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Gene and genotypic diversity of *Phytophthora cinnamomi* in South Africa and Australia revealed by DNA polymorphisms

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Abstract

Phytophthora cinnamomi isolates from South Africa and Australia were compared to assess genetic differentiation between the two populations. These two populations were analysed for levels of phenotypic diversity using random amplified polymorphic DNAs (RAPDs) and gene and genotypic diversity using restriction fragment length polymorphisms (RFLPs). Sixteen RAPD markers from four decanucleotide Operon primers and 34 RFLP alleles from 15 putative loci were used. A few isolates from Papua New Guinea known to posses alleles different from Australian isolates were also included for comparative purposes. South African and Australian *P. cinnamomi* populations were almost identical with an extremely low level of genetic distance between them ($D_m = 0.003$). Common features for the two populations include shared alleles, low levels of phenotypic/genotypic diversity, high clonality, and low observed and expected levels of heterozygosity. Furthermore, relatively high levels of genetic differentiation between mating type populations (D_m South Africa $= 0.020$ and D_m Australia $= 0.025$ respectively), negative fixation indices, and significant deviations from Hardy–Weinberg equilibrium, all provided evidence for the lack of frequent sexual reproduction in both populations. The data strongly suggest that both the South African and Australian *P. cinnamomi* populations are introduced.

Introduction

Phytophthora cinnamomi Rands is a host-nonspecific, soilborne, fungal pathogen of many plant species (Zentmyer, 1980). In South Africa it is most important as a pathogen of *Eucalyptus* (Wingfield and Knox-Davies, 1980), avocado trees (Darvas et al., 1984; Wager, 1942), grapevines (Van der Merwe et al., 1972) and members of the Proteaceae family (Von Broembsen and Brits, 1985). It is especially important in the South African forestry industry where 90% mortality has been experienced in some of the cold tolerant *Eucalyptus* spp. such as *E. fraxinoides*. In Australia, *Eucalyptus* spp., especially *E. marginata*, are severely affected by *P. cinnamomi* (Podger et al., 1965; Weste, 1994). *P. cinnamomi* infects its hosts mainly through its motile zoospores (Zentmyer, 1961) and can survive for many years in soil and plant material in the form of survival structures such as chlamydospores (Zentmyer and Mircetich, 1966) or oospores (Kassaby et al., 1977).

P. cinnamomi has two mating types, A1 and A2, which can interact to produce sexual spores (oospores). Both mating types have a global distribution (Zentmyer, 1980, 1988) and both occur in South Africa (Linde et al., 1997; Von Broembsen, 1989), Australia (Pratt and Heather, 1973) and Papua New Guinea (Arentz and Simpson, 1986). The A2 mating type is found throughout South Africa while the A1

mating type is most commonly encountered in southern South Africa (Cape region) (Linde et al., 1997; Von Broembsen, 1989). The presence of both mating types in these regions may provide opportunities for sexual reproduction and thus acquisition of new genotypes that give rise to more pathogenic genotypes.

A recent isozyme study on the South African *P. cinnamomi* population indicated low levels of gene and genotypic diversity and the absence or rare occurrence of sexual recombination (Linde et al., 1997). This population study showed low levels of genetic differentiation between regional and temporal *P. cinnamomi* populations. Isozyme studies on *P. cinnamomi* in Australia (Old et al., 1988, 1984) also indicated low levels of genetic diversity, a high frequency of A2 and rare occurrence of A1 mating type isolates, and the absence of sexual reproduction. It was thus hypothesised that the fungus was introduced into Australia (Old et al., 1988, 1984). In contrast, a high level of genetic diversity was found among *P. cinnamomi* isolates from Papua New Guinea (Old et al., 1984).

Population genetic studies on fungi using isozymes are often limited by the number of isozyme loci available and in the number of alleles at each locus (Michelmore and Hulbert, 1987). This is in contrast to higher eucaryotic organisms where such markers have been used very successfully. These limitations can be overcome by using DNA based markers such as RAPDs (Williams et al., 1990) and RFLPs. Multilocus RFLP markers can be obtained by using multilocus RFLP probes (Botstein et al., 1980), often referred to as DNA fingerprint probes. Such techniques offer a virtually unlimited number of selectively neutral markers from which to randomly select a sufficient number to conduct population genetic analyses. Detailed genetic and population genetic studies have been conducted on the heterothallic *Phytophthora infestans* (Drenth et al., 1993, 1994; Fry et al., 1992, 1993; Goodwin et al., 1992, 1995) and the homothallic *P. sojae* (Drenth et al., 1996; Förster et al., 1994), using DNA fingerprint and low copy RFLP probes, respectively. Despite the importance of *P. cinnamomi* as a plant pathogen, very little effort has been made to apply DNA based markers to elucidate the genetic structure of the pathogen population. One such attempt has been made by Chang et al., 1996, who applied RAPDs to 26 *P. cinnamomi* isolates from Taiwan.

The availability of DNA based genetic markers provides multiple opportunities to accurately determine the levels of gene and genotypic diversity in *P. cinnamomi* populations. In order to directly compare the structure of the South African and Australian *P. cinnamomi* populations, both should ideally be analysed using the same set of RAPD and RFLP markers. If *P. cinnamomi* was introduced into South Africa, a similar population structure to the Australian population would be expected. Strong evidence to support the view that *P. cinnamomi* was introduced into Australia is provided by the irreversible destruction *P. cinnamomi* causes in native vegetation and the low levels of gene diversity (Shepherd, 1975; Old et al., 1988, 1984).

