DOTHISTROMA PINI GENETIC DIVERSITY IS LOW IN NEW ZEALAND

P. HIRST,

Institute of Molecular BioSciences, Massey University,
Private Bag 11222, Palmerston North, New Zealand

T. E. RICHARDSON,

New Zealand Forest Research Institute,
Private Bag 3020, Rotorua, New Zealand

S. D. CARSON,

Fletcher Challenge Forests Ltd,
State Highway 30, Te Teko, RD 2, Whakatane, New Zealand

and R.E. BRADSHAW*

Institute of Molecular BioSciences, Massey University,
Private Bag 11222, Palmerston North, New Zealand

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ABSTRACT

The risk of severe Dothistroma needle blight in *Pinus radiata* D. Don forests depends on many factors, including the genetic diversity of the pathogen population, and so molecular methods were used to estimate the genetic diversity of the population of *Dothistroma pini* Hulbary in New Zealand. Samples of *D. pini* were collected from infected *P. radiata* trees using two sampling regimes. One involved collections from three forests, using a hierarchy of populations approach. The other involved collections from a Forest Research Institute field trial for Dothistroma resistance in which each tree was of known parentage. In addition to these, four *D. pini* samples isolated in New Zealand during the 1960s, and DNA from a Central American strain of the teleomorph (sexual form) *Mycosphaerella pini* E. Rostrup apud Monk, were analysed. Using the PCR-based techniques of RAPD (Random Amplification of Polymorphic DNA) and RAMS (Random Amplified Microsatellites), no genetic diversity was detected within the New Zealand samples. These results suggest that a single strain of *D. pini* was introduced into New Zealand and that this spread throughout the country. The New Zealand strain of *D. pini* is easily distinguishable from the Central American teleomorph, using both RAPD and RAMS techniques. The current study does not specifically address virulence levels, but this work implies that although the current New Zealand strain of *D. pini* is unlikely to overcome the current level of *P. radiata* resistance, the introduction of an overseas strain into New Zealand could present a threat to forest health.

* Corresponding author

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INTRODUCTION

With the increasing trend towards clonal forestry in New Zealand it is important to evaluate the risks that increased genetic uniformity may bring. A clonal forest may not contain the diversity of mechanisms required to maintain resistance to all races of a pathogen. Moreover, pathogens which are not causing significant losses at present may acquire increased virulence by mutation or recombination, or by expansion of the gene pool with the immigration of isolates from overseas (Burdon 1993). A knowledge of both the mode of reproduction and the genetic diversity of pathogen populations is important for evaluating disease risks (Carson & Carson 1991a).

Dothistroma needle blight (caused by the fungus *Dothistroma pini*) is one of the most significant foliage diseases in New Zealand's *Pinus radiata* forests. Needle loss is kept in check by regular aerial applications of copper-based fungicides, but the overall cost of this disease is considerable (Dick 1989). *Pinus radiata* seedlots with increased Dothistroma resistance have been developed (Carson 1989) but although mean infection levels in such stands are reduced by >12%, this is generally at the expense of growth and form.

Dothistroma needle blight was first seen in New Zealand in 1962 (Gilmour 1967). The teleomorph (*Mycosphaerella pini*, syn. *Scirrhia pini* Funk and Parker) has not been observed in New Zealand (M. Dick, New Zealand Forest Research Institute, pers. comm.). The absence of the teleomorphic stage from New Zealand has important implications for disease spread since, apart from reassorting genetic traits, the ascospores of the teleomorph are forcibly ejected and wind-dispersed whereas the conidia of the anamorph are distributed through a water-splash mechanism. It is not known whether the lack of a sexual cycle in New Zealand *D. pini* is due to genetic or environmental influences, or whether the fungus is heterothallic and only one mating type is present.

The main aim of this work was to determine the level of genetic variability in the New Zealand *D. pini* population. Two major sampling strategies were used. Firstly, to determine whether there is a correlation between pathogen genotype and host resistance, *D. pini* samples were collected from a "clones within families" field trial of *P. radiata* progeny from crosses made between individuals with high resistance to Dothistroma needle blight and those with low resistance. Secondly, to get an indication of whether pathogen genotype varies with geographical location, samples were taken from *P. radiata* growing in different New Zealand forests. In addition, isolates were available from collections made in the 1960s, which enabled an assessment of whether the New Zealand *D. pini* gene pool has changed over time. DNA from a Central American isolate of the teleomorph *M. pini* served as a control to test that the DNA-based methods had sufficient resolution to discriminate between strains within the species.

