) AR173*

The chromosome segment duplications *Dp(IL) 39311*, *Dp(IR) AR173* and *Dp(IR) P7442* partition the *mat - cr-1*

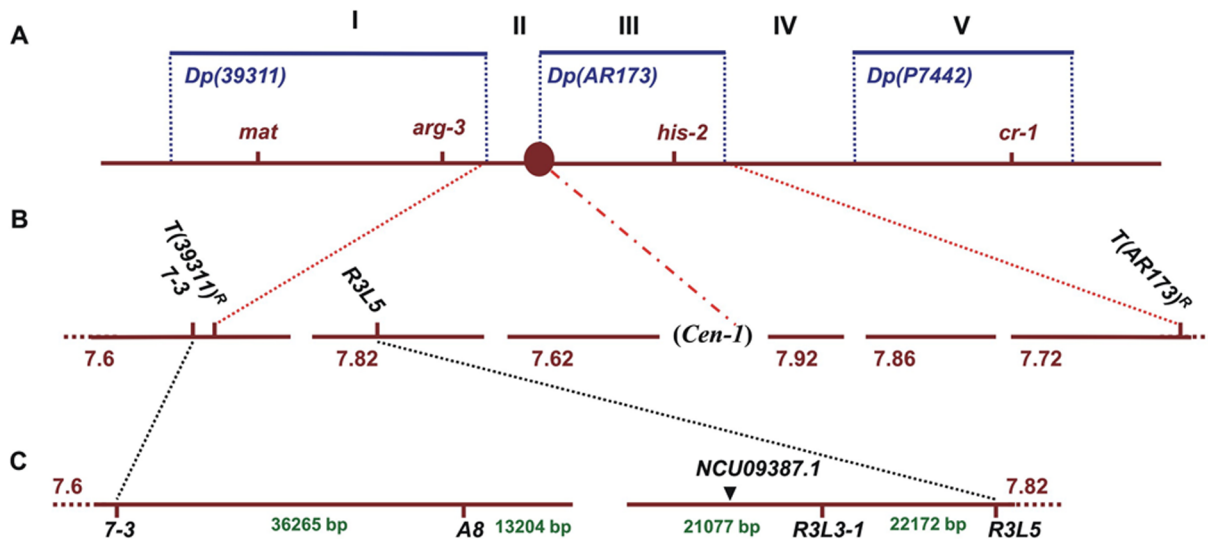
interval into five segments (I–V; figure 1). To determine whether *fmf-1* is covered by any of these *Dp*'s, we crossed the translocation strains *T(AR173) a* and *T(P7442) a* with the heterokaryon [*fmf-1*; *pyr-3 A*] and the *T(39311) a* translocation strain with the heterokaryon [*fmf-1*; *tol pan-1 A*] (see Materials and Methods for strain descriptions). Among the progeny from crosses of these translocations with normal sequence strains, a subset is duplicated for the translocated segment (i.e., *Dp(AR173)*, *Dp(P7442)* and *Dp(39311)*), and the duplication progeny (*Dp*) can be identified in a subsequent cross by the barren phenotype characteristic of *Dp* × euploid crosses (Perkins 1997). Barren crosses make normal looking perithecia, but produce exceptionally few ascospores. Barrenness is caused, at least in part, by a presumably RNAi-based process called meiotic silencing by unpaired DNA, that silences duplication-borne genes, including those required for the completion of meiosis and ascus development (Shiu *et al.* 2001). The semi-dominant *Sad-1* mutation suppresses meiotic silencing and, consequently, *Dp* × *Sad-1* crosses show increased productivity (Shiu *et al.* 2001). Thus *Dp* progeny are barren in crosses with OR but relatively more fertile in crosses with *Sad-1*.