Knowledge about the population genetics of *P. cinnamomi* will be useful for disease resistance and selection programmes, and will give further information regarding the origin of *P. cinnamomi* in South Africa. Various population genetic questions pertaining to *P. cinnamomi* already partially answered in an isozyme study (Linde et al., 1997), were further addressed in this study. *P. cinnamomi* populations used in this study include populations from South Africa and Australia, as well as a few isolates from Papua New Guinea included for comparative purposes. RAPD and RFLP markers were used to specifically address these population genetic questions.

The specific aims of the current investigation were to: (i) identify the extent of clonality in the South African and Australian *P. cinnamomi* populations, (ii) assess the level of genotypic diversity in the respective *P. cinnamomi* populations, (iii) assess the level of gene diversity in the respective *P. cinnamomi* populations, (iv) compare A1 and A2 mating type populations from the South African and Australian *P. cinnamomi* populations, (v) test for goodness of fit to Hardy–Weinberg equilibrium in the South African and Australian *P. cinnamomi* populations, and (vi) quantify the genetic differentiation between the South African and Australian *P. cinnamomi* populations.

Materials and methods

Collection of P. cinnamomi *isolates*

P. cinnamomi isolates were collected in South Africa between 1977–1986 and 1991–1993 from two discrete geographical regions (Cape and Mpumalanga) (Table 1). These different regional and temporal subpopulations have previously been shown to be genetically similar (Linde et al., 1997), and in this study

Table 1. Mating type, RAPD phenotype, and RFLP genotype characteristics of *P. cinnamomi* isolates from South Africa, Australia and Papua New Guinea

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype[a]	Host
C8	South Africa	A1	51	Ψ	*Vitis vinifera*
C9	South Africa	A2	40	2	*Eucalyptus fraxinoides*
C11	South Africa	A1	54	Ψ	*Leucadendron argenteum*
C19	South Africa	A2	47	1	*Leucadendron rubrum*
C44	South Africa	A2	14	1	*Leucospermum reflexum*
C69	South Africa	A1	53	Ψ	*Leucospermum cordiifolium*
C73	South Africa	A2	3	1	*Leucadendron argenteum*
C161	South Africa	A1	51	Ψ	*Leucadendron laureolum*
C167	South Africa	A2	24	1	*Leucadendron argenteum*
C168	South Africa	A1	53	Ψ	*Leucospermum* sp.
C174	South Africa	A1	51	3	*Hakea sericeae*
C177	South Africa	A1	53	Ψ	*Protea* sp.
C180	South Africa	A2	3	1	*Leucadendron argenteum*
C196	South Africa	A2	45	2	*Cryptomeria* sp.
C202	South Africa	A1	53	Ψ	*Eucalyptus fraxinoides*
C208	South Africa	A2	60	1	*Widdringtonia nodiflora*
C210	South Africa	A1	57	3	*Protea magnifica*
C213	South Africa	A2	15	Ψ	*Widdringtonia cypressoides*
C214	South Africa	A1	54	Ψ	*Erica patersonia*
C215	South Africa	A1	61	3	*Leucospermum cordiifolium*
C218	South Africa	A2	23	1	*Araucaria angustifolia*
C220	South Africa	A1	53	Ψ	*Leucadendron argenteum*
C223	South Africa	A2	42	1	*Leucospermum patersonii*
C226	South Africa	A2	7	Ψ	*Pinus pinaster*
C227	South Africa	A2	19	1	*Pinus pinaster*
C228	South Africa	A2	20	2	*Eucalyptus* sp.
C274	South Africa	A2	25	1	*Pinus pinaster*
C284	South Africa	A1	54	Ψ	*Leucadendron tinctum*
C371	South Africa	A1	52	3	*Serruria florida*
C403	South Africa	A1	53	Ψ	*Mimetus capitulatus*
C410	South Africa	A1	55	Ψ	*Leucospermum pluridens*
C411	South Africa	A2	59	5	*Pinus radiata*
C412	South Africa	A2	3	Ψ	*Pinus radiata*
C414	South Africa	A1	51	Ψ	*Leucospermum conocarpodendron*
C415	South Africa	A1	54	Ψ	*Leucospermum praecox*
C418	South Africa	A1	62	3	*Cunninghamia casuarina*
C419	South Africa	A1	54	Ψ	*Banksia burdenii*
C432	South Africa	A1	53	Ψ	Water
C435	South Africa	A2	10	1	*Pinus radiata*
C453	South Africa	A2	8	1	*Pinus patula*
C504	South Africa	A2	4	1	*Pinus radiata*
CP80	South Africa	A2	37	2	*Persea americana*
CP81	South Africa	A2	43	Ψ	*Persea americana*
CP227	South Africa	A1	51	Ψ	*Casuarina* sp.
CP233	South Africa	A1	62	3	*Casuarina* sp.
CP467	South Africa	A2	45	2	*Eucalyptus fastigata*
CP468	South Africa	A2	44	2	*Eucalyptus fastigata*
CP470	South Africa	A2	15	Ψ	*Eucalyptus smithii*
CP477	South Africa	A2	16	1	*Eucalyptus smithii*
CP481	South Africa	A2	15	1	*Eucalyptus smithii*
CP488	South Africa	A2	45	Ψ	*Eucalyptus macarthurii*
CP490	South Africa	A2	3	1	*Pinus radiata*
CP491	South Africa	A2	3	1	*Pinus radiata*
CP492	South Africa	A2	3	Ψ	*Pinus patula*
CP494	South Africa	A2	17	1	*Pinus patula*