The PCR-based methods of RAPD (Williams *et al.* 1990) and RAMS (Hantula *et al.* 1996) DNA profiling were used to assess genetic variability in *D. pini*. These methods have been used to identify intraspecific variation in many plant pathogenic fungi, including forest pathogens (Wang 1997) and to distinguish between fungal pathotypes (Jungehulsing &

Tudzynski 1997; Nicholson & Rezanoor 1994). The New Zealand isolates showed no evidence of genetic diversity using these methods. This suggests that a single strain of *D. pini* was introduced into New Zealand which, if heterothallic, has been unable to reproduce sexually due to the absence of a mating partner. A Central American strain of the sexual form, *M. pini*, was shown by RAPD and RAMS analysis to be genetically distinct from the New Zealand *D. pini*.

MATERIALS AND METHODS

Collection of *D. pini* Samples

Field trial samples

Dothistroma pini was sampled from 5-yr-old trees in a field trial in which progeny from controlled crosses exhibited a wide range of Dothistroma resistance. The "clones within families" *P. radiata* field trial (Cpt 324 of Kaingaroa Forest) had 10 offspring from each of 36 crosses, derived from four sets of three-parent factorial crosses. From each of the 360 offspring, six genetically identical cuttings (ramets) were taken and planted in randomised blocks in 1990. Information about current disease levels (percentage of crown infected) was available for each offspring, determined as outlined by Carson (1989). The aim was to obtain *D. pini* samples from the two individual progeny with the most extreme (highest and lowest) Dothistroma resistance from at least four families, with samples from at least four ramets per individual and two samples per ramet.

National samples

For the national study, several forests were chosen on the basis of similar, severe, *D. pini* infection levels: Kinleith and Kaingaroa Forests (both in the mid North Island), Golden Downs (Nelson, South Island), and Nemona, Mokihinui, Victoria, and Paparoa Forests (West Coast of the South Island). All trees were less than 15 years old at the time of sampling. Sampling was carried out with a completely nested design, to enable any observed pathogen variation to be partitioned into "between forests", "between sites within a forest", and "between trees within a site". The aim was to obtain samples from at least one North Island and one South Island forest, with at least two sites per forest (5–50 km apart), two trees per site (at least 10 m apart), and two samples per tree. All trees sampled from Kinleith and Kaingaroa Forests were 850 Gwavas seedlot GF14 (open-pollinated seed orchard 3/3/87/01). This seedlot had not been planted in the South Island, hence South Island samples were selected on the basis of infection levels rather than host genotype.

Needles infected with *D. pini* were collected from the outer foliage of the tree at a height as close to 2 m from the ground as possible. Approximately 100 infected needles were collected for each sample. Infected needles were placed in 50-ml Falcon tubes and stored at –20°C until required for culture isolation.

Four additional purified isolates of *D. pini,* obtained from New Zealand forests in the 1960s, were supplied by Dr P. Gadgil of New Zealand Forest Research Institute. DNA from *M. pini* isolated from *Pinus tecumumannii* Schwerdtfeger in Guatemala, IMI 281626 (Evans 1984), was also analysed (culture grown and DNA extracted by Dr R. Bradshaw in Britain). All *D. pini* isolates used in the analysis are listed in Table 1.

TABLE 1A–Origins of *Dothistroma pini* and *Mycosphaerella pini* isolates: collections made from *Pinus radiata* field trial (collected in 1995 from Cpt 324 of Kaingaroa Forest)

Family (*Pinus radiata* cross)	Isolations from most dothistroma-resistant *P. radiata* progeny from crosses				Isolations from most dothistroma-sensitive *P. radiata* progeny from crosses			
Mean/Range of progeny DR*	*D. pini* isolate†	Host (progeny #)	Ramet	Branch	*D. pini* isolate†	Host (progeny #)	Ramet	Branch
Cross 1	DP 101	490	3	1	DP 138	494	2	2
DR Mean 17					DP 139	494	3	1
Range 20					DP 141	494	3	2
Cross 2	DP 102	327	2	1	DP 145	320	1	2
DR Mean 23	DP 103,104	327	4	2	DP 146,147	320	2	2
Range 24	DP 105	327	5	2	DP 148	320	3	2
	DP 106	327	6	1	DP 149	320	4	2
					DP 150	320	6	1
Cross 3	DP 107	175	1	1	DP 151,152	180	1	1
DR Mean 25	DP 111	175	1	2	DP 154	180	2	1
Range 28	DP 113,114	175	2	1	DP 155	180	3	1
	DP 115	175	3	1	DP 156	180	3	2
	DP 118	175	4	1	DP 157,158	180	5	2
	DP 119	175	5	1	DP 161	180	6	2
	DP 120	175	5	2				
Cross 4	DP 126	257	1	1	DP 163	259	2	1
DR Mean 30	DP 127	257	2	1	DP 164	259	2	2
Range 20	DP 128	257	2	2	DP 166	259	3	1
	DP 129	257	3	1	DP 168	259	3	2
	DP 130	257	4	2	DP 170	259	4	1
	DP 131	257	6	2	DP 172	259	4	2
					DP 173	259	5	1
					DP 174	259	5	2

* Dothistroma resistance expressed as percentage infection (mean and range) of all progeny in family.
† Where two isolates are listed together, they originate from different spores in the same fruiting body.