Sixty-three *f*<sub>1</sub> progeny from *T(AR173) a* × [*fmf-1*; *pyr-3 A*] were crossed with OR strains of opposite mating type and 27 were phenotypically *Fmf*<sup>+</sup> *a* (i.e. fertile in crosses with OR *A*), whereas 36 were *Fmf*<sup>−</sup> *A* (i.e. arrested in crosses with OR *a*). Of the *Fmf*<sup>−</sup> *A* strains, 28 were *Ura*<sup>−</sup> and could be used to select heterokaryons with the *helper-1* strain (see

Materials and methods). Four heterokaryons were barren in crosses with OR *a* but more productive with *Sad-1 a*, suggesting that their non-*helper* component was genotypically *Dp(AR173)*, and since this component as a homokaryon had the *Fmf*<sup>−</sup> mutant phenotype, it followed that *Dp(AR173)* does not cover *fmf-1*. The remaining 24 heterokaryons tested gave fertile crosses with OR *a*; therefore, their non-*helper* component must be of the non-duplication *fmf-1 A* genotype.

Of 34 *f*<sub>1</sub> segregants examined from [*fmf-1*; *pyr-3 A*] × *T(P7442) a*, 11 were *Fmf*<sup>+</sup> *a* and 23 were *Fmf*<sup>−</sup> *A*. Of the latter, 14 were *Ura*<sup>−</sup> and used to make heterokaryons with *helper-1* and crossed with OR *a* and *Sad-1 a*. One [(*Fmf*<sup>−</sup>; *Ura*<sup>−</sup> *A*) + *helper-1*] heterokaryon was barren in the cross with OR *a*, but fertile with *Sad-1 a*, thus indicating that its non-*helper* component was *Dp(P7442)* in genotype, and allowing us to conclude that *Dp(P7442)* does not cover *fmf-1*. Of 38 *f*<sub>1</sub> segregants examined from [*fmf-1*; *tol pan-1 A*] × *T(39311) a*, 19 were *Fmf*<sup>+</sup> *a*, eight were *Fmf*<sup>−</sup> *A* and 11 had the 'square' morphology characteristic of strains duplicated for *mat* and whose mating type incompatibility is suppressed by *tol*. By PCR we confirmed that the 11 strains contained both *mat* idiomorphs (data not shown), as would be expected for strains with the *Dp(39311)*, *tol* genotype. These strains were phenotypically *Fmf*<sup>−</sup>; therefore, we could conclude that *Dp(39311)* does not cover *fmf-1*.

Since *fmf-1* was not covered by any of the three duplications, the mutation must lie either in interval II or IV shown in figure 1. The *arg-3* and *his-2* loci are, respectively, the



**Figure 1.** Genetic localization of *fmf-1*. (A) *Dp(IL) 39311*, *Dp(IR) AR173* and *Dp(IR) P7442* partition the *mat* - *cr-1* interval into the segments I–V. (B) The proximal breakpoint of *Dp(39311)* (i.e., *T(39311)<sup>R</sup>*), and the distal breakpoint of *Dp(AR173)* (i.e., *T(AR173)<sup>R</sup>*), were localized onto contigs 7.6 and 7.72 (dotted red lines, see text for details). Inverse PCR from *T(AR173)<sup>R</sup>* and sequencing revealed that the proximal breakpoint of *Dp(AR173)* is in a centromeric repeat sequence (*Cen-1*) (dotted and dashed red line). Since *fmf-1* is not covered by the *Dp*'s, nor is it distal to *his-2* (see text), it must lie in II, i.e., proximal to *T(AR173)<sup>R</sup>*. (C) Crosses that produced prototrophic progeny from *arg-3 fmf-1 a* × *fmf-1<sup>+</sup> his-2 A* were mapped with respect to the RFLP markers 7.3, A8, *R3L3-1* and *R3L5* on contigs 7.6 and 7.82 and this localized *fmf-1* to the 34281 bp segment (plus a gap) bounded by A8 and *R3L3-1*. The *fmf-1* strain displayed a T to A mutation in the *NCU 09387.1* gene within this region.