Table 1. Continued

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype[a]	Host
CP499	South Africa	A2	12	2	*Persea americana*
CP503	South Africa	A2	38	2	*Ocotea bullata*
CP504	South Africa	A2	6	1	*Prunus* sp.
CP506	South Africa	A2	34	Ψ	*Ocotea bullata*
CP507	South Africa	A2	36	2	*Ocotea bullata*
CP508	South Africa	A2	43	2	*Ocotea bullata*
CP509	South Africa	A2	43	Ψ	*Ocotea bullata*
CP510	South Africa	A2	49	2	*Ocotea bullata*
CP511	South Africa	A2	29	2	*Ocotea bullata*
CP513	South Africa	A1	53	Ψ	*Ocotea bullata*
CP514	South Africa	A2	37	2	*Ocotea bullata*
CP517	South Africa	A2	35	2	*Ocotea bullata*
CP518	South Africa	A1	58	3	*Ocotea bullata*
CP522	South Africa	A2	3	1	*Ocotea bullata*
CP525	South Africa	A2	38	2	*Ocotea bullata*
CP526	South Africa	A2	38	Ψ	*Ocotea bullata*
CP527	South Africa	A1	53	Ψ	*Ocotea bullata*
CP528	South Africa	A2	35	Ψ	*Ocotea bullata*
CP529	South Africa	A2	11	2	*Ocotea bullata*
CP530	South Africa	A2	34	2	*Ocotea bullata*
CP531	South Africa	A1	53	Ψ	*Ocotea bullata*
CP532	South Africa	A1	54	Ψ	*Ocotea bullata*
CP533	South Africa	A1	53	Ψ	*Ocotea bullata*
CP534	South Africa	A2	40	7	*Ocotea bullata*
CP537	South Africa	A2	39	2	*Ocotea bullata*
CP538	South Africa	A2	39	2	*Ocotea bullata*
CP541	South Africa	A2	35	2	*Ocotea bullata*
CP542	South Africa	A2	32	2	*Ocotea bullata*
CP544	South Africa	A2	35	Ψ	*Ocotea bullata*
CP545	South Africa	A2	38	Ψ	*Ocotea bullata*
CP546	South Africa	A2	35	Ψ	*Ocotea bullata*
CP548	South Africa	A2	3	1	*Ocotea bullata*
CP550	South Africa	A2	3	Ψ	*Ocotea bullata*
CP551	South Africa	A1	54	Ψ	*Ocotea bullata*
T2	South Africa	A2	17	1	*Ananas comosum*
T3	South Africa	A1	54	Ψ	*Leucadendron argenteum*
T4	South Africa	A2	26	1	*Leucospermum cordiifolium*
T5	South Africa	A1	53	Ψ	*Leucospermum comosum*
T6	South Africa	A2	31	2	*Persea americana*
T7	South Africa	A1	53	Ψ	*Vitis vinifera*
T8	South Africa	A2	7	1	*Eucalyptus fraxinoides*
T11	South Africa	A1	53	3	*Serruria krausii*
T12	South Africa	A1	53	Ψ	*Priestleya* sp.
T13	South Africa	A1	55	3	*Leucospermum reflexum*
T14	South Africa	A1	54	3	*Orothamnus zeiheri*
T16	South Africa	A1	54	Ψ	*Hakea sericea*
T17	South Africa	A1	53	Ψ	*Protea* sp.
T18	South Africa	A1	53	Ψ	*Mimetus splendidus*
T19	South Africa	A2	29	Ψ	*Cryptomeria liebertiana*
T21	South Africa	A1	53	3	Water
T22	South Africa	A1	54	Ψ	Water
T24	South Africa	A1	53	Ψ	Water
T25	South Africa	A1	53	Ψ	Water
T26	South Africa	A1	53	Ψ	Water
T27	South Africa	A1	53	3	Water
T28	South Africa	A1	53	Ψ	Water