Isolation and Growth of *D. pini*

Dothistroma pini was isolated by incubating sections of the infected needles in a damp-chamber, picking the conidia off, placing them in a drop of water, and streaking them on to a plate of Dothistroma medium (5% (w/v) Oxoid malt extract, 2.3% (w/v) Oxoid nutrient agar). After 10–14 days' incubation at 20°C, *D. pini* colonies were identified by visual analysis of spore morphology and dothistromin toxin secretion. To improve sporulation, cultures were grown on Dothistroma sporulation medium (2% (w/v) Oxoid malt extract, 0.5% (w/v) Oxoid yeast extract, 1.5% (w/v) agar) at 20°C for 10 days. All cultures were purified by growing from single colonies obtained using a standard spore suspension streaking technique (to obtain colonies derived from a single spore or cluster of spores) and were stored on agar plates at 4°C and as mycelium blocks at –80°C.

TABLE 1B–Origins of *Dothistroma pini* and *Mycosphaerella pini* isolates: National study of *D. pini* (all collected 1995)

D. pini isolate	Forest site (Cpt)	Progeny #	Tree (block)	Branch
DP 177	Kaingaroa 1 (324)	601	1 (19F)	1
DP 178	Kaingaroa 1 (324)	601	1 (19F)	2
DP 179	Kaingaroa 1 (324)	601	2 (19I)	1
DP 180	Kaingaroa 1 (324)	601	2 (19I)	2
DP 181, 182	Kaingaroa 2 (1276)	X914	1 (1C)	1
DP 183	Kaingaroa 2 (1276)	X914	2 (2C)	1
DP 184	Kaingaroa 2 (1276)	X914	2 (2C)	2
DP 185	Kaingaroa 3 (1286)	X914	1 (7B)	1
DP 186	Kaingaroa 3 (1286)	X914	1 (7B)	2
DP 187	Kaingaroa 3 (1286)	X914	2 (5A)	2
DP 188	Kaingaroa 3 (1286)	X914	2 (5A)	3
DP 303, 304	Kinleith 1 (D6257)	Rep. 2	1	1
DP 305	Kinleith 1 (D6257)	Rep. 2	1	2
DP 306	Kinleith 1 (D6257)	Rep. 2	2	1
DP 307	Kinleith 1 (D6257)	Rep. 2	2	2
DP 401	Gn. Downs 1 (Pascoe)	Rep. 1	2	2
DP 402	Gn. Downs 2 (925)	Rep. 1	2	2
DP 403,404	Gn. Downs 3 (TeHepe)	Rep. 1	1	1

TABLE 1C–Origins of *Dothistroma pini* and *Mycosphaerella pini* isolates: *M. pini* and 1960s *D. pini*

D. pini/M. pini isolate	Forest	Source/ Alternative No.	Year of isolation
MP 001	Guatemala	IMI 281626	1983
DP003	Karioi, NZ	NZFRI/16E	1965
DP005	FRI nursery	NZFRI/16J	1969
DP100	Kaingaroa*	NZFRI/16G	1967
DP301	Kinleith	NZFRI/16B	1964

* *Pseudotsuga menziesii* host

DNA Extraction and PCR Amplification

To grow mycelium for genomic DNA extraction, homogenised mycelium or spores were spread over cellophane discs in a plate containing Dothistroma medium. After 7 days at 20°C, mycelium was harvested by scraping it off the cellophane into a sterile tube; it was then freeze-dried overnight. DNA was extracted using the FastDNA Kit H (Bio101). To minimise any effects of batch differences between kits, samples were randomised prior to DNA extraction. DNA quantitation was performed using a GeneQuant spectrophotometer.

For PCR amplification, genomic DNA was freshly diluted to 3 ng/μl. A Beckman Biomek 2000 Laboratory Automation Station was programmed to set up the reactions, with each 25-μl reaction containing a final concentration of 1 × Taq PCR buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM primer, along with 0.8 units *Taq* polymerase (Boehringer Mannheim) and 15 ng template DNA. Primers were random 10-mer

oligonucleotides (Operon Technologies, Alameda, CA, USA) for RAPD (Williams *et al.* 1990), or microsatellite/M13 primers (kindly supplied by Alan Goldstein) for RAMS (Hantula *et al.* 1996; Vassart *et al.* 1987) as shown in Table 2. Amplification was performed in 96 well polycarbonate plates with a paraffin oil overlay in a Techne PHC-3 thermocycler. For RAPD-PCR the cycling conditions were: an initial cycle of 94°C for 180 s, 37°C for 60 s, 72°C for 120 s, followed by 39 cycles of 95°C for 60 s, 37°C for 60 s, 72°C for 90 s, and then 72°C for 8 min. For RAMS-PCR the cycling conditions were: 93°C for 120 s, followed by 40 cycles of 93°C for 20 s, 50°C for 60 s, 72°C for 20 s, and then 72°C for 6 min. Amplification products were analysed by electrophoresis of 20 μl of each reaction in a 1.5% agarose, 1 × TBE gel followed by ethidium bromide staining.

