AVF REPORT

February 2002

I. Project Title:

Understanding the sexual life-cycle of *Eutypa lata*

II. Principal Investigators:

Dr Peter G Long

Institute of Natural Resources
Massey University
Palmerston North
New Zealand

Phone 0064 6 350 5920
Fax 0064 6 350 5679
E-mail P.G.Long@massey.ac.nz

Dr Rosie E Bradshaw

Institute of Molecular Biosciences
Massey University,
Palmerston North,
New Zealand.

0064 6 350 5515 ex. 2567
0064 6 350 5688
R.E.Bradshaw@massey.ac.nz

Address all correspondence to:
Dr Peter Long, Institute of Natural Resources, Massey University,
Private Bag 11222, Palmerston North, New Zealand

Massey Agriculture

Massey University

Table of Contents

	Page
III. Summary	3
IV. Summary of Major Research Accomplishments together with Objectives and Experiments Conducted to Meet Stated Objectives	4
Objective 1	**4**
Objective 2	11
Objective 3	12
V. Research Success Statement	12

Formatted: Bullets and Numbering

Summary

Objective 1 - To isolate the mating type gene from *E. lata*

Exhaustive attempts were made to clone fragments of *Eutypa lata* mating type genes (idiomorphs *MAT1-1* or *MAT1-2*) using degenerate PCR with primers designed to regions that are conserved in pyrenomycete fungi. In total more than 100 PCR experiments were performed (each with an average of 14 reactions, including controls) using various combinations of seven different primers specific for pyrenomycete *MAT1-1* and seven specific for pyrenomycete *MAT1-2*. Genomic DNA templates from a total of 34 independent *E. lata* isolates from 3 countries were used in the PCR reactions, along with DNA from *Neurospora crassa* as a positive control. PCR products within the expected size ranges were purified and cloned. More than 50 of these were sequenced but none showed sufficient (translated) sequence identity to pyrenomycete mating type genes to suggest that cloning of *MAT1-1* or *MAT1-2* fragments had been achieved.

Attempts were made to identify the presence of mating type genes in the genome of *E. lata* by hybridization. Southern blots were probed with gene fragments from *N. crassa* mating type genes, as well as with end-labelled oligonucleotides designed to highly conserved regions. Despite using low-stringency conditions, no hybridization was detected.

There is a possibility that *E. lata* does not contain orthologs of mating type genes commonly found in other pyrenomycetes. If *E. lata* has an alternative type of mating control system this will be very interesting indeed. In addition to continued attempts to clone *MAT1-1* and *MAT1-2* orthologs we suggest an increased focus on elucidation of methods to complete the sexual cycle of *E. lata* in axenic culture (which will allow us to identify mating types). In addition it may be prudent to search for orthologs of mating type genes from a wider range of ascomycetes and even from other fungal groups such as basidiomycetes that could have been obtained by *E. lata* through lateral gene transfer.

Objective 2 – Production of the sexual stage in culture

An experiment to produce the sexual stage in culture has been running for 6 months and will be completed in May 2002.

Objective 3 - To use the tools developed in objectives 1 and 2 to determine the distribution of mating types in the natural environment.

Work on this objective has not yet commenced since it is entirely dependant on identification of the MAT genes in Objective 1.

Summary of Major Research Accomplishments together with Objectives and Experiments Conducted to Meet Stated Objectives

Formatted: Bullets and Numbering

Objective 1

Background

The AVF final report dated 1 June 2001 listed several possibilities to explain why we had so far been unsuccessful in cloning a *MAT1-2* idiomorph from *Eutypa lata*:

1. The *E. lata* isolates tested may all have been of the opposite mating type (*MAT1-1*) and more isolates from different regions (and countries) need to be tested.

2. The *MAT1-2* idiomorph in *E. lata* may not be homologous (conserved) in the regions used for priming,

3. The mating type genes are unrecognisable by comparison with those of other ascomycetes.

4. *E. lata* is homothallic (and all perithecial isolates tested were *MAT1-1*)

5. *E. lata* has a different type of mating control system altogether, as found recently in some *Glomerella* species.