Table 1. Continued

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype[a]	Host
T30	South Africa	A1	53	Ψ	*Mimetus splendidus*
T31	South Africa	A1	13	3	*Ocotea bullata*
T32	South Africa	A1	53	Ψ	*Ocotea bullata*
T33	South Africa	A1	55	3	*Ocotea bullata*
T34	South Africa	A1	53	3	*Cunnonia capensis*
T35	South Africa	A1	54	Ψ	*Ocotea bullata*
T36	South Africa	A2	40	Ψ	*Ocotea bullata*
T37	South Africa	A1	51	Ψ	*Ocotea bullata*
T38	South Africa	A2	28	2	*Protea roupelliae*
T44	South Africa	A2	5	1	*Ocotea bullata*
UQ629	Australia	A2	39	2	*Telopea* sp.
UQ633	Australia	A1	55	3	Unknown
UQ640	Australia	A2	3	1	*Lychee chinensis*
UQ642	Australia	A2	39	2	*Oryza sativa*
UQ665	Australia	A2	3	10	*Leucospermum* sp.
UQ732	Australia	A2	3	10	*Banksia* sp.
UQ733	Australia	A2	3	1	*Darwinia oxylepis*
UQ734	Australia	A2	3	1	*Allocasuarina fraseriana*
UQ735	Australia	A2	3	10	*Pinus radiata*
UQ736	Australia	A2	3	10	*Adenanthos* sp.
UQ737	Australia	A2	33	2	*Hibbertia subvaginata*
UQ738	Australia	A2	3	1	*Banksia grandis*
UQ739	Australia	A2	3	1	*Banksia* sp.
UQ740	Australia	A2	3	1	*Banksia quercifolia*
UQ741	Australia	A2	3	1	*Pinus radiata*
UQ742	Australia	A2	1	1	*Xanthorrhoea preissii*
UQ743	Australia	A2	3	1	*Myrtaceae*
UQ771	Australia	A2	46	1	*Persea americana*
UQ787	Australia	A2	63	2	*Eucalyptus globoidea*
UQ788	Australia	A2	3	1	*Castenea sativa*
UQ789	Australia	A1	55	3	*Eucalyptus gummifera*
UQ790	Australia	A1	55	3	*Pinus radiata*
UQ791	Australia	A2	10	4	*Banksia marginata*
UQ792	Australia	A1	55	3	*Pinus elliottii*
UQ794	Australia	A2	3	1	*Persea americana*
UQ795	Australia	A1	56	3	Soil
UQ817	Australia	A2	30	2	*Aotus ericoides*
UQ818	Australia	A2	22	9	*Dillwynia floribunda*
UQ822	Australia	A2	3	1	Soil
UQ823	Australia	A2	3	1	*Monotoca glauca*
UQ824	Australia	A2	21	2	Soil
UQ827	Australia	A1	27	6	*Allocasuarina littoralis*
UQ828	Australia	A1	50	3	Unknown
UQ871	Australia	A2	2	1	*Castanea* sp.
UQ873	Australia	A2	3	1	Soil
UQ877	Australia	A2	3	1	Soil
UQ879	Australia	A2	3	Ψ	Soil
UQ891	Australia	A2	3	1	*Persea americana*
UQ819	Papua New Guinea	A2	38	2	Soil
UQ820	Papua New Guinea	A2	3	1	Soil
UQ821	Papua New Guinea	A2	7	1	Soil
UQ831	Papua New Guinea	A1	48	8	Soil
UQ832	Papua New Guinea	A2	18	11	Soil
UQ835	Papua New Guinea	A1	41	13	Soil
UQ836	Papua New Guinea	A1	9	12	Soil

[a] Ψ = Not identified.

they are, therefore, regarded as a single population. Isolates represent both mating types and were obtained from various host species. A2 mating type isolates were predominantly isolated from forestry species such as *Eucalyptus* and *Pinus* spp., whereas A1 mating type isolates were predominantly isolated from native vegetation. Details pertaining to isolation, maintenance of cultures, determination of mating type, and isozyme analysis have been provided elsewhere (Linde et al., 1997). Australian and Papua New Guinea isolates were obtained from M.J. Dudzinski, Division of Forestry, CSIRO, Canberra, Australia. A total of 166 *P. cinnamomi* isolates from South Africa, Australia, and Papua New Guinea, were analysed using RAPDs. A subset of isolates for each population was randomly chosen for RFLP analysis (Table 1).

DNA extraction

Mycelium for DNA extraction was grown in clarified V8 broth in 250 ml Erlenmeyer flasks for 1 week. Mycelium was harvested through a Büchner funnel, freeze-dried, and ground into a fine powder in liquid nitrogen. DNA extraction procedures followed those used for *P. infestans* (Drenth et al., 1993). DNA concentration was measured on a fluorometer and adjusted in milli-Q water to the required concentration for analysis. All DNA samples were stored at −20 °C.

RAPD analysis

Each RAPD reaction was done in a total volume of 25 µl and comprised of 250 µM each dNTPs, 25 ng primer, 4 mM $MgCl_2$, 60 ng *P. cinnamomi* DNA, 1.6 units of *Tth* Plus DNA polymerase (Biotech International, Australia), 2.5 µl of 10× buffer supplied by the manufacturer, and ultra-pure water. Thermocycling was carried out at 39 cycles of 1 min at 94 °C, 1 min at 37 °C, and 2 min at 72 °C. Then, 1 cycle of 1 min at 94 °C, 1 min at 37 °C, and 10 min at 72 °C was performed. Four decanucleotide primers were used to analyse the *P. cinnamomi* populations (Table 2). The total volume of amplified DNA fragments was size fractionated on 1.5% agarose gels in 1× TBE buffer (Sambrook et al., 1989) at 120 V and maximum current for 6 h. The gels were stained in an ethidium bromide solution for 20 min, and destained in deionised water before visualisation on a UV transilluminator.

Table 2. Nucleotide sequence of decanucleotide Operon primers used to characterise *Phytophthora cinnamomi* populations and the number of polymorphic polymerase chain reaction fragments analysed for each primer

Primer	Nucleotide sequence	No. of polymorphic fragments
OPAC-01	TCCCAGCAGA	3
OPM-10	TCTGGCGCAC	6
OPT-07	GGCAGGCTGT	5
OPZ-04	AGGCTGTGCT	2