TABLE 2A–Primers used for PCR analysis: RAPD and RAMS* primers used in Stages 1–3.

Name	Sequence (5' to 3')
OPA-8	GTGACGTAGG
OPB-4	GGACTGGAGT
OPB-11	GTAGACCCGT
OPC-5	GATGACCGCC
OPC-15	CATCCGTGCT
OPD-3	GTCGCCGTCA
$(GTG)_5$*	GTGGTGGTGGTGGTG
M13core*	GAGGGTGGNGGNTCT

TABLE 2B–Primers used for PCR analysis: RAPD and RAMS* primers used only in Stage 1

Name	Sequence (5' to 3')	Name	Sequence (5' to 3')
OPA-9	GGGTAACGCC	OPA-10	GTGATCGCAG
OPA-15	TTCCGAACCC	OPB-1	GTTTCGCTCC
OPB-5	TGCGCCCTTC	OPB-7	GGTGACGCAG
OPB-8	GTCCACACGG	OPB-10	CTGCTGGGAC
OPB-12	CCTTGACGCA	OPB-15	GGAGGGTGTT
OPB-17	AGGGAACGAG	OPB-18	CCACAGCAGT
OPB-19	ACCCCCGAAG	OPC-3	GGGGGTCTTT
OPC-6	GAACGGACTC	OPC-7	GTCCCGACGA
OPC-11	AAAGCTGCGG	OPC-14	TGCGTGCTTG
OPC-19	CTGGGGACTT	OPC-20	ACCCGGTCAC
OPD-1	ACCGCGAAGG	OPD-4	TCTGGTGAGG
OPD-6	ACCTGAACGG	OPD-8	GTGTGCCCCA
$(CAC)_5$*	CACCACCACCACCAC	OPE-1	CCCAAGGTCC
$(CA)_8$*	CACACACACACACACA	OPF-6	GGGAATTCGG
$(GACA)_4$*	GACAGACAGACAGACA		

To test the reproducibility of the RAPD markers, all RAPD-PCR reactions were performed in duplicate. For 11 of the samples, independent DNA extractions were used for duplicates. The PCR typing was carried out in three stages. In Stage 1, an initial screen was made with 37 different primers to find which produced clear, consistent, and polymorphic amplification profiles. This screen included DNA from two sets of *D. pini* isolates, each consisting of five isolates from the broadest possible range of backgrounds (*see* Table 1 for origins of *D. pini* isolates). The first set (Set A) included DP 301 (1960s sample), DP 402

(Golden Downs), DP 303 (Kinleith), DP 101 (Kaingaroa field trial—from the most resistant clone in the most resistant family sampled), DP 168 (Kaingaroa field trial—from the most susceptible clone in the most susceptible family). Representing the same groups as the first set, the second set of five isolates (Set B) was (respectively): DP 100, DP 401, DP 306, DP 102, DP 163. The only reason for using two sets instead of one was availability of DNA at the time of the screening procedure. Stage 2 involved further experiments, using four primers (OPA-8, OPD-5, M13 core, $(GTG)_5$) selected during Stage 1, with 30 *D. pini* isolates. These isolates included all 21 samples from the national study (four from Golden Downs, 12 from Kaingaroa, and five from Kinleith), eight from the field trial study (an isolate from one of the most resistant and one of the most susceptible trees in each of the four families), and one *M. pini*. Stage 3 involved amplification of all 71 isolates (70 *D. pini* + 1*M. pini*), as listed in Table 1, with three primers (OPB-4, OPB-11, OPC-5).

RESULTS

Collection and Isolation of *D. pini*

The overall efficiency of pathogen isolation from infected needles was poor. Of 144 samples collected for the field trial study, *D. pini* was successfully isolated and purified from 61. Of these, only 45 were informative in that they represented infection on both resistant and susceptible progeny from the same family. The 45 samples used were from four families with two individuals/family and between one and eight samples/individual (two from different branches of four ramets where possible). The host trees of the 45 *D. pini* isolates used for further study are indicated in Table 1A, with, for each cross (family) the most Dothistroma-resistant host (on the left) and the most Dothistroma-sensitive host (on the right).

Of the 84 samples collected for the national study, *D. pini* was isolated from 21, representing each of three stands sampled from Kaingaroa and Golden Downs Forests, but only one stand from Kinleith Forest. All 21 samples (Table 1B) were used in subsequent analysis. No *D. pini* isolates were isolated from needles collected from the West Coast forests in the South Island.