Although it is possible that *E. lata* does not have a conserved *MAT1-2* gene, several experiments were suggested to complete the current investigation:

a) **Test different *E. lata* isolates from overseas** for the presence of the *MAT1-2* idiomorph to compensate for the limited and geographically restricted sample size used so far. Use all sets of *MAT1-2* specific primers, optimised PCR parameters and *N. crassa* as a positive control.

b) **Test at least two more perithecia from New Zealand** for reproducible polymorphic differences between single-ascospore isolates. If polymorphism is found, test the isolates for amplification of a *MAT1-2* gene fragment as above.

c) **Continue analysis of clones obtained so far**. Perform nested PCR with the uncharacterised 640 bp SmHMG2/SmHMG3 gene product and internal sets of *MAT1-2* primers (NcHMG1 and SmHMG1). Clone and sequence the 640 bp product. Design specific primers for any putative *MAT1-2* gene fragments and use these to test for the presence or absence of these sequences in overseas and single-ascospore isolates using high stringency PCR.

d) Attempt to PCR-amplify a region of the *MAT1-1* idiomorph using existing isolates with primers designed to the α1 domain (Pöggeler 1999).

Experimental

Test different *E. lata* isolates from overseas

Overview:
In further attempts to isolate a mating type gene, DNA was obtained from overseas isolates of *Eutypa lata*: three of diverse origin from Australia and eight from a single perithecium in the USA. PCR amplification using a range of primer pairs designed to amplify fragments of pyrenomycete *MAT1-2* genes did not yield clear and distinct products of the expected size. Some weakly amplified products within the expected size range were cloned and sequenced but none showed sequence identity to known fungal mating type genes.

Materials and methods:
Genomic DNA from eleven *E. lata* isolates was obtained from the USA and Australia. Three sent by Richard Lardner (South Australia) and eight sent by Cheryl Blomquist (UC Davis):
Aus 1 - M280 (Victoria, late 1990s)
Aus 2 - N04 (Barossa Valley, June 2000)
Aus 3 - SS6 (Adelaide plains, 1981)
USA1 through 8 - VFR2V15 1-8 (all from a perithecium in California)

DNA from the new isolates was tested, alongside New Zealand isolates, for PCR amplification of a putative *MAT1-2* fragment. Primers and amplification conditions were those outlined in the June 2001 report, except that annealing temperatures varied from 47 - 51^{0}C.

Results:
Confirmation that these genomic DNA samples were of *E. lata* origin was achieved using *E. lata* -specific ITS primers in a diagnostic PCR assay. No clear single PCR product of the size expected for a *MAT1-2* HMG box (200-350 bp depending on intron size) was seen with any of the new DNA templates or with any of the sets of primers used. However some reactions produced faint PCR products within this size range in addition to other products. A selection of these were shotgun-cloned and sequenced. None were found with significant sequence identity to any fungal mating type genes, except in primer regions.

Selected sample of sequenced clones:

Date	PCR	Clone	primers*strain	insert (bp)	BLASTX (E value & match)		
13/9	H-4	4a	Sm3/Sm2	Aus1	400	5.1	ABC transporter
13/9	J-7	6a	Nc1/mix2	USA7	200	6.7	*toc* gene Drosophila
19/9	E-10	2b	Nc1/Pa2 Aus2	300	5.6	orf72 Podospora (mt)	
19/9	H-4	4b	Sm3/Sm2	Aus1	400	1.0	malate dehydrogenase
19/9	J-7	6c	Nc1/mix2	USA7	200	No sig. similarity found	
19/9	K-10	8c	Nc1/mix2	Aus2	300	1.2	bacteriochlorophyll

* Nc1 = NcHMG1, etc; mix2 = equimolar mix of NcHMG2, PaHMG2 & GfHMG2

These experiments were repeated using touch-down PCR, and with a new primer (PWHMG1; 5'-CCTCGYCCTCCYAATGCCTACAT-3') that was designed to the same region as NcHMG1 but with reduced degeneracy (4-fold instead of 256-fold). However no *MAT1-2* gene fragments were obtained using these approaches.

Test at least two more perithecia from New Zealand

In view of the lack of success in obtaining MAT gene fragments from isolates both within New Zealand and from Australia and the U.S.A., this approach was not considered promising at this stage and all efforts were put into other approaches.