RFLP analysis

Restriction digestion was conducted using 3 µg of total DNA digested overnight at 37 °C with 30 units of restriction enzymes *Hin*dIII, *Xho*I, and *Pst*I (Boehringer Mannheim, New England Biolabs, Beverly, MA). Restricted DNA fragments were separated on 0.8% agarose gels for 14–16 h at 40 V (600 Vh) before alkaline transfer to Hybond N^+ (Amersham) hybridisation membrane. Membranes were prehybridised at 65 °C in 15 ml of hybridisation solution (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM disodium ethylenediaminetetraacetic acid, 7% sodium dodecyl sulfate, pH 7.2), 50 µg salmon sperm DNA per ml, and 15 µl poly A for 4 h. Probes were labelled with [α^{32}P] dCTP following manufacturer's instructions (Amersham, Australia). Blots were hybridised overnight and subjected to stringency washes (twice with 5× SSPE, twice with 1× SSPE, 0.1% sodium dodecyl sulfate, and once with 0.1× SSPE, 0.1% sodium dodecyl sulfate). Filters were exposed at −70 °C to Kodak X-Omat™ film. Stripping of blots for reuse was carried out at 45 °C for 30 min in 0.4 M NaOH and 30 min in (0.2 M Tris-HCl pH 7.5, 0.1× SSC, 0.1× sodium dodecyl sulfate) (20× SSPE = 350.6 g NaCl, 55.2 g NaH_2PO_4, 14.8 g EDTA, pH 7.4) (20× SSC = 346.6 g NaCl, 76.4 g Na_3 Citrate, pH 7.0).

Selection of RFLP probes

Fifteen probes (Table 3) were screened for single copy RFLP patterns by hybridisation to a sub-set of *Hind*III, *Xho*I, and *Pst*I digested *P. cinnamomi* DNA. On the basis of this screen, eight probes were selected to characterise *P. cinnamomi* populations. These probes were selected because they showed clearly identifiable single copy loci using *Hind*III, *Xho*I, or *Pst*I as restriction enzyme. In the case of *pPi*120 at *Hind*III and *Pst*I, two fragments were observed that were confirmed to

be allelic after simultaneous digestion with *Hind*III and *Pst*I.

Data analysis

RAPD data were scored for the absence or presence of fragments which was used to derive a multicharacter phenotype for each isolate. Isolates with the same overall multicharacter phenotype were considered clonal. The clonal fraction of each population was calculated. Clonal fraction $= (N - C)/N$ where N is the sample size and C is the number of distinct genotypes. RFLP data were scored based on the presence of RFLP fragments with similar sequence but different size, and each fragment of a particular size was assumed to represent a specific allele at single genetic locus. A multilocus RFLP genotype was derived for each isolate studied, based on all fragments observed with eight selected RFLP probes. Isolates with the same overall RFLP genotype were considered clonal.

Genotypic diversity ($\hat{G}$) (Stoddard and Taylor, 1988) was calculated on the basis of the number of multilocus RAPD phenotypes and RFLP genotypes within each *P. cinnamomi* population studied. To compare levels of genotypic diversity between regions, diversity values of each region were corrected for sample size (N) (McDonald et al., 1994), to calculate the percentage maximum possible diversity obtained ($\hat{G}/N\%$). The significance of differences between the percentages of maximum diversity ($100 * \hat{G}/N$) obtained with RAPD and RFLP analyses for South African and Australian populations was calculated using a t-test (Chen et al., 1994; Stoddard and Taylor, 1988).

The mean number of alleles per locus was calculated for each population to give an estimate of the level of gene diversity. Gene diversity for each *P. cinnamomi* population studied was calculated using Nei's gene diversity (H_{exp}) (Nei, 1973). The probable mating system was determined by comparing the observed proportion of heterozygotes with the expected level of heterozygosity by Wright's fixation index, $F = 1 - (H_{obs}/H_{exp})$, where H_{obs} is the observed mean heterozygosity per locus and H_{exp} is the expected mean heterozygosity in the population which is the same as Nei's gene diversity (Brown, 1979).

Regional differentiation between *P. cinnamomi* populations was determined using differences in allele frequencies. Based on allele frequencies of the 15 RFLP loci, population differentiation between A1 and

Table 3. Fifteen probes screened for RFLP's of *P. cinnamomi* populations

Name	Probe			Restriction enzyme[z]		
	DNA of origin	Species of origin	Obtained from	*Hind*III	*Xho*I	*Pst*I
β-tubulin	cDNA	*P. cinnamomi*	L.P. Lehnen and A.R. Hardham, unpubl.	−	+	+
*BipBh*5	Genomic DNA[y]	*P. cinnamomi*	L.P. Lehnen and A.R. Hardham, unpubl.	+	+	−
*LPV*18	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−
pADACT	Genomic DNA[y]	*P. infestans*	Unkles et al., 1991	−	+	−
*pPi*120[x]	cDNA	*P. infestans*	Pieterse et al., 1993	++	+	++
*pNia*7-Ss	Genomic DNA[y]	*P. infestans*	Pieterse et al., 1995	−	−	−
*pPc*2	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	+
*pPc*3	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	+
*pPc*5	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	+	+	−
*pPc*7	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−
*pPc*9	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−
*pPc*10	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	+
*pPc*11	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−
*pPc*14	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−
*pPc*15	cDNA	*P. cinnamomi*	J. Marshall and A.R. Hardham, unpubl.	−	−	−

[x]In the case of *pPi*120 at *Hind*III and *Pst*I, two fragments were observed that were confirmed to be not allelic after simultaneous digestion with *Hind*III and *Pst*I. Therefore, two loci *pPi*120α-1 and *pPi*120α-2 were scored when genomic *P. cinnamomi* DNA was digested with *Hind*III and *Pst*I.

[y]Represent coding regions of known genes.

[z](+) = polymorphic; (−) = monomorphic, or (++) = multiple.

A2 mating type populations of South Africa and Australia, and between the South African, Australian and Papua New Guinea populations was determined using an unbiased minimum genetic distance (D_m) (Nei, 1978). The occurrence of sexual reproduction in *P. cinnamomi* was tested using frequencies of genotypes in the six polymorphic RFLP loci in South African and Australian populations. These genotypic frequencies were tested for deviations from expected Hardy–Weinberg equilibrium using the Biosys-1 Statistical Package (Swofford and Selander, 1981). No correction for clonal genotypes was conducted as this would lead to an unacceptably small population size ($n = 6$).