proximal-most auxotrophic markers on LG IL and IR, and they are covered, respectively, by *Dp(IL) 39311* and *Dp(IR) AR173*. We used repeat-induced point mutation (RIP) to introduce a *his-2* mutation into the *fmf-1* background (see Materials and methods), and the resulting (*fmf-1 his-2*) A strain was made into a heterokaryon with *helper-1* and crossed with *arg-3 a*. If *fmf-1* is distal to *his-2*, then all the prototrophic crossover progeny from this cross should be *Fmf*<sup>+</sup>. Of 62 prototrophic segregants examined, seven were *Fmf*<sup>-</sup> A, 53 *Fmf*<sup>+</sup> A and two *Fmf*<sup>+</sup> a. Since 11% of the progeny were *Fmf*<sup>-</sup> in phenotype, it followed that *fmf-1* is not distal to *his-2* and therefore it must map to interval II shown in figure 1, i.e. between the proximal breakpoints of *Dp(IL) 39311* and *Dp(IR) AR173*, a region operationally defined to be pericentromeric on LG I (Rosa et al. 1997). The two *Fmf*<sup>+</sup> a segregants must represent double crossovers with one crossover between *mat* and *arg-3* and the other between *arg-3* and *fmf-1*.

#### *Dp(39311) and Dp(AR173) proximal breakpoints localized onto the genome sequence*

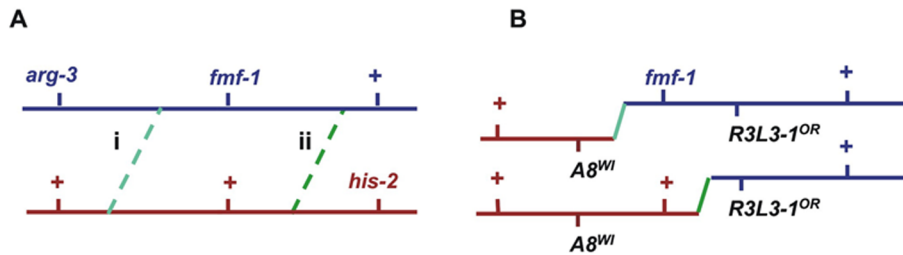
The *arg-3 – his-2* genome segment is partially sequenced (Galagan et al. 2003); *arg-3* is on contig 7.6, 305.4 kbp from the contig's proximal end, whereas *his-2* is on contig 7.72, ~74.4 kbp from the proximal end. Four other contigs; 7.82 (~107.5 kbp), 7.62 (~181 kbp), 7.92 (69.4 kbp) and 7.86 (~95.6 kbp) are located between contigs 7.6 and 7.72. Thus, the *arg-3* and *his-2* interval contains 833.3 kbp of sequence and five gaps of unknown lengths (figure 1). To localize the proximal breakpoints of *Dp(IL) 39311* and *Dp(IR) AR173* onto this sequence, we obtained a *T(39311)*, *tol pan-1 a* segregant from [*fmf-1*; *tol pan-1 A*] × *T(39311) a*, and then crossed it and the *T(AR173) a* translocation (*T*) strain with the wild-isolated (*W*) *mat A* strains Mughalsarai and Chemax. The *Dp(IL) 39311* progeny were identified by their 'square' morphology and the *Dp(IR) AR173 A* progeny by the barrenness of their crosses with OR *a*. *Dp* segregants from a *T* × *W* cross contain both the *T* and *W* alleles of any RFLPs that are within the translocated segment but, barring crossovers, only the *W* allele for RFLPs outside this segment. By testing a series of linked RFLP markers for coverage by the *Dp*, we identified covered and uncovered markers bracketing the *Dp* breakpoint, and then progressively narrowed down the 'breakpoint interval' by iterating this process with additional RFLPs from within the interval (data not shown; see Vyas et al. 2006 for a figure describing the method). The *Dp(IL) 39311* proximal breakpoint was further localized to between a pair of oligonucleotides that failed to PCR amplify a product when DNA from the *T* strain was used as template but could amplify a ~1.5 kbp fragment from OR (and *W*) DNA, thus suggesting that the *Dp(IL) 39311* proximal breakpoint lay within this amplicon. This amplicon shared ~200 bp overlap with a more distal amplicon which was amplified from both OR and *T* DNA. Therefore the breakpoint was in the 1345 bp interval bounded by the proximal primers of the

two amplicons, the 5' bases of which were, respectively, nucleotides 38240 and 36895 on the '+' strand of contig 7.6.