Continue analysis of clones obtained so far.

Overview:

A 640 bp PCR product that was mentioned as a candidate *MAT1-2* idiomorph in the June 2001 report failed to amplify with nested primers. Furthermore the original PCR that gave rise to this fragment did not give consistent results when repeated.

Materials, methods & results:
On page 11 of the June 2001 report it was noted that a faint 640 bp product had been obtained using SmHMG3/SmHMG2 primers designed to an alternative region of the *MAT1-2* idiomorph. Nested PCR was performed using this 640 bp PCR product as a template with the primers SmHMG3 and SmHMG1, but the expected 331 bp nested product was not obtained. The intention was to clone and sequence the 640 bp fragment but attempts to re-amplify this product by repeating the original PCR from a range of *E. lata* isolates were not successful. The 640 bp fragment was therefore probably a non-specific amplification product rather than a genuine *MAT1-2* fragment.

Attempts to PCR-amplify a region of the *MAT1-1* idiomorph

Overview:
Two sets of primers were designed to the most conserved regions of the *MAT1-1* idiomorph. The first set was designed from alignments of a range of pyrenomycete *MAT1-1* genes. Exhaustive attempts were made to PCR amplify and clone a *MAT1-1* fragment but DNA sequence analysis revealed no convincing evidence of success, and a low level of contamination with the positive control *Neurospora crassa* DNA. A second set of primers was modeled on those of Pöggeler (1999) who used a more limited range of pyrenomycete *MAT1-1* genes on which to base the primer design. Fragments of the expected size were obtained with fresh reagents and freshly extracted DNA templates, although nested PCR with these fragments did not give products of the expected size. Fragments were cloned and have been sent for sequencing in case there are insertions or deletions that prevent annealing of the nesting primers.

MAT1-1 primer set A

Materials and methods
Four primers were designed to the α1 domain of the *MAT1-1* idiomorph. This region is not so conserved as the *MAT1-2* HMG domain and with some species Pöggeler (1999) had to use nested PCR to achieve success with amplification in this region.

Primer sequences 5' - 3' (see schematic below to show orientations of primers and appendix for alignment showing precise positions of these primers):

PJMAT1-1	TTY ATG CAN ATH YTN TGG CA
PJMAT1-2	CAY AAN GAR TGG RAY TTY ATG
PJMAT1-3	RCA IAR ICC RTK CAT IGG YTG
PJMAT1-4	IGC IAC IRR RTG IKG RTT

Results
Ten separate attempts were made at PCR amplification with an extensive range of *E. lata* genomic DNA templates (from NZ, USA and Australia) with each of the four possible combinations of primers and with nested PCR. *Neurospora crassa* was used as a positive control. Optimisation parameters included PCR annealing temperature (including the use of touch-down PCR), cycle number, and the concentrations of *Taq* polymerase, DNA template, $MgCl_2$, dNTPs and primers. In several reactions PCR products of the expected size were obtained for *E. lata*. These were purified by gel extraction, or with a PCR clean-up kit (Qiagen), cloned into the pGEM-T easy vector (Promega), sequenced and analysed using BLASTX and BLASTN programs at http://www.ncbi.nlm.nih.gov/. All sequences either gave no match to fungal mating type genes, or a perfect match to the *N. crassa MAT1-1* sequence,

suggesting a trace contamination from the positive control. No convincing evidence was obtained for an *E. lata* *MAT1-1* gene fragment.

Fresh genomic DNA was isolated in a separate location where *N. crassa* cultures are not held and PCR is currently being repeated with fresh reagents.

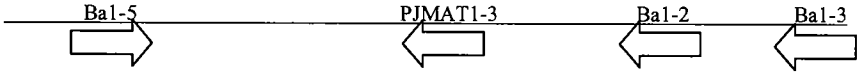
MAT1-1 primer set B

Materials and methods

Three primers were designed to alternative regions of the α1 domain of the *MAT1-1* idiomorph following the methods of Pöggeler (1999) except that Pöggeler's Ba1-2 primer was re-designed by removing a nucleotide corresponding to a degenerate 3rd position from the 5' end of the primer and by adding a conserved 1st codon position nucleotide to the 3' end. Pöggeler's primers are designed to the *Neurospora, Sordaria* and *Podospora* spp *MAT1-1* genes rather than to a broader range of pyrenomycete genes as for primer set A. PJMAT1-3 was also used for nesting with these primers.

