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Project Title: DNA-based identification of needle pathogens

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EXECUTIVE SUMMARY

Commercially available DNA extraction kits are generally as reliable as a "homemade" lab-based method for extracting DNA from needle lesions infected with Dothistroma. However in experiments to determine the minimum number of lesions required for extraction the success was variable with all methods, although sufficient DNA for PCR amplification could be obtained from as few as one or two lesions. A "whole-genome amplification" method is being trialed which may improve success rates with DNA extraction from single lesions. If successful, this method will be especially useful for herbarium samples.

PCR primers have been developed that amplify a dothistromin biosynthetic gene. These NZ-developed primers mean we now have three sets of primers which can all be used to identify *D. septosporum*, but can be used for different purposes and with different levels of resolution (species-specific, mating-type specific, etc.). The dothistromin primers are currently being checked for specificity to ensure they do not detect other common fungi, and real-time PCR experiments are being planned.

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1. INTRODUCTION

The previous progress report showed validation of mating type primers, developed overseas, for species-specific, and mating-type specific, detection of *D. septosporum* and *D. pini*. A DNA extraction method developed in our laboratory was used to obtain "PCR-quality" DNA from Dothistroma lesions on infected needles. Initial results in extracting DNA from lesions stored on dried needles from a herbarium met with limited success.

The aims of the current part of this project have been to:

- Evaluate commercially available DNA extraction kits and to compare these alongside the new lab-developed method. The use of these kits would make the methods more transferable to other laboratories than an in-house DNA extraction method.
- Identify species-specific differences in DNA sequences based on dothistromin genes. These differences may turn out to be biologically relevant as they may influence virulence, and will provide a "made in New Zealand" species-specific assay for *Dothistroma* spp.

2. MATERIALS AND METHODS

Only materials and methods that are new since the March 2007 progress report are described here.

2.1 DNA extraction from needles using commercial kits

DNA was extracted from infected needles firstly using either a Dneasy Plant Mini Kit (Qiagen) or a ZymoResearch Plant/Seed DNA Kit (Ngaio Diagnostics). Subsequently a magnetic bead-based ChargeSwitch plant gDNA kit (Invitrogen) became available for trial. Extraction methods followed the manufacturer's instructions.

2.2 Comparison of dothistromin gene sequences from *D. septosporum* and *D. pini*

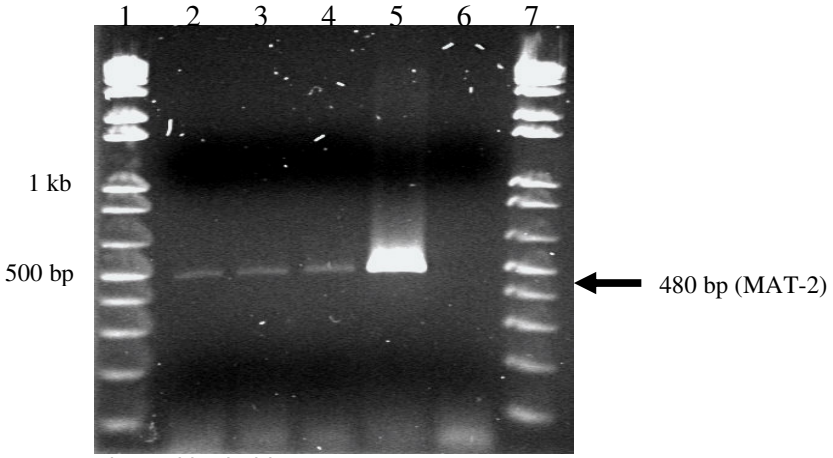
PCR was carried out using primers specific for dothistromin genes (*pksA*, *dotA*, *dotC* or *epoA*; reference). Each 25 µl PCR reaction contained 4 ng of genomic DNA template, 1x amplification buffer, 0.4 µM dNTPs, 1.5 mM $MgSO_4$, 0.4 µM of each primer and 0.5 U of Taq Polymerase (Invitrogen). The cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C (30 s), 51°C (30 s) and 72°C (60 s), followed by 72°C (5 min). The PCR products were visualised on an agarose gel, then purified and cloned in *E. coli* in a pCR II vector (Invitrogen) using standard methods and sequenced at the Allan Wilson Centre Genome Analysis Service, Massey University, Palmerston North. Note: The first part of this work was commenced by a Bio-Protection CoRE summer student.

3. RESULTS AND DISCUSSION

3.1 DNA extraction from needles using commercial kits

DNA was extracted from freshly-collected Dothistroma-infected needles (sample Waimahia 632/8) using two commercial plant DNA extraction kits and a lab-based method. The resulting DNA was subjected to PCR using the *Mat1-2* gene species specific primers. The lab-based extraction and *Mat1-2* PCR specifications were previously described in the March 2007 FHRC report. Figure 1 shows the PCR results for all three DNA extraction methods. PCR products of the expected size were obtained from DNA extracted using all three methods, using approximately 12 lesions per extraction. These results have since been validated in other experiments and suggest that extraction of DNA of sufficient quality for PCR amplification can be easily obtained from infected needles using a variety of methods. However the Qiagen kit is more reliable on the whole than the Zymo kit.