The *Dp(IR) AR173* strains were duplicated for all RFLP markers examined on contigs 7.72, 7.86 and 7.92, but not for any of the markers tested on contigs 7.62 and 7.82 (data not shown). In parallel, the distal breakpoint of *Dp(IR) AR173* was localized to a 102 bp interval bounded by two oligonucleotide primers (INV-R1 and INV-R2) whose 5' bases were at positions 41534 and 41432 on the '-' strand of contig 7.72 (data not shown). Then, using *T(AR173) a* genomic DNA as template and primers INV-R2 and INV-F that anneal to sequences immediately distal to the *Dp(IR) AR173* distal breakpoint, we performed an inverse PCR to 'jump' into the sequence immediately proximal to the proximal breakpoint. The breakpoint-proximal sequence (accession number EU815636; see figure 1 in electronic supplementary material) was almost identical to a repeat sequence found in the centromeric regions of LG VII and LG III, which suggested that the proximal breakpoint of *Dp(IR) AR173* was in a copy of this repeat in the LG I centromeric/pericentromeric region. That the *T(IR) AR173* breakpoint is in putative *Cen-1* sequences is consistent with inferences from earlier studies that this breakpoint is the most proximal landmark on LG IR (Rosa et al. 1997) and implied that contigs 7.86 and 7.92 represent LG IR sequences proximal to 7.72, whereas contigs 7.62 and 7.82 were probably sequences from LG IL (figure 1). This suggested that the *fmf-1* candidate region was now wholly in LG IL, in the genome segment proximal to the proximal breakpoint of *Dp(IL) 39311*. This candidate region contains ~327 kbp of sequence and three gaps (figure 1).

#### *Further localization of fmf-1 and identification of a candidate gene*

We targeted RIP to the *his-2* locus in LG I of the wild-isolated strains Colonia Paraiso and Mauriceville-1c (see Methods) so that the resulting *his-2 A* strains retained the wild-isolate genetic background in the LG I pericentromeric region. These strains were then crossed with the heterokaryon (*arg-3 fmf-1 a*), thereby creating crosses that were heterozygous for *fmf-1*, as well as for RFLPs between OR and Colonia Paraiso (CP) or Mauriceville-1c (M) in the *fmf-1* candidate region (figure 2). The progeny from these crosses will henceforth be referred to, respectively, as CP or M type. Most of the progeny were auxotrophic for either arginine or histidine, but one could easily select prototrophs resulting from rare crossovers between *arg-3* and *his-2* (figure 2). The prototrophs were phenotypically *Fmf*<sup>-</sup> or *Fmf*<sup>+</sup> depending on whether the crossover occurred between *arg-3* and *fmf-1* (region i; figure 2) or between *fmf-1* and *his-2* (region ii; figure 2). Of the 58 prototrophs examined, 43 were *Fmf*<sup>-</sup> (26 CP + 17 M) and 15 were *Fmf*<sup>+</sup> (5 CP + 10 M). Each crossover was mapped with respect to the RFLP markers 7.3, A8, *R3L3-1* and *R3L5* on contigs 7.6 and 7.82 (figure 1). Among the prototrophs, the OR versus wild-isolate allele fraction should increase going from markers on the left (7.3) to those on the



**Figure 2.** Prototrophic progeny produced in the cross *arg-3 fmf-1 a* × *fmf-1<sup>+</sup> his-2 A*. (A) Crossovers in region i produce progeny with the *fmf-1* phenotype, whereas crossovers in region ii produce progeny with the *fmf-1<sup>+</sup>* phenotype. The blue line represents the OR genetic background and the brown line the genetic background of the wild-isolate (Colonia Paraiso or Mauriceville-1c). (B) The ‘right-most’ region i crossover was between the Colonia Paraiso allele of *A8* and the OR allele of *R3L3-1* and suggested that *fmf-1* was to the right of *A8* (top panel). The ‘left-most’ region ii crossover occurred between the Mauriceville-1c allele of *A8* and the OR allele of *R3L3-1* and suggested that *fmf-1* was to the left of *R3L3-1*. Thus *fmf-1* was localized between *A8* and *R3L3-1*.