Primer sequences 5' - 3' (see schematic below to show orientations of primers and appendix for alignment showing precise positions of these primers):

Ba1-5	AAGAAGAAGGTCAACGGCTTCATG
Ba1-3	CCAGAGCCATGTTGTAGGAATCATT
Ba1-2	TCGTAGCTAGGATCTGAAAGCTT



Expected sizes of PCR products (variable depending on introns):
Using outside primers Ba1-5 and Ba1-3: 740-800 bp,
Using nested primer Ba1-2 with Ba1-5: 440-500 bp
Using nested primer PJMAT1-3 with Ba1-5: 380-440 bp

Pöggeler, S. (1999) Phylogenetic relationships between mating-type sequences from homothallic and heterothallic ascomycetes. Current Genetics 36:222-231.

Results (NZ, USA and Australian isolates)

PCR was attempted using the exact conditions of Pöggeler (1999), which included a 50°C annealing step in the first round of PCR and 55°C annealing in the subsequent nested PCR. A large number of PCR products were obtained, including a distinct product within the expected size range (740 bp) with Ba1-3 and Ba1-5. This product was seen even more clearly when the initial PCR was carried out at the more stringent annealing temperature of 55°C. Furthermore, the products were seen in some isolates and not others, consistent with expectations if it was a MAT gene fragment.
However fragments of 740-800 bp were also seen when combinations of primers expected to give shorter products (Ba1-5 with Ba1-2 or PJMAT1-3) were used. Furthermore nested PCR with internal primers gave inconsistent results, with no clear evidence for nesting within the 740 bp fragment even when gel-purified prior to nesting.

Results (five NZ isolates from one perithecium)

Using genomic DNA freshly isolated from NZ perithecial isolates, isolate SS9 gave the most promising result with products of approximately the expected size in lanes 12, 13 and (faintly) 14 (Fig. 1). Isolate SS13 had products of about 750 bp in all three lanes while isolate SS3 had a product of 750 bp in lane 7 only – a lane where the expected size was only 380-440 bp. . These results were further investigated by using the reaction mix of lanes 12 and 15 as templates for a nested PCR.

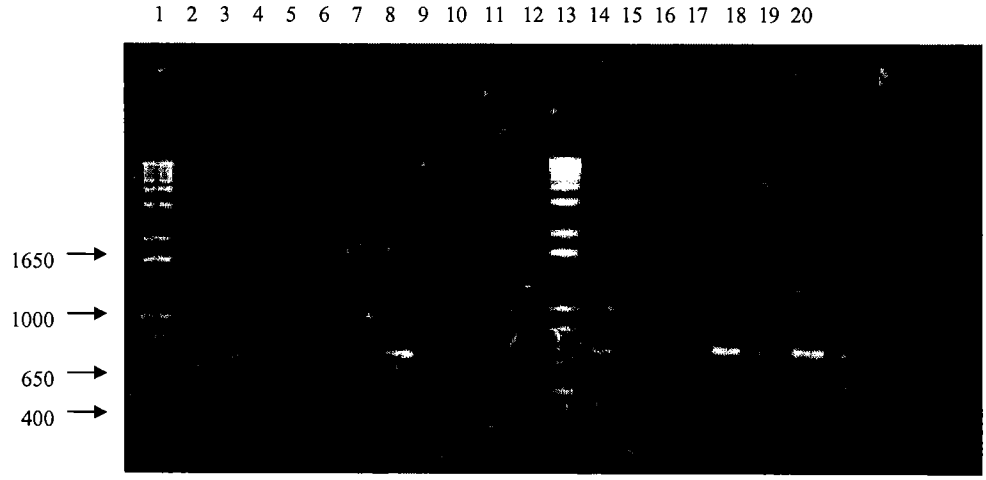


Fig. 1 Gel of PCR products from 5 NZ isolates of E. lata with 3 primer combinations.
Negative controls (lanes 2-4), SS1 (lanes 5-7), SS3 (lanes 8-10), SS9 (lanes 12-14), SS13 (lanes 15-17), SS14 (lanes 18-20) and 1 kb plus ladder (lanes 1 and 11; sizes in bp). Primers Ba1-5/Ba1-3 (lanes 2, 5, 8, 12, 15 and 18), Ba1-5/Ba1-2 (lanes 3, 6, 9, 13, 16 and 19) and Ba1-5/PJMAT1-3 (lanes 4, 7, 14, 17 and 20).

