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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Nested polymerase chain reaction–based detection of *Dothistroma septosporum*, red band needle blight of pine, a tool in support of phytosanitary regimes

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Abstract

Red band needle blight is one of the most important foliar diseases of *Pinus* species and is of increasing international forest health and biosecurity concern. To provide a rapid identification technique for this pathogen in support of official control measures, a nested polymerase chain reaction–based diagnostic assay that employs species-specific primer sets has been developed. The assay is able to detect the presence of the pathogen direct from pine needles, irrespective of host species, to within 10 fg of target DNA, the equivalent of approximately 2–3 ascospores or hyphael cells.

Keywords: diagnostic nested PCR, *Dothistroma septosporum*, *Dothistroma pini*, red band needle blight, quarantine organism, forest biosecurity, forest health, risk management

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Dothistroma septosporum (Dorog.) M. Morelet *sensu stricto* (teleomorph: *Mycosphaerella pini* Rostr.) is the causal agent of red band needle blight of pine and has a recorded host range of over 70 different pine species (Ivory 1994; Barnes *et al*. 2004; Bednářová *et al*. 2006), including, under high infection pressure and favourable conditions, certain species of spruce, larch and Douglas fir (Watt *et al*. 2009). Red band needle blight has been particularly damaging to plantation forestry in southern hemisphere countries, where its impacts have been considerable. Gibson (1974) attributed the rapid intercontinental spread of *D. septosporum* to anthropogenic activities, principally the movement of live plants or contaminated seed stocks. However, although reported from over 60 different countries from Europe, Africa, Oceania and the Americas (CABI/EPPO 1990; Bednářová *et al*. 2006), it is widely considered that *D. septosporum* has not achieved its full potential geographic/global range (Watt *et al*. 2009).

In addition to a complicated taxonomic history, red band needle blight has been proposed as comprising two divergent lineages: *D. septosporum* (Dorog.) M. Morelet (lineage I), with a worldwide distribution, and *D. pini* Hulbary (no known teleomorph) (lineage II), currently only known from a limited host and geographic range within the United States mid-west (Barnes *et al*. 2004). As general host range, aetiology, morphology and disease symptoms are the same, including similar impacts, this work subsequently treated both lineages as a single taxonomic entity, i.e. *D. septosporum sensu stricto*, with regard to assay development in support of official control purposes.

Of increasing phytosanitary concern in Europe, where it has also been spreading, particularly north into the Baltic region (e.g. Estonia, Latvia, Lithuania and Finland (Bednářová *et al*. 2006; Drenkhan & Hanso 2009; Markovskaja & Treigienė 2009), *D. septosporum* is recognized as a quarantine organism under Annex III, Section II of Commission Directive 29/2000/EC, under the teleomorphic synonym *Scirrhia pini* Funk & Parker. Here, the Directive specifically recognizes *D. septosporum* as a pathogen known to occur in the region and relevant for the entire community, thus subject to official controls with respect to plants of *Pinus* L. intended for planting, other than seeds. *D. septosporum* is further identified as a quarantine organism in other legal instruments from across the world, including Australia, where measures against interstate movement also apply.

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Early detection and identification is a prerequisite to successful/enhanced management strategies of plant health risks. In the event of latent infections, particularly in the absence of condiomata, conventional morphological diagnosis of *D. septosporum* is slow and difficult, taking up to several weeks. With respect to the growing international and intercontinental quarantine importance attached to this forestry disease, a rapid, technically simplistic and easily applicable nested PCR-based diagnostic assay for the direct and sensitive detection of *D. septosporum* from pine needles was developed in further support of risk management approaches and possible incursion response needs.

Diagnostic PCR primers detailed in this study were developed from areas of the rRNA internal transcribed spacer (ITS) regions exhibiting interspecific sequence divergence, but intraspecific conservation, revealed through multiple sequence comparisons between both *D. septosporum sensu stricto*, and most closely related species. These species were further selected based on known pathological or ecological importance within forest ecosystems, and species revealed through BLAST searches using ClustalW (Thompson *et al.* 1994). A total of 67 *D. septosporum sensu stricto* ITS sequences (Data deposited at Dryad: http://dx.doi.org/10.5061/dryad.8296), representing both phylogenetic lineages as proposed by Barnes *et al.* (2004), either generated in this study (employing universal ITS primers (White *et al.* 1990), or accessed via public databases, were used in the identification and further evaluation of primers.