right (*R3L5*). The fraction of prototrophic *Fmf<sup>-</sup>* progeny with the OR allele was 35/43 at 7.3 (22 CP + 13 M), 42/43 at *A8* (25 CP + 17 M), and 43/43 at *R3L3-1* and *R3L5*. Thus, the ‘right-most’ region i crossover was between the Colonia Paraiso allele of *A8* and the OR allele of *R3L3-1* (figure 1); therefore *fmf-1* must map to the right of *A8*. The fraction of prototrophic *Fmf<sup>+</sup>* progeny with the OR allele was 0/15 at 7.3 and *A8*, 1/15 at *R3L3-1* (M) and 2/15 at *R3L5* (1 CP + 1M). That is, the ‘left-most’ region ii crossover lay between the Mauriceville-1c allele of *A8* and the OR allele of *R3L3-1*; therefore *fmf-1* must map to the left of *R3L3-1*. The *A8-R3L3-1* interval (i.e., the new candidate region) contained ~34.2 kbp of sequence and one (potentially treacherous) gap (figure 1).

Hardly had we sequenced ~8 kbp of this candidate region in the mutant strain, than we found a T to A transversion mutation that altered the 3’ acceptor signal of a putative intron of the *NCU 09387.1* gene in contig 7.82. The wild type *NCU 09387.1* allele has the sequence TATAG-C (the ‘-’ denotes the splice site), which agrees well with the *Neurospora* splice acceptor consensus (Bruchez *et al.* 1993), whereas in the *fmf-1* mutant this signal was TAAAG-C (accession number EU624416). This result identified *NCU 09387.1* as a candidate for *fmf-1<sup>+</sup>*, and suggested that the mutant phenotype was due to a splicing defect.

#### Evidence that *NCU 09387.1* is *fmf-1*

An *NCU 09387.1KO a* strain (*NCU 09387.1* knockout; FGSC 13541), generated in the *Neurospora* Genome Project, is available from the Fungal Genetics Stock Center. We found that perithecial development was blocked in crosses of this strain with OR A (data not shown), just as in the *fmf-1* × OR crosses. Conjecturing that the knockout nuclei might be able to participate in a cross if they are derived from a heterokaryon with *helper-1*, we crossed *his-1 A* mycelia with *NCU 09387.1KO a* mycelia to which we had added a sus-

pension of *helper-1* conidia. This ‘triparental’ cross was fertile, presumably because formation of the heterokaryon [*NCU 09387.1KO a* + *helper-1*] fostered successful mating between the *NCU 09387.1KO a* and *his-1 A* nuclei. From such mating we obtained ten *NCU 09387.1KO; his-1 a* progeny and used them to construct [*NCU 09387.1KO; his-1 a*] + (*arg-3 fmf-1 a*) heterokaryons. Crosses of all ten heterokaryons with OR A were blocked in perithecial development. Therefore *NCU 09387.1KO* and *fmf-1* did not appear to complement in the heterokaryons, consistent with the idea that both *NCU 09387.1KO* and *fmf-1* affect the same gene.

In other experiments we had found that [*helper-1* + *fmf-1 A*] × [*helper-1* + *fmf-1 a*] crosses also were blocked in perithecial development (data not shown), which suggested that FMF-1 protein has an ascus-autonomous function that cannot be fulfilled by FMF-1 protein from the parental mycelia. We constructed 10 [*NCU 09387.1KO; his-1 a* + *helper-1*] heterokaryons and crossed each with [*helper-1* + *fmf-1 pyr-3 A*]. Again, all ten crosses were blocked in perithecial development, thus demonstrating that *NCU 09387.1KO* and *fmf-1* also do not complement for the presumptive ascus-autonomous function. Once again, noncomplementation between *NCU 09387.1KO* and *fmf-1* was consistent with the idea that *NCU 09387.1KO* and *fmf-1* mutate the same gene.