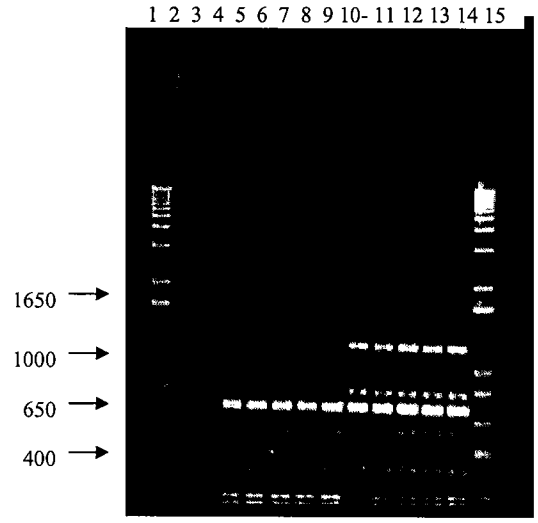


Fig. 2 Gel of nested PCR products from 2 NZ isolates of E. lata
Negative controls (lanes 2-3), SS9 (lanes 4-8), SS13 (lanes 9-13), and 1 kb plus ladder (lanes 1 and 14). Templates undiluted (lanes 4 and 9), 1:100 (lanes 5, 6, 10 and 11) and 1:1000 (lanes 7, 8, 12 and 13). Primers Ba1-5/Ba1-3 (lanes 4 and 18), Ba1-5/Ba1-2 (lanes 2, 5, 7, 10 and 12) and Ba1-5/PJMAT1-3 (lanes 3, 6, 8, 11 and 13).

The nested PCR gel had the same bands in all lanes of SS9 and two additional bands in SS13. To check that products from the original template were not interfering with the reaction or masking new products on the gel, new templates were prepared by cutting out the 750 bp product of lanes 4 and 9 and the 800 bp product of lane 9 and using them as templates for a new nested primer PCR (Fig. 3).

1 2 3 4 5 6 7 8 9 10 11 12 13

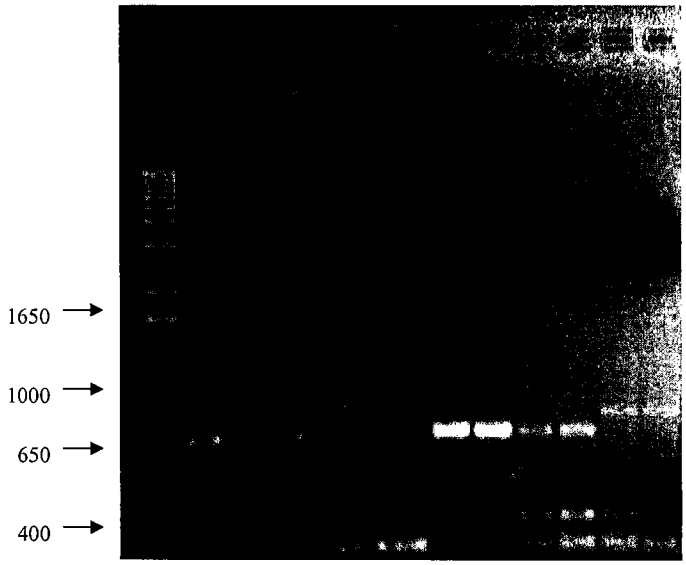


Fig. 3 Gel of nested PCR products obtained using gel-purified products of PCR shown in Fig. 2 as templates. SS9 750 bp product (lanes 2,3,8 and 9), SS13 750 bp product (lanes 4, 5, 10 and 11), SS13 800 bp product (lanes 6,7,12 and 13), and 1 kb plus ladder (lane 1). 13). Primers Ba1-5/Ba1-2 (lanes 2, 4, 6, 8, 10 and 12) and Ba1-5/PJMAT1-3 (lanes 3, 5, 7, 9, 11 and 13).