Morphological Observations

No obvious morphological differences (colony size, growth rate, shape, texture, or colour) were seen between isolates, including those from different geographic locations (results not shown). However, there was some morphological variation between some field trial isolates: three isolates taken from different ramets of the same tree exhibited different characteristics (Fig. 1). The production of dothistromin was observed in all cultures, which confirmed that contamination with another fungus had not occurred. Single purified isolates of *D. pini* have also been noted to exhibit variable morphologies, often displayed as sectoring in culture. Sectoring was observed in four out of 210 purified colonies plated in this study (Fig. 2).

Stage 1 PCR Typing: Screening of Primers with Five *D. pini* Isolates

Altogether 32 different RAPD and five microsatellite primers were used to PCR-amplify DNA from *D. pini* sample sets A or B, but there was no evidence of polymorphism between

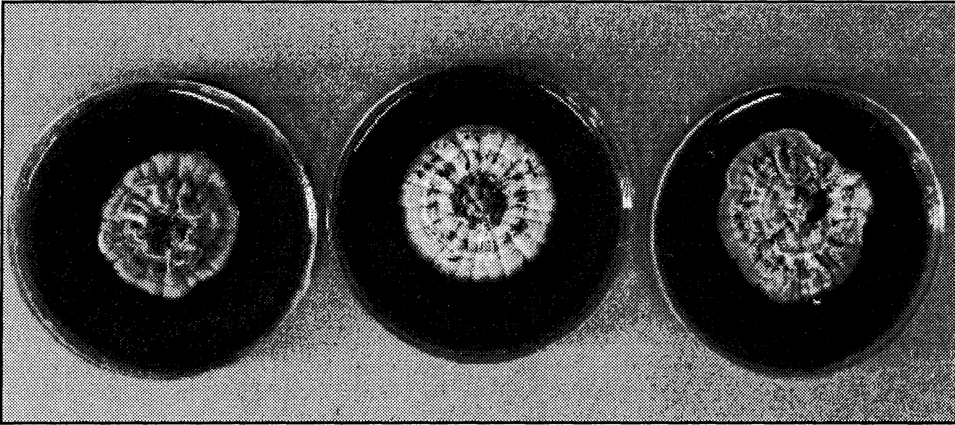

FIG. 1–Three *D. pini* isolates (left to right: DP 148, DP 146, DP 150) from different ramets of susceptible *P. radiata* host (progeny No. 320), grown on DM for 9 weeks at 20°C.

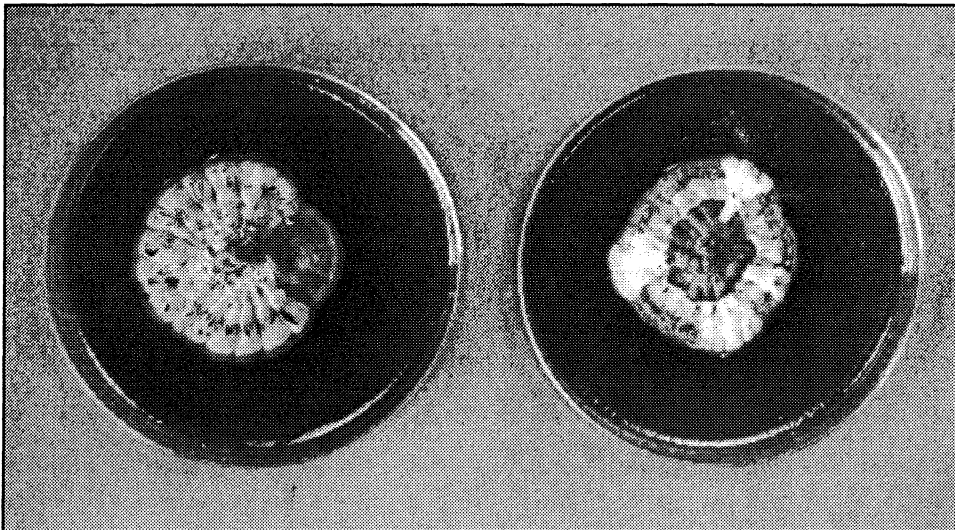

FIG. 2–Two *D. pini* isolates (left to right: DP 307, DP 106) showing sectors.

any of the isolates, in either of the sets, with any of the primers. Between one and 22 reproducible products (consistent between replicates) were amplified with each of the 37 primers. A sample of these results is shown in Fig. 3, for one of the RAPD primers (OPC-15) and *D. pini* set B. The seven brightest PCR products are visible in all samples (sizes of 2.65, 2.25, 1.85, 1.32, 0.94, 0.78, 0.51 kb). The product at 0.72 kb (immediately below the brightest band) does not appear to be present in all isolates; however, it was clearly seen in all reactions in the original photograph. The 3.0 kb products which are visible in lanes 2 and 3 are also present in lanes 6 and 8 (but not in the corresponding duplicate lanes 7 and 9), indicating poor reproducibility of amplification between duplicates, rather than polymorphisms. A product of 1.02 kb is also seen only in one duplicate of some isolates (lanes 3, 5, and 8).