Results

RAPD analysis

Overall diversity

Sixteen polymorphic RAPD fragments revealed 63 RAPD phenotypes among a total of 166 *P. cinnamomi* isolates analysed (Table 5). South African *P. cinnamomi* isolates were resolved into 48, Australian isolates into 15, and Papua New Guinea isolates into seven different RAPD phenotypes (Table 5).

Regional diversity

Only one RAPD phenotype, phenotype 3 (A2 mating type), occurred in isolates from all the countries analysed (South Africa, Australia, and Papua New Guinea). RAPD phenotypes 10 (A2 mating type), 39 (A2 mating type) and 55 (A1 mating type) occurred in both the South African and Australian populations, whereas RAPD phenotypes 7 (A2 mating type) and 38 (A2 mating type) occurred in both the South African and Papua New Guinea populations. No RAPD phenotypes other than phenotype 3 occurred in both the Australian and Papua New Guinea populations. The overall levels of phenotypic diversity as determined using RAPDs for the different *P. cinnamomi* populations were low for South Africa ($\hat{G}/N = 11.2$) and Australia ($\hat{G}/N = 8.7$), compared to the high level found in the small Papua New Guinea population ($\hat{G}/N = 100.0$) (Table 5). Genotypic diversity for South African ($\hat{G} = 13.6$) and Australian ($\hat{G} = 3.3$) *P. cinnamomi* populations did not differ significantly ($t = 0.138$) from each other.

RFLP analysis

RFLP probe screening and assessment of fragment patterns

Eight of the 15 RFLP probes tested in the preliminary screening showed clearly identifiable polymorphisms, or monomorphic identifiable single or low copy loci, using *Hind*III, *Xho*I, or *Pst*I as restriction enzymes. Some of these loci are illustrated in Figure 1. The eight probes were used in different probe enzyme combinations with the three restriction enzymes to yield a total of 15 loci for the analysis of all isolates. Eleven RFLP loci showed polymorphisms and four were monomorphic with a total of 31 different alleles among a total of 109 *P. cinnamomi* isolates analysed from South Africa, Australia, and Papua New Guinea (Table 4). Based on these analyses, a total of 13 multilocus RFLP genotypes were identified.

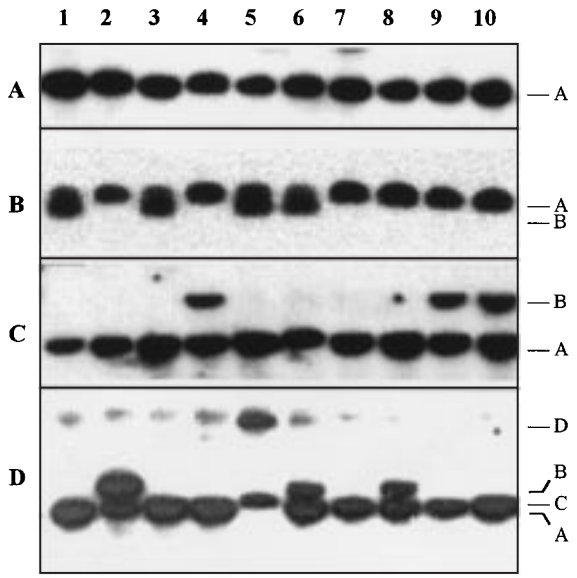

Figure 1. Autoradiograph of Southern blots containing 10 isolates of *Phytophthora cinnamomi* in four probe/enzyme combinations. A, Genomic DNA cut with *Hind*III and hybridised with probe *pPi*120. Only the first locus is shown, pPi120α-1. B, Genomic DNA cut with *Pst*I and hybridised with *pPc*2. C, Genomic DNA cut with *Pst*I and hybridised with *pPc*3. D, Genomic DNA cut with *Hind*III and hybridised with *BipBh*5. Isolates represented in 1–10 in panel A, B, and C are identical: UQ734, T13, C504, CP80, C218, C167, T31, T33, CP517, and CP529. Isolates represented in lines 1–10 of D are: CP468, UQ835, UQ821, UQ820, UQ832, UQ831, UQ819, UQ836, T44, and CP548. On the right, the various alleles are indicated by A, B, C and D and these correspond to the alleles given in Table 4.

Table 4. Alleles present in South African, Australian and Papua New Guinea isolates of *P. cinnamomi* in each of 13 different RFLP genotypes

Genotype	Locus															Mating type	No. of isolates		
	*Hind*III				*Xho*I					*Pst*I							SA[x]	Aus[y]	PNG[z]
	*BipBh*5	*pPc*5	*pPi*120α-1	*pPi*120α-2	*BipBh*5	β-*tubulin*	*pPi*120α	*pADACT*	*pPc*5	β-*tubulin*	*pPc*3	*pPc*2	*pPi*120α-1	*pPi*120α-2	*pPc*10				
1	AA	AA	AA	BB	AA	AA	AA	AB	AA	AA	AA	AB	AA	BB	AB	2	25	18	2
2	AA	AA	AA	BB	AB	AA	AA	BB	AA	AA	AB	AA	AA	BB	AA	2	23	6	1
3	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	AA	AA	BB	AA	1	15	6	0
4	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AB	AA	BB	AB	2	0	1	0
5	AA	AA	AA	BB	AA	AA	AA	AB	AA	AA	AA	AB	AA	BC	AB	2	1	0	0
6	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	BB	AA	BC	AA	1	0	1	0
7	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AB	AA	AA	BB	AA	2	1	0	0
8	AB	AA	AA	BB	AA	AB	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
9	AA	AA	AA	BB	AB	AA	AA	BB	AA	AA	AA	AA	AA	BB	AA	2	0	1	0
10	AA	AA	AA	BB	AC	AA	AA	AB	AA	AA	AA	AB	AA	BB	AB	2	0	4	0
11	CD	BB	AA	BC	AC	CD	AA	BB	BC	AA	AA	AB	AA	BC	AB	2	0	0	1
12	AB	AB	AA	BB	AC	BC	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
13	AB	AA	AA	BB	AC	BB	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
Total no. of isolates																	65	37	7