There were no products in the 450-550 bp range (expected if nesting had occurred) although the template was re-amplified in most lanes

Despite indications that the PCR products might not be from a *MAT1-1* gene, 740 bp products and several other possible (nested) products from the experiments outlined above were cloned and sent for DNA sequence analysis. None of them show sequence identity to fungal MAT gene sequences. Sequence analysis revealed that several of the products had been primed by Ba1-5 at both ends, indicating non-specific priming.

Hybridisation with Oligonucleotide probes.

Overview:
Due to difficulties in obtaining a *MAT1-1* or *MAT1-2* gene product from *E. lata* by PCR amplification, a Southern blot was attempted to check out the possibility of using an oligonucleotide hybridization probe to identify the idiomorphs.

Materials and methods:

Genomic DNA from five strains of *E. lata* (E11PDA2, EO1, SSI2, SSI4 and SSI9) was digested with *Eco*RI + *Bam*HI, separated by electrophoresis on a 0.8% agarose gel and blotted onto nylon membrane. Oligonucleotide probes were end-labelled with [γ-^{32}P] dATP (3000 Ci/mmol) and hybridized to the blots with 3xSSC hybridization buffer at 51^{0}C overnight. Replicate blots were hybridized: one with a mixture of the *MAT1-1* primers PJMAT1-2 and PJMAT1-3, the other with the *MAT1-2* primer PWHMG1.

Results:
N. crassa genomic DNAs from each of the two known mating types were successfully used as positive controls but there was no evidence of probe hybridization to *E. lata* DNA.

Objective 2 – to produce the sexual stage in culture

Overview:
The ability to produce the sexual stage in culture is a pre-requisite for many studies on genetic variability in the fungus. The sexual stage has not been produced in culture and without defined mating strains a large number of combinations must be superimposed on the factors to be investigated. For this reason, only one experiment has been set up to date. To increase the chances of success, all incubation periods have been set at longer periods than desirable on the basis that if the sexual stage is produced then incubation periods can be reduced to the minimum after further experimentation.

Materials and methods:

Six isolates (SS1, SS3, SS9, SS13, SS14 and SS18) from a single perithecium are being tested on three media (autoclaved old grapevine stems, young blackcurrant stems, and malt agar) with each replicate in an individual McCartney bottle. Each isolate was grown at 15°C in the dark for 8 weeks followed by 10°C in the dark for 8 weeks before attempted fertilization.
Fertilisation inocula were prepared by growing the isolates individually on a flask shaker at 20°C for 3-4 weeks, until fungal pellets were well grown. The fungal pellets were macerated and used to inoculate the established colonies either with macerate of the same isolate or with a mixture of the other isolates.
After inoculation, the cultures were incubated at 1°C in the dark for eight weeks and were transferred to a 15°C light/10°C dark regime where they will be held for at least 16 weeks to monitor possible production of the sexual stage. They are regularly inspected and sterile distilled water added when required to maintain moisture levels.

Results:

If the sexual stage is not produced within the next 16 weeks the method will not be considered successful.

Objective 3 – To use the tools developed in objectives 1 and 2 to determine the distribution of mating types in the natural environment.