Similar results to those shown in Fig. 3 were obtained with each of the 37 primers tested (results not shown). Most of the primers gave some amplification products which were not reproducible between duplicates. These products were therefore not used in analysis. Reproducibility was independent of the number of products amplified with each primer and may have been influenced by DNA quality. Occasionally, a profile shift was observed where, for example, the larger molecular-weight products would not amplify and the smaller products would feature more prominently. These profiles were often reproducible in the duplicate samples, but these bands were also not used in the analyses. In general, products of an intermediate size (1.0 to 2.0 kb) and stronger intensity were more reproducible. Altogether, 427 reproducible RAPDs and RAMS bands were obtained.

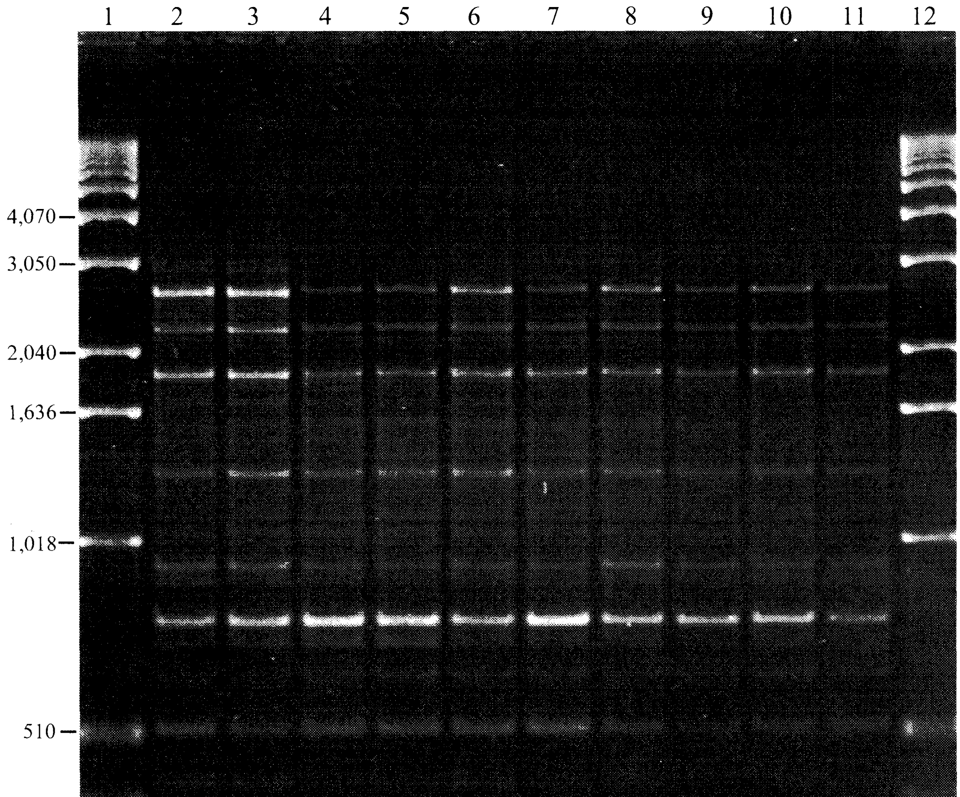

FIG. 3—Stage 1 RAPD amplification of Set B isolates using primer OPC-15. Amplification products are shown for DP 100 (lanes 2 and 3), DP 401 (lanes 4 and 5), DP 306 (lanes 6 and 7), DP 102 (lanes 8 and 9), and DP 163 (lanes 10 and 11). Numbers at the left of the Figure indicate sizes (bp) of the BRL 1 kb ladder used as a standard (lanes 1 and 12).

Stage 2 PCR Typing: Testing 30 *D. pini / M. pini* Isolates

To determine whether the two sets of five isolates used to screen primers in Stage 1 were representative of the New Zealand population, a broader sample of 30 isolates was taken which was representative of the entire collection. The primers used in these experiments (two

RAPD primers OPA-8 and OPD-3, and two microsatellite primers M13 core and $(GTG)_5$) were chosen because they had reproducibly amplified a minimum of eight products in the screening run.

No reproducible differences were observed between any New Zealand *D. pini* isolates using any of the primers. With all primers, however, differences were seen between *M. pini* and the *D. pini* samples (results not shown).