[x]SA = South Africa; [y]Aus = Australia; [z]PNG = Papua New Guinea.

Table 5. Summary statistics for *P. cinnamomi* populations from South Africa, Australia and Papua New Guinea, based on 16 RAPD markers and 34 RFLP alleles from 15 putative loci

	South Africa	Australia	Papua New Guinea
RAPDs			
No. of isolates	121	38	7
No. of phenotypes	48	15	7
$\hat{G}$[t]	13.6	3.3	7.0
$\hat{G}/N(\%)$[u]	11.2	8.7	100.0
Clonal fraction	60.3	60.5	0
RFLPs			
No. of isolates	65	37	7
Genotypes	1–3, 5, 7	1–4, 6, 9, 10	1, 2, 8, 11–13
$\hat{G}$[t]	3.1	3.3	5.5
$\hat{G}/N(\%)$[u]	4.7	8.9	77.8
A[v]	1.4	1.5	2.1
H_{obs}[x]	0.129	0.155	0.308
H_{exp}[y]	0.105	0.118	0.288
F[z]	−0.229	−0.313	−0.069

[t] $\hat{G}$ = Phenotypic/genotypic diversity (Stoddard and Taylor, 1988).
[u] $\hat{G}/N(\%)$ = The percentage of maximum possible diversity obtained.
[v] A = Mean number of alleles per locus.
[x] H_{obs} = Observed heterozygosity.
[y] H_{exp} = Expected heterozygosity [= Nei's gene diversity index (Nei, 1973)].
[z] F = Wright's fixation index.

Among the 65 South African *P. cinnamomi* isolates analysed, all 15 A1 mating type isolates were of multilocus RFLP genotype 3. The A2 mating type isolates were separated into four multilocus RFLP genotypes, with genotypes 1 and 2 being the most common. The seven A1 mating type isolates analysed from Australia were resolved into two multilocus RFLP genotypes with genotype 3 being the most common. Australian A2 mating type isolates were resolved into five different multilocus RFLP genotypes with genotypes 1 and 2 being the most common. Six of the seven Papua New Guinea isolates were resolved into different multilocus RFLP genotypes, with two isolates belonging to multilocus RFLP genotype 1 (Table 4).

All RAPD phenotypes identified in this study represent their own specific RFLP genotype except RAPD phenotypes 3 (RFLP genotype 1 and 10) and 10 (RFLP genotype 1 and 4). Levels of genotypic diversity as determined using RAPD and RFLP markers are similar in these populations of different size. Combining RAPD and RFLP data does not increase the overall number of phenotypes found.

Regional diversity

P. cinnamomi RFLP genotypes 1 and 2 (A2 mating type) were identified in isolates from all three countries considered (South Africa, Australia and Papua New Guinea). RFLP genotype 3 (A1 mating type) occurred in South Africa and Australia only, whereas RFLP genotypes 4 to 13 were specific to one country only. In total, five RFLP genotypes were identified in South Africa giving rise to a low overall level of genotypic diversity ($\hat{G}/N = 4.7$), seven RFLP genotypes in Australia and a low level of genotypic diversity ($\hat{G}/N = 8.9$), and seven RFLP genotypes in Papua New Guinea ($\hat{G}/N = 77.8$) (Table 5). Levels of genotypic diversity of the South African and Australian *P. cinnamomi* populations did not differ significantly ($t = 0.055$). Levels of gene diversity were low for South Africa ($H_{exp} = 0.105$) and Australia ($H_{exp} = 0.118$) compared to those of the Papua New Guinea isolates ($H_{exp} = 0.288$) (Table 5). Genetic distance between South African and Australian populations was low ($D_m = 0.003$). Genetic distances were larger between the South African and Papua New Guinea population ($D_m = 0.022$), as well as between the Australian and Papua New Guinea population ($D_m = 0.020$).

Sexual reproduction in P. cinnamomi

P. cinnamomi populations from South Africa and Australia appeared to contain a large fraction of clonal lines, as analysed using RAPD and RFLP markers. In contrast, the seven isolates analysed from Papua New Guinea were not clonal at all, with a clonal fraction of zero (Table 5). Levels of observed heterozygosity of South African ($H_{obs} = 0.129$) and Australian ($H_{obs} = 0.155$) *P. cinnamomi* populations were lower than that of Papua New Guinea ($H_{obs} = 0.308$). Fixation indices for all three populations were negative (Table 5). Genetic distances between A1 and A2 mating type populations were similar for South African ($D_m = 0.020$) and Australian ($D_m = 0.025$) populations, but higher for the Papua New Guinea population ($D_m = 0.060$).