Two sets of nested species-specific primers were developed: Mpini1 (forward 5′-GGATCATTACTGAGTGAGGG-3′) and Mpini4 (reverse 5′-CTCTTCAGCGAAATATATGC-3′) (first round/outer), and Mpini2 (forward 5′-GCCGTTTCGGCGACGGCGCC-3′) and Mpini3 (reverse 5′-CTTTAAGGCGCGCGGAACCG-3′) (second round/nested), for *D. septosporum sensu stricto*, using the GeneFisher, PRIMER (version 0.5; Whitehead Institute) and PrimerSelect (DNAStar, Madison, WI, USA) software programs, following the general primer design concepts as outlined by Dieffenbach *et al.* (1995). As a convention, primers were named with respect to their teleomorphic nomenclature. From the sequence information, the predicted diagnostic nested amplification size product was 288 bp. Amplification conditions for first- and second-round (nested) PCR were as follows: in 50-μL volume comprising the following components: 100 μM each of dNTP, 0.5 units *Taq* DNA polymerase, 10× PCR reaction buffer, (200 mM Tris–HCL (pH 8.4), 500 mM KCL and 1.5 mM $MgCl_2$), 0.1 mM of each forward and reverse primer and either 5 ng of fungal genomic DNA (at a standard concentration of 0.5 ng/μL), for first-round nested PCR, or 5 μL of unquantified total freeze/thawed polyvinylpolypyrrolidone-purified (PVPP; Sigma) DNA elutes prepared direct from needle material. The cycling profile comprised an initial denaturation (3 min at 94 °C) followed by 30 cycles of 1 min at 94 °C, 1 min at 62 °C and 1 min at 72 °C with a final postextension of 72 °C at 10 min. A 50^{-1} dilution of first-round product served as template in second-round (nested) amplification (conditions and cycling parameters as described earlier). All experiments were conducted with aerosol-resistant tips in a designated PCR laminar flow cabinet. Appropriate controls were routinely included in each step of all experiments. Amplification products were assessed by electrophoresis on 1.5% agarose gels, with a positive result indicated by the presence of the predicted 288 bp band.

To assess the specificity of both primer sets, a total of 54 isolate accessions of *D. septosporum sensu stricto* (either as culture, herbarium accession or DNA extract material), of diverse geographic and host origins, and a range of other closely related species revealed either through BLAST searches or of known pathological or ecological association within pine [e.g. *Lecanosticta acicola* (teleomorph: *Mycosphaerella dearnessii*), *M. gibsoni*, *M. confusa*, *M. ellispoidae*, *Cyclaneusma minus*, *Cladosporium fulvum*] and a range of other fungal tree/pinus-infecting pathogens, including those associated with pine (e.g. *Fusarium circinatum*, *Neonectria galligena*), and healthy pine needle (*P. radiata*, *P. pinaster* and *P. sylvestris*) DNA extracts [e.g. see Fig. 1, row (a), lanes 14–18] were tested with the nested primer sets. No cross-reactivity with either primer set was observed with any of the empirically tested material.

The sensitivity of the nested PCR assay and the ability of the nested PCR approach to detect *D. septosporum sensu stricto* within naturally infected pine needle tissue were assessed. The lower limit of detection of the nested assay was empirically determined against serial dilutions of *D. septosporum* DNA, revealing a minimum detection level of 10 fg/μL. No potential masking effect of *Pinus* DNA on assay sensitivity was observed through the addition of 10 ng/μL pine DNA to each dilution concentration. This approximates to an ascospore, or hyphael cell with two to three nuclei assuming a genome size of 10–100 Mbp (currently, the genome size of *D. septosporum* is estimated at 30.21 Mbp (http://genome.jgi-psf.org/Dotse1/Dotse1.info.html). Further, no cross-reactivity with *P. radiata* DNA (nor with *P. pinaster* or *P. sylvestris* DNA in other tests) was observed. Both specificity and sensitivity results were 100% reproducible over multiple replicates.

The assay was able to detect the presence of *D. septosporum sensu stricto* directly from the total DNA extracts of infected needle material representing seven different host species of wide geographic provenance (eight global locations, representing three continents) (Table 1, Fig. 1, row (c)). Typically, DNA was extracted from varying amounts of needle material according to the method