We used the oligonucleotide primers *fmf-F* 5’ CCGGTTTGACCAAGAAGACAG 3’ and *fmf-R* 5’ CCTGTCCTATCCTCGTTCCA 3’ to PCR amplify a 3557 bp segment of wild-type DNA that included the *NCU09387.1* ORF together with sequences 947 bp upstream and 228 bp downstream of the predicted start and stop codons and cloned it into the *EcoRV* site of the vector pCSN44. We transformed the resulting plasmid (*pSI9387*) by electroporation into conidia of strain *arg-3 fmf-1a* and selected several transformants on hygromycin medium. We performed crosses of 32 primary transformants with the strains *ORA* and *Sad-1 A*. Ordinarily, crosses of *arg-3 fmf-1a* with *ORA* and *Sad-1 A*

are sterile, but at least ten transformants yielded ascospores in crosses with both *ORA* and *Sad-1 A*, thereby demonstrating that *pSI9387* contains sequences that can complement the *fmf-1* mutation. This established that NCU 09387.1 and *fmf-1*<sup>+</sup> are identical.

## Discussion

This work has established that *fmf-1* is *NCU09387.1*. The genome sequence facilitated the identification of PCR based RFLPs between the OR and wild-isolate strains and by using RIP to target *his-2* mutations in the wild-isolate backgrounds we could retain RFLP heterozygosity in the *arg-3 fmf-1* × *fmf-1*<sup>+</sup> *his-2* crosses and thus localize the *fmf-1* mutation to a genome segment small enough to be easily sequenced in the mutant. Also, the *helper-1* strain and the *Sad-1* mutation made it easier to test for coverage of *fmf-1* by chromosome segment duplications. Tools such as the genome sequence, RIP, *helper-1* and *Sad-1* have become available in *Neurospora* only since 1979.

Interestingly, in the mapping studies to establish that *fmf-1* is not distal to *his-2*, we had obtained 53 single crossovers in the interval *arg-3 - fmf-1* and only seven in the interval *fmf-1 - cen I - his-2*, despite the fact that the former interval was only 317 kbp in size and had only one gap, whereas the latter was 513.4 kbp and had four gaps. That is, fewer crossovers occurred per kbp in the putatively larger interval than in the putatively smaller one, possibly because the larger interval includes the centromere and therefore might be more heterochromatinized.

*NCU 09387.1* is an orthologue of the *Schizosaccharomyces pombe ste11* gene (Borkovich et al. 2004). Therefore it was likely that *fmf-1* and *ste11* share similar functions, and that the unusual *fmf-1* mutant phenotype might be explainable in terms of the properties uncovered for its fission yeast homologue. Ste11 protein (Ste11p) is a highly unstable HMG-box transcription factor that is induced by starvation and, in turn, activates a number of genes required for sexual differentiation (Sugimoto et al. 1991; Mata and Bahler 2006). Ste11p also stimulates the transcription of its own gene, thus setting up a positive feedback loop (Kunitomo et al. 2000). During vegetative growth Ste11p is localized primarily in the cytoplasm (Qin et al. 2003) and its DNA-binding activity is inhibited by Cdk-phosphorylation (Kjærulff et al. 2007); this presumably safeguards against inappropriate activation of the sexual developmental pathway. As cells starve, dephosphorylation of Ste11p makes it available for nuclear import and leads to increased expression of genes required for mating pheromone signalling and sexual differentiation of both *matP* and *matM* cells (Qin et al. 2003). If FMF-1 plays a corresponding role in mating pheromone signalling in *Neurospora*, then it is likely to be required both for expression in protoperithecial trichogynes of receptors for mating pheromones secreted by the conidia, as well as in the conidia for the synthesis and secretion of the