Stage 3 PCR Typing: Testing all 71 Collected *D. pini/M. pini* Isolates

The results from Stages 1 and 2 suggested that all the New Zealand *D. pini* samples collected would give the same genetic profile with RAPD- or RAMS-PCR. To verify this, amplification was performed with three primers (OPB-4, OPB-11, OPC-5) using all 71 isolates in duplicate. Representative results (for the OPC-5 primer with nine isolates) are shown in Fig. 4 in which amplification products from the four 1960s isolates appear alongside those from the Central American *M. pini* and four "national study" isolates. Again, some lack of reproducibility was noticed between replicates, but no reproducible differences were observed between any of the *D. pini* isolates using any of the primers, suggesting that genetic diversity is very low. Some DNA profiles of *M. pini* and *D. pini* shared some bands (Fig. 4, lanes 10 and 11), but there were also several bands which were clearly unique to *M. pini*. With each of the primers used, products were observed in the *M. pini* isolate which were not amplified in *D. pini*. As well as showing that there are obvious genetic differences between the New Zealand *D. pini* population and a strain from overseas, this also illustrated that RAPD analysis can be used to detect genetic variability where polymorphisms exist to

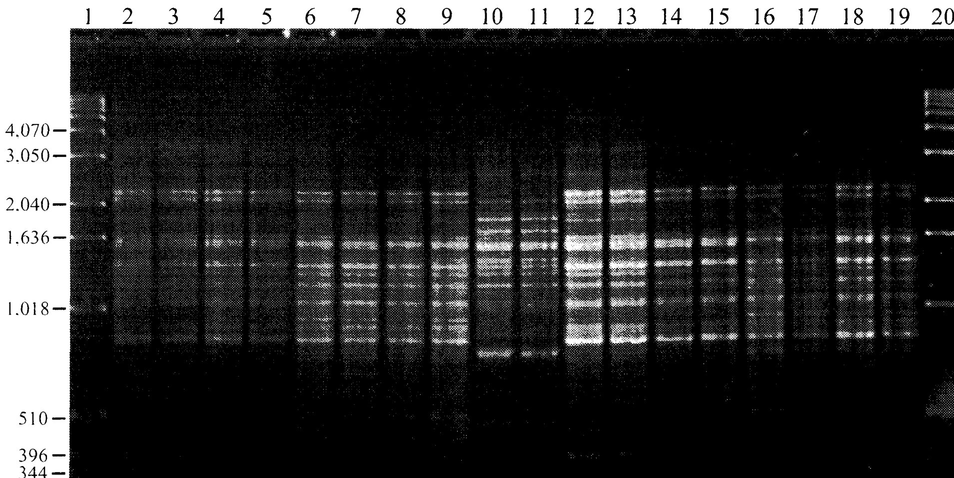

FIG. 4—Sample of Stage 3 RAPD amplification of 71 isolates using primer OPC-5. Amplification products are shown for 1960s isolates DP 301 (lanes 2 and 3), DP 003 (lanes 4 and 5), DP 100 (lanes 6 and 7), DP 005 (lanes 8 and 9), *M. pini* MP 001 (lanes 10 and 11), and Golden Downs Forest isolates DP 401 (lanes 12 and 13), DP 402 (lanes 14 and 15), DP 403 (lanes 16 and 17), and DP 404 (lanes 18 and 19). Numbers at the left of the Figure indicate sizes (bp) of the BRL 1 kb ladder used as a standard (lanes 1 and 20).

be identified. Electronic copies of gel photographs of all RAPD and RAMS amplifications are available from the corresponding author on request.

DISCUSSION

The reliability and sensitivity of the RAPD and RAMS methods for detecting polymorphisms was verified by performing all amplifications in duplicate and by obtaining different profiles with the Central American *M. pini* isolate. The assumption was made that, for each set of reactions, co-migrating products were also equivalent in sequence. PCR analysis of all 70 *D. pini* isolates (used in Stage 3 of this study) showed the same numbers and sizes of products for each of three primers. This suggested that the smaller number of *D. pini* isolates used (in Stage 1) to screen 37 different primers was a representative sample. It was concluded that continued testing of all available isolates with further primers would not reveal significant DNA polymorphisms.

The amplification of 427 reproducible bands with 37 primers constitutes a direct measure of 9.2 kb of priming sites (which would be affected by any nucleotide substitutions, insertions, or deletions) and 645 kb of total amplicon length (which would be affected by any insertions or deletions). Although these represent only a small proportion of the genome (based on an estimated genome size of 4×10^4 kb), the PCR-based methods used here are extremely sensitive. For example, pathotypes of the rape pathogen *Phoma lingam* were distinguished using four RAPD-type primers (Schäfer & Wöstemeyer 1992) and a single primer was sufficient to discriminate between field isolates of the broad host-range pathogen *Claviceps purpurea* (Jungehülsing & Tudzynski 1997). Moreover, in a comprehensive review of reproduction and population structure in phytopathogenic fungi Brygoo *et al.* (1998) concluded that, compared to sexual species, "small samples and few molecular markers are sufficient to survey the structure of asexual populations". Hence, even though our sample was limited, on the basis of our results we propose that the genetic diversity of the *D. pini* population in New Zealand is very low.