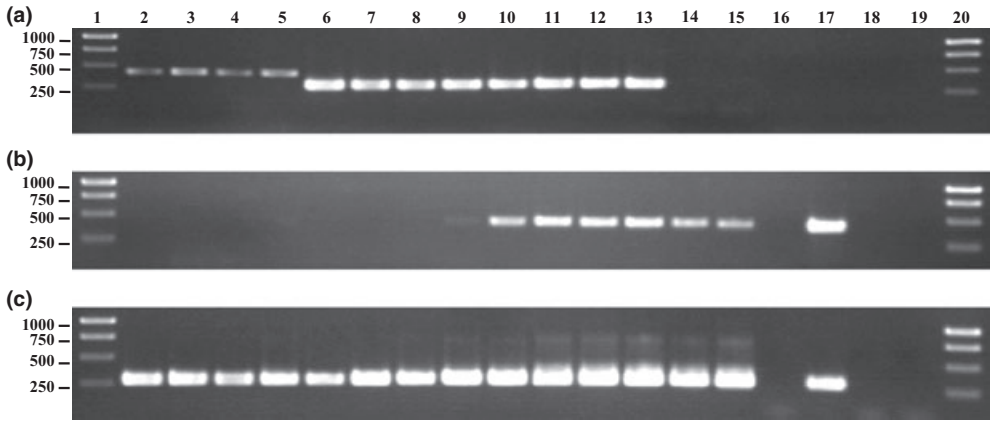

Fig. 1 Row (a) Species specificity of the *Dothistroma septosporum sensu stricto* assay. Lanes 2–5 (Canada, Austria, USA and New Zealand isolates, respectively), first-round amplification *D. septosporum* DNA with Mpini1 & 4 (419-bp product), 6–9 (same isolates as lanes 2–5), with Mpini2 & 3 (288-bp product), 10–13, nested PCR of first-round products (Victoria, New South Wales, Australian Capital Territory and Queensland isolates, respectively), 14–18, nested PCR against *Lecanosticta acicola*, *F. circinatum*, *Cyclaneusma minus*, *M. gibsoni* and *Pinus* sp. DNA, respectively. Rows (b) and (c) Application of the *D. septosporum sensu stricto* assay to infected needle material (*in planta* evaluation). Row (b) first-round amplification with Mpini1 & 4, row (c) second-round/nested PCR of the same needle material with nested primers Mpini2 & 3. Lanes 2–4, New South Wales, 5–6, Victoria, 7–8, Queensland, and 9, Australian Capital Territory (all dried herbarium material, see Table 1), lanes 10, Canada, 11–12, Austria, 13–14, USA, and 15, New Zealand (all fresh needle material, see Table 1). Lanes 16 and 19, negative controls, 17, *D. septosprum* DNA (positive control), 18, *M. dearnessii* DNA. Lanes 1 and 20, 1-kb ladder (Promega).

described by Langrell (2005). Alternatively, a rapid procedure where DNA was eluted directly into extraction buffer from 4–6, 1- to 1.5-cm segments of needle material via a repeated freeze/thaw cycle between *c*. −196 °C and *c*. 100 °C was used. In both approaches, DNA extracts, or elutes, were subject to PVPP chromatography purification prior to PCR. In all cases, the nested PCR diagnostic band size of 288 bp was observed, confirming the presence of *D. septosporum* [e.g. see Fig. 1, row (c)]. The application of the assay, including preparation of template, is fast, with a documented result attainable in approximately 4 h. With respect to application, the assay technology has already been transferred to at least one other forest research laboratory in support of forest health research and controls.

Red band needle blight is a pathogen of major global economic and ecological concern and is extending beyond its known geographic range with increasing rapidity where natural and plantation forests from large parts of the world are at risk of incursion and overall threat of invasion (with negative consequences). However, despite its importance to global pine forestry plantations worldwide, red needle band blight has received relatively limited molecular diagnostic attention. Pehl *et al*. (2004) have used a PCR-based ITS-RFLP technique to differentiate *M. pini* (the teleomorph of *D. septosprum sensu stricto*) from *M. dearnessii* (and forms the basis of the EPPO standards PM 7/47 and PM 7/46 for *M. pini* and *M. dearnessii*, respectively (Anonymous 2005a,b).

Table 1 Sample size and geographic provenance of fresh and dried herbarium needle material used to evaluate *in planta* detection capability of the *Dothistroma septosporum sensu stricto* nested PCR assay

Sampling locality	D*	V†	Mpini‡
Canada§	1	1	+
Austria¶	6	10	+
USA	1	2	+
New Zealand**	7	7	+
Victoria††	1	22	+
New South Wales††,‡‡	8	20	+
Australian Capital Territory††,§§	1	1	+
Queensland††	1	8	+

*D = number of sampling sites per location (i.e. country, state or territory).

†V = total number of individual samples used for evaluation per location (i.e. country, state or territory).

‡Nested PCR amplification with *D. septorsporum sensu stricto* specific primer sets Mpini1 and Mpini4 and Mpini2 & Mpini3. +, indicates positive diagnostic nested PCR signal of predicted size of 288 bp.

§*P. contorta* var. *latifolia*.

¶*P. mugo*, *P. nigra*, *P. sylvestris* (seven of the 10 individual samples were dried herbarium material).