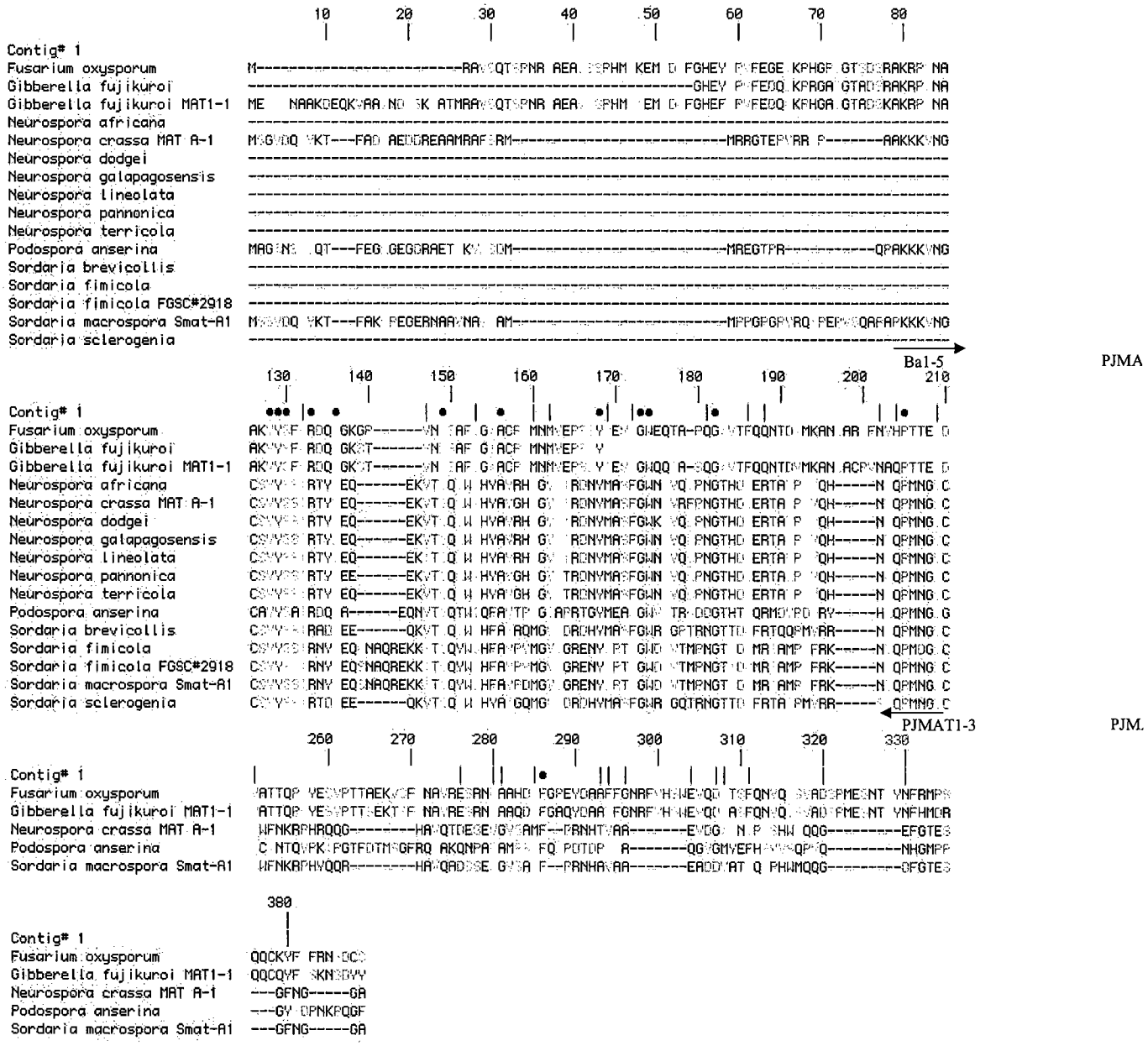
Work on this objective has not yet commenced since it is entirely dependant on identification of the MAT genes in Objective 1.

V. Research Success Statement

A start has been made on determining the basis for sexual reproduction and hence of variability in the fungal pathogen *Eutypa lata*. This will contribute to a better understanding of the basis of pathogenic variation and of the basis for selecting stable forms of resistance in breeding new varieties and of other approaches to managing this major, worldwide disease problem.

Reference:

Pöggeler , S. 1999 Phylogenetic relationshipsbetween mating type sequences from homothallic and heterothallic ascomycetes. Current Genetics. 36: 222-231


Ba1-5
PJMA
PJMAT1-3
PJM
Contig# 1
Fusarium oxysporum
Gibberella fujikuroi
Gibberella fujikuroi MAT1-1
Neurospora africana
Neurospora crassa MAT A-1
Neurospora dodgei
Neurospora galapagosensis
Neurospora lineolata
Neurospora pannonica
Neurospora terricola
Podospora anserina
Sordaria brevicollis
Sordaria fimicola
Sordaria fimicola FGSC#2918
Sordaria macrospora Smat-A1
Sordaria sclerogenia