The observation that *D. pini* samples isolated in the 1960s share the same DNA profiles as those isolated in the 1990s suggests that only one strain has been present in New Zealand since its first introduction and that relatively little genetic change has occurred. One implication of this is that plant quarantine measures have so far been adequate to prevent further entry of overseas strains. Another implication is that the lack of sexuality seen in New Zealand *D. pini* may be due to the absence of a mating type partner (assuming heterothallism).

Our inability to detect genetic diversity does not mean there is no genetic diversity in New Zealand *D. pini*. Firstly, it is possible that there are polymorphisms in strains of *D. pini* from areas not sampled in this study. Secondly, only part of the *D. pini* genome has been screened for genetic polymorphisms in this study and even within the PCR-amplified regions some polymorphisms (e.g., single base substitutions) may remain undetected. Asexual fungi usually show at least some population diversity due to mutation (Burdon 1993) and it seems very likely that this will have occurred if the same strain has been in New Zealand for over 30 years. We cannot rule out the possibility that there is some heterogeneity in virulence of the *D. pini* population in New Zealand.

The *D. pini* strain which appears to be ubiquitous in New Zealand grows with a variety of different morphologies and appears to be able to switch between these morphologies

(displayed as sectoring of colonies on plates). The mechanism by which these observed changes in morphology occur is unknown. The uniformity of the RAPD and RAMS data suggest the changes are not due to genetic alterations, although this remains a possibility. Heterokaryosis or mixed cultures remain formal explanations for the appearance of morphologically distinct sectors in our studies. It is also possible that an epigenetic mechanism such as methylation may have altered gene expression, and thus morphology. Alternatively, the observed changes may be due to transposable elements in the *D. pini* genome. These elements can cause chromosome rearrangements and alterations in gene expression leading to phenotypic instability, and are present in many fungal pathogens (Daboussi 1997).

The discovery that the genetic diversity of New Zealand *D. pini* is very low has important implications for the *P. radiata* Dothistroma-resistance breeding programme. Breeding for resistance to one asexually reproducing strain of pathogen means that resistance is likely to be more durable than if the pathogen population was sexual and genetically diverse. Our results suggest that *D. pini* has not changed much over 30 years in New Zealand and durability for this strain is likely to remain high. However, a further implication is that the Dothistroma-resistance breeding programme thus far has selected for resistance to only one strain of *D. pini*. New Zealand Dothistroma-resistant trees may not be resistant to strains which are found overseas. Furthermore, if the New Zealand strain is heterothallic and exists only in the anamorphic state because of the absence of a mating type partner, an introduced overseas strain may also sexually recombine with the New Zealand strain.

So far, however, only low-moderate gains in Dothistroma resistance have been achieved and most of the genetic variance for this trait is of the additive type (Carson & Carson 1991b). These observations suggest that current resistance is not due to a race-specific ("gene-for-gene") interaction. This is not too surprising since most plant-fungal pathogen relationships proven to have a gene-for-gene pattern of resistance involve biotrophic rather than necrotrophic pathogens (Heath 1996). *Dothistroma pini* appears to be a predominantly necrotrophic pathogen, since lateral spread of the fungus within a needle is preceded by death of host tissue (Gadgil 1967; Franich *et al.* 1986). Although the apparent absence of a gene-for-gene interaction implies that the Dothistroma-resistant breed may confer resistance to other races of the pathogen, any overseas isolates of *D. pini* should be considered a potential threat until the precise basis for resistance is known.

Although only one overseas strain (isolated from *P. tecumumannii*) was examined in this study as a control, genetic differences from the New Zealand strain were apparent. Further studies with overseas strains carried out in England have shown some to be extremely high producers of the toxin dothistromin (Bradshaw *et al.* in press) compared to the New Zealand strain. The implications of these results for New Zealand quarantine are very important. It will be vital to maintain vigilance in preventing the introduction of overseas strains into New Zealand. Given the variable morphology of the New Zealand *D. pini*, a DNA-based method should be developed to monitor severe outbreaks of Dothistroma needle blight for the appearance of overseas strains. If new strains are detected, measures can then be taken to contain the infection before it spreads further.

In summary, DNA profiles of a wide range of *D. pini* isolates collected in New Zealand suggest the population genetic diversity is extremely low. The resistance of current

Dothistroma-resistant *P. radiata* to the New Zealand *D. pini* is expected to be durable. However, there is now a valid concern that *D. pini* may increase in virulence if further strains are introduced to New Zealand. This heightens the importance of preventing further introductions as well as developing more effective strategies to confine and/or combat Dothistroma needle blight.

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