**P. radiata*, *P. ponderosa*, *P. torreyana* (two of the seven individual samples were dried herbarium material).

††All *P. radiata*.

‡‡Eight of the 20 individual samples were dried herbarium material.

§§Single dried herbarium sample.

Similarly, Barnes *et al.* (2004) provide a similar PCR-based ITS-RFLP assay to distinguish between lineage I and lineage II (*D. pini* and *D. septosporum*) based on a distinguishing *Alu*I restriction site exclusive to the ITS2 region in *D. pini* (yielding PCR restriction fragments of ~170 and 350 bp). However, both the EPPO standards and Barnes *et al*. (2004) rely on isolation of the fungus in the first instance. More recently, Loos *et al.* (2010) reported the development of a quantitative (q) PCR test for both *D. septosporum* lineages targeting the *β-tub2* and *EFI-α* genes as target loci. Although a solid approach, qPCR is not as yet a ubiquitous and accessible technological platform as conventional PCR in all research and official national plant protection control laboratories, particularly in developing and newly emerging states.

Although this work recognized *D. septosporum sensu stricto*, as a single entity, as the nested PCR amplicon of lineage II contains an *Alu*I restriction polymorphism, as identified by Barnes *et al*. (2004), this could be exploited as an extension of the nested diagnostic PCR to differentiate the intraspecific lineages as proposed by them (where lineage II amplicons exclusively yield a distinctive *Alu*I RFLP pattern of 251 and 37 bp). The addition of the proposed detection assay to the limited spectrum of diagnostic tools will assist in the enhanced management of such forest health and sustainability risks, particularly with respect to early incursion responses, when the success of eradication efforts is dependent, and in the improved ecological and epidemiological understanding of red band needle blight disease.

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References

Anonymous (2005a) *Mycosphaerella pini*. *EPPO Bulletin*, **35**, 303–306.

Anonymous (2005b) *Mycosphaerella dearnessii*. *EPPO Bulletin*, **35**, 299–302.

Barnes I, Crous PW, Wingfield BD, Wingfield MJ (2004) Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*. *Studies in Mycology*, **50**, 551–565.

Bednářová M, Palovčíková D, Jankovský L (2006) The host spectrum of Dothistroma needle blight *Mycosphaerella pini* E. Rostrup – new hosts of Dothistroma needle blight observed in the Czech Republic. *Journal of Forest Science*, **52**, 30–36.

CABI/EPPO (1990) Data sheets on Quarantine Pests. *Mycosphaerella dearnessii* and *Mycosphaerella pini*. Prepared by CABI and EPPO for the EU under Contract 90/399003.

Commission Directive 29/2000/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. O.L.J 169, 10.7.2000:1.

Dieffenbach CW, Lowe TMJ, Dveksler GS (1995) General concepts for PCR primer design. In: *PCR Primer: A Laboratory Manual* (eds Dieffenbach CW & Dveksler GS), pp. 133–142. Cold Spring Harbor Laboratory Press, New York, NY.

Drenkhan R, Hanso M (2009) Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. *Forestry Studies*, **51**, 49–64.

Gibson IAS (1974) Impact and control of Dothistroma blights of pines. *European Journal of Forest Pathology*, **4**, 89–100.

Ivory MH (1994) Records of foliage pathogens of *Pinus* species in tropical countries. *Plant Pathology*, **43**, 511–518.

Langrell SRH (2005) Development of a nested PCR detection procedure for *Nectria fuckeliana* direct from Norway spruce bark extracts. *FEMS Microbiology Letters*, **242**, 185–193.

Loos R, Fabre B, Saurat C, Fourrier C, Frey P, Marçais B (2010) Development, comparison, and validation of real-time and conventional PCR tools for the detection of the fungal pathogens causing brown spot and red band needle blights of pine. *Phytopathology*, **100**, 105–114.

Markovskaja S, Treigienė A (2009) New data on invasive pathogenic fungus *Dothistroma septosporum* in Lithuania. *Botanica Lithuanica*, **15**, 41–45.

Pehl L, Burgermeister W, Wulf A (2004) [Mycosphaerella needle blights of pine – identification by ITS-RFLP patterns.]. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, **56**, 239–244.

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal-W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**: 4673–4680.

Watt MS, Kriticos DJ, Alcaraz S, Brown AV, Leriche A (2009) The hosts and potential geographic range of Dothistroma needle blight. *Forest Ecology and Management*, **257**, 1505–1519.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A guide to Methods and Applications* (eds Innes MA, Gelfand DH, Sninsky JJ & White TJ), pp. 315–322. Academic Press, San Diego, USA.