pheromone. Conidial trichogyne attractants and their protoperithecial receptors are mating type-specific (Bistis 1983; Kim and Borkovich 2004, 2006). Therefore, in an *fmf-1* × *fmf-1*<sup>+</sup> cross, *fmf-1* protoperithecia would fail to express receptors for the pheromone of the *fmf-1*<sup>+</sup> conidia, and *fmf-1* conidia would fail to express the pheromone to signal to the *fmf-1*<sup>+</sup> trichogynes. Consequently, neither the *fmf-1*<sup>+</sup> nor the *fmf-1* protoperithecia are fertilized, and perithecial development is arrested at the same stage in both cases, regardless of whether the mutant strain participates in the cross as the male or female parent.

Johnson (1979) had reported that the [(*fmf-1*<sup>+</sup>; *tol A*) + (*fmf-1*; *tol a*)] heterokaryon was ‘self-fertile’ whereas the [(*fmf-1*; *tol A*) + (*fmf-1*; *tol a*)] heterokaryon was ‘self-sterile’. That is, if mating type heterokaryon incompatibility is overcome by use of *tol*, then the FMF-1 protein from a single *fmf-1*<sup>+</sup> allele can satisfy the requirement of this protein for sexual differentiation of ‘fmf-1’ protoperithecia and conidia and fertilization is not hampered. In other words, the unusual ‘perithecium-dominant’ and ‘heterokaryon-recessive’ *fmf-1* mutant phenotype emerges from the fact that mating type heterokaryon incompatibility prevents the diffusion of FMF-1 protein between the parental *mat A* and *mat a* mycelia.

The [*fmf-1* + *helper-1*] heterokaryon bears some analogy to mixed cultures of *fmf-1* and *N. intermedia*, *N. sitophila*, *N. tetrasperma* or *N. discreta* strains of the same mating type; each component of the mixed culture is sterile in its cross with OR and, presumably, if any heterokaryon were to form, it would not be subjected to mating type heterokaryon incompatibility. However, in contrast to the ready productivity of [*fmf-1* + *helper-1*] × OR crosses, attempts to ‘cross’ the mixed cultures with OR remained nonproductive (SVI and DPK, unpublished results). This provided experimental evidence against the occurrence of interspecies heterokaryons, possibly because other kinds of vegetative heterokaryon incompatibilities also can prevent diffusion of the FMF-1 protein.

In summary, this work has identified *fmf-1* as the *Neurospora* homologue of *S. pombe ste11*<sup>+</sup>. The Ste11p is known from other studies to be required for mating pheromone signalling in *S. pombe matP* and *matM* cells. Our results suggest that the FMF-1 protein might play a corresponding role in mating pheromone signalling in *Neurospora*. This hypothesis enables us now to envision how perithecial development might be arrested in an *fmf-1* × *fmf-1*<sup>+</sup> cross.

## Acknowledgements

Charges for strains obtained from the Fungal Genetics Stock Center (FGSC) were generously waived. The FGSC is supported by National Science Foundation (USA) grant BIR-9222772. SVI and MR were supported by Research Fellowships from the Council for Scientific and Industrial Research, Government of India. An Indo-

Portuguese collaborative grant was awarded to DPK and Arnaldo Videira (Instituto de Biologia Molecular e Celular, Porto, Portugal) as partial support for this study.