Figure 1 *Mat1-2* PCR of DNA extracted from infected needles



Lanes 1 and 7: 1 kb+ ladder
Lane 2: Zymo kit
Lane 3: QIAgen kit
Land 4: Lab-based method
Lane 5: Positive control (NZE7)
Lane 6: Negative control

To determine the sensitivity of these DNA extraction and PCR amplification methods, DNA extractions have been attempted using smaller numbers of Dothistroma-infected needle lesions. The March report showed extraction of PCR-amplifiable DNA from 4 lesions or more, using the lab-based method, but the results were inconsistent. Subsequent experiments using 1, 2, 4, or 8 lesions per DNA extraction, and commercial kits (Qiagen or Invitrogen), have likewise shown variable results, although PCR-amplifiable DNA has been extracted from samples containing only 1 or 2 lesions using ITS primers. There is likely to be inherent variability in the amount of intact DNA present in each lesion, which could account for the variable success.

3.2 PCR of fungi from herbarium specimens

Barnes et al (2004) attempted to isolate DNA for PCR amplification from two herbarium specimens using the Qiagen DNeasy Plant Mini Kit outlined above. They were unable to extract usable DNA from a 66 year old specimen but a 47 year old sample gave some PCR products using primers that target the multicopy ribosomal ITS region of the genome. Our aim is to be able to extract DNA that can be amplified using primers that have only a single target in the genome, so that genes for mating types, dothistromin biosynthesis, and other informative regions of the genome can be examined.

In this work, our lab-based method and the first two commercial plant DNA extraction kits (Zymos and Qiagen) as outlined above (section 2.1) were used to extract DNA from Forest Research Herbarium samples, NZFR(M) 1634 (collected 1972) and NZFR(M) 1580 (collected 1966). The DNA was then subject to PCR as previously described (results not shown). No band was obtained for either herbarium sample for either of the commercial kits for the single target copy *Mat1-2* primers, but PCR was successful with multicopy-target ribosomal ITS primers. However, with the lab-based method *Mat1-2* PCR products were obtained.

We are currently evaluating use of a whole-genome amplification method that should in theory allow us to obtain large amounts of genomic DNA from very small quantities of starting material. Amplified DNA was obtained in our first trials with this method. Adaptation of this technique for filamentous fungi was carried out in a Massey University laboratory (Foster and Monahan 2005).

3.3 Comparison of dothistromin gene sequences from *D. septosporum* and *D. pini* & development of primers.

PCR products were obtained for fragments of *D. pini pksA, dotC* and *epoA* dothistromin genes. The *D. pini* sequences showed 9.8%, 12.6% and 17.4% percentage divergence (sequence differences) from the corresponding *D. septosporum* genes respectively (total length of sequence aligned: 2.5 kb). This compares to 9.7% divergence between housekeeping genes of *D. pini* and *D. septosporum* (total length of sequence aligned: 2.2 kb) compared by Barnes *et al* (2004). The dothistromin *pksA* alignment is shown in the appendix (Fig. 2) and has been used as the basis for designing further sets of species-specific PCR primers. The primers are designed to be used in real-time PCR and to amplify both *D. septosporum* and *D. pini* DNA. A standard procedure in real-time PCR is to perform a "melting curve" analysis of the products. Using the primers we designed, the PCR products from these two species contain 8 nucleotide differences which affect their %GC content, and thus their melting temperatures. Hence the species will be able to be distinguished on the basis of their "melting curves". Since the ribosomal ITS regions of *D. pini* and *D. septosporum* only differ in 3 nucleotides the ITS region is not so suitable for development of a species-specific PCR assay.

Standard PCR has been performed to confirm that the new *pksA* gene primers are able to amplify Dothistroma DNA (results not shown), and validation of negative controls (with other fungal species) is in progress.

These NZ-developed primers mean we now have three sets of primers which can all be used to identify *D. septosporum* and *D. pini*, but can be used for different purposes:

- Ribosomal ITS primers (based on South African work): target multi-copy genes, useful where DNA targets are of poor quality or in small amounts. We have designed Dothistroma (genus-specific) primers based on these sequences).
- Mating type primers (Netherlands work): target a single-copy gene and are specific for both species and mating type. PCR using four primer sets would be required if species and mating type were unknown.
- Dothistromin (*pksA*) primers (New Zealand work): target a single-copy gene and will amplify both *D. septosporum* and *D. pini,* but these species can be distinguished by a simple melting curve procedure following real-time PCR.

4. FUTURE WORK

DNA extraction from fresh and herbarium needles

- Work will continue with the Qiagen and Invitrogen DNA extraction kits to optimise success rates with single lesion extractions from fresh and herbarium material.
- Work will continue with the whole genome amplification method, particularly with herbarium samples, for which material is very limited.

PCR amplification

- The new *pksA* PCR primers will be checked for lack of amplification of other fungal forest species.
- Real-time PCR will be carried out with the pksA, mating-type and ITS primers.
- The sensitivity of real-time PCR will be compared alongside conventional PCR.

ACKNOWLEDGEMENTS

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APPENDIX

Figure 1. **Partial alignment of the *pksA* gene sequences from *D. pini* (DP) and *D. septosporum* (DS). The pksA sequences** show 90.2% sequence identity over the full 1.3 kb sequenced (not all sequence is shown here). The positions and orientations of PCR primers are indicated by arrows. For real-time PCR product sizes of 150-200 bp are optimal. Several primers have been designed for each end and the best pair will eventually be selected. Note that in the regions amplified by these primers, nucleotide differences between the two species are mainly G or C in *D. pini* and A or T in *D. septosporum*, which means the PCR products will have different melting temperatures.