## References

- Bhat A., Tamuli R. and Kasbekar D. P. 2004 Genetic transformation of *Neurospora tetrasperma*, demonstration of repeat-induced point mutation (RIP) in self crosses, and a screen for recessive RIP-defective mutants. *Genetics* **167**, 1155–1165.
- Bistis G. N. 1983 Evidence for diffusible, mating type-specific trichogyne attractants in *Neurospora crassa*. *Exp. Mycol.* **7**, 292–295.
- Borkovich K. A., Alex L. A., Yarden O., Freitag M., Turner G. E., Read N. D. *et al.* 2004 Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. *Microbiol. Mol. Biol. Rev.* **68**, 1–108.
- Bruchez J. J. P., Eberle J. and Russo V. E. A. 1993 Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, introns, poly (A) tail formation sequences. *Fung. Genet. Newslett.* **40**, 89–96.
- Centola M. and Carbon J. 1994 Cloning and Characterization of centromeric DNA from *Neurospora crassa*. *Mol. Cell Biol.* **14**, 1510–1519.
- Davis R. H. and De Serres F. J. 1970 Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* **17**, 79–143.
- Galagan J. E., Calvo S. E., Borkovich K. A., Selker E. U., Read N. D., Jaffe D. *et al.* 2003 The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**, 859–868.
- Galagan J. E. and Selker E. U. 2004 RIP: the evolutionary cost of genome defense. *Trends Genet.* **20**, 417–423.
- Johnson T. E. 1979 A *Neurospora* mutation that arrests perithecial development as either male or female parent. *Genetics* **92**, 1107–1120.
- Kim H. and Borkovich K. A. 2004 A pheromone receptor gene, *pre-1*, is essential for mating type specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa*. *Mol. Microbiol.* **52**, 1781–1798.
- Kim H. and Borkovich K. A. 2006 Pheromones are essential for male fertility and sufficient to direct chemotropic polarized growth of trichogynes during mating in *Neurospora crassa*. *Eukaryot. Cell* **5**, 544–554.
- Kjærulff S., Andersen N. R., Borup M. T. and Nielsen O. 2007 Cdk phosphorylation of the Ste11 transcription factor constrains differentiation-specific transcription to G<sub>1</sub>. *Genes Dev.* **21**, 347–359.
- Kunitomo H., Higuchi T., Iino Y. and Yamamoto M. 2000 A zinc-finger protein, Rst2p, regulates transcription of the fission yeast *ste11<sup>+</sup>* gene, which encodes a pivotal transcription factor for sexual development. *Mol. Biol. Cell* **11**, 3205–3217.
- Mata J. and Bahler J. 2006 Global roles of Ste11p, cell type, and pheromone in the control of gene expression during early sexual differentiation in fission yeast. *Proc. Natl. Acad. Sci. USA* **103**, 15517–15522.
- Newmeyer D. 1970 A suppressor of the heterokaryon incompatibility associated with mating type in *Neurospora crassa*. *Can. J. Genet. Cytol.* **12**, 914–926.
- Perkins D. D. 1997 Chromosome rearrangements in *Neurospora* and other filamentous fungi. *Adv. Genet.* **36**, 239–398.
- Perkins D. D., Radford A. and Sachs M. S. 2001 The *Neurospora* compendium: chromosomal loci. Academic Press, San Diego.
- Qin J., Kang W., Leung B. and McLeod M. 2003 Ste11p, a high-mobility-group box DNA-binding protein, undergoes pheromone- and nutrient-regulated nuclear-cytoplasmic shuttling. *Mol. Cell Biol.* **23**, 3253–3264.
- Rosa A. L., Haedo S. D., Temporini E. D., Borioli G. A. and Mautino M. R. 1997 Mapping chromosome landmarks in the centromere I region of *Neurospora crassa*. *Fung. Genet. Biol.* **21**, 315–322.
- Shiu P. K., Raju N. B., Zickler D. and Metzberg R. L. 2001 Meiotic silencing by unpaired DNA. *Cell* **107**, 905–916.
- Staben C., Jensen B., Singer M., Pollock J., Schechtman M., Kinsey J. and Selker E. 1989 Use of a bacterial Hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. *Fung. Genet. Newslett.* **36**, 79–81.
- Sugimoto A., Iino Y., Maeda T., Watanabe Y. and Yamamoto M. 1991 *Schizosaccharomyces pombe ste11<sup>+</sup>* encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. *Genes Dev.* **5**, 1990–1999.
- Vyas M., Ravindran C. and Kasbekar D. P. 2006 Chromosome segment duplications in *Neurospora crassa* and their effects on repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA. *Genetics* **172**, 1511–1519.

Received 7 July 2008, in revised form 22 September 2008; accepted 24 September 2008

Published on the Web: 3 April 2009