The South African and Australian *P. cinnamomi* populations were analysed to test for goodness of fit to an expected Hardy–Weinberg equilibrium at the six polymorphic RFLP loci for each population. In the South African population, five out of six, and in the Australian population, three out of six Hardy–Weinberg analyses, deviated significantly from the assumption of random mating.

Discussion

This study, using RAPD and RFLP markers, revealed low levels of gene and genotypic diversity in the South African and Australian *P. cinnamomi* populations. The levels of gene and genotypic diversity in the South African *P. cinnamomi* populations were similar to those found in a previous study using isozymes (Linde et al., 1997). These are lower than would be expected from a heterothallic, outbreeding organism. Similar results have been obtained for the Australian *P. cinnamomi* population, although levels of observed heterozygosity were slightly lower using isozymes (Goodwin, 1997; Old et al., 1988). These differences in levels of heterozygosity may be partially attributed to the use and/or selection of different markers in this and other studies (Linde et al., 1997; Old et al., 1988).

There are several lines of evidence that indicate the absence or rare occurrence of sexual reproduction in the South African and Australian *P. cinnamomi* populations. These include: (i) low levels of observed heterozygosity, (ii) negative fixation indices which seemed to be fixed for asexual reproduction of heterozygotes, (iii) high levels of genetic distance between mating type populations which is higher than the genetic distance between South African and Australian populations, (iv) populations are highly clonal as determined using RAPDs, and (v) of the six polymorphic loci tested for the South African and Australian populations, five loci in the South African and three loci in the Australian population deviated significantly from Hardy–Weinberg equilibrium and represent non-randomly mating populations. In a previous study using isozymes, it was also suggested that reproduction in the South African *P. cinnamomi* population is predominantly asexual (Linde et al., 1997). Similar results were found for Australian *P. cinnamomi* isolates (Old et al., 1988, 1984).

Negative fixation indices for the South African and Australian populations, indicate a possible excess of heterozygosity in populations. This could be either from disassortive mating or by the predominance of one, or a few, particularly fit heterozygous clonal lines. Asexual reproduction of heterozygous clonal lines is the most likely explanation for the negative fixation index, as was the case for *P. infestans* populations from outside Mexico (Goodwin, 1997; Tooley et al., 1985). Although the fixation index for the Papua New Guinea isolates analysed was also negative, no meaningful conclusions can be drawn from this observation, as very few isolates have been analysed.

Distribution of RFLP genotypes revealed that two A2 mating type RFLP genotypes seem to be widespread and that they occur in all three countries analysed. Common A1 mating type RFLP genotypes occurred only in the South African and Australian *P. cinnamomi* populations. A1 mating type isolates analysed from Papua New Guinea all represented unique RFLP genotypes. Unfortunately, very few isolates from Papua New Guinea have been analysed and it is possible that isolates representing RFLP genotype 3 also occur in Papua New Guinea.

South African and Australian *P. cinnamomi* populations show a similar structure and close relationship. Firstly, the low number of different alleles in the Australian and South African *P. cinnamomi* population found in this and previous studies (Linde et al., 1997; Old et al., 1988, 1984), are indicative of introduced pathogen populations. Secondly, both populations are reproducing predominantly asexually. Thirdly, South African and Australian *P. cinnamomi* populations share many alleles, which is reflected in their low genetic distance ($D_m = 0.003$), indicating the similarity of the two populations. This is even more evident, if it is considered that the genetic distances between mating type populations within both regional *P. cinnamomi* populations is considerably higher than the genetic distance between the populations from the two different continents. South African and Australian *P. cinnamomi* populations also show remarkable similarities when analysed using RAPDs. Low levels of phenotypic/genotypic diversity as well as the high clonal fractions were almost identical for the two populations analysed. In contrast, none of the seven *P. cinnamomi* isolates studied from Papua New Guinea had the same RAPD genotype.

Using RFLPs, genotypic diversity for the South African population was slightly lower, but not significantly different from that for Australia. However, genotypic diversities for both populations were significantly

lower than those for Papua New Guinea. Furthermore, low observed and expected levels of heterozygosity for both regional populations were similar. Different alleles and allelic combinations were found among the seven Papua New Guinea isolates using RFLPs. This suggests that these isolates belong to a different gene pool than the South African and Australian populations. The similarity between the Australian and South African *P. cinnamomi* populations is of direct practical importance to the forestry plantation industry. Disease management by means of host resistance in the two countries, could be similar (Leung et al., 1993) because pathogen populations are similar.

Based on low levels of gene and genotypic diversity, results of the RFLP analyses confirm previous reports that *P. cinnamomi* has been introduced, in recent times, into South Africa (Linde et al., 1997) and Australia (Shepherd, 1975; Old et al., 1984). In addition, based on the striking similarity between the two populations in both countries, we speculate that related introduction or migration events have occurred. However, populations in other parts of the world need to be analysed to confirm or reject this hypothesis in an unambiguous manner. Isolates from Papua New Guinea showed no clonality using RAPDs and high levels of gene and genotypic diversity using RFLPs. This confirms the hypothesis of Old et al. (1984), that Papua New Guinea is probably within the centre of origin for *P. cinnamomi*. Similarly, Shepherd (1975) suggested that the New Guinea/Celebes area is the most likely centre of origin for *P. cinnamomi*, based on mating types occurring in equal proportions and general resistance of native plants to disease. A New Guinea–Malaysia–Celebes region was then later suggested by Zentmyer (1988) as the centre of origin for *P. cinnamomi*. However, a detailed study including large population samples from the New Guinea–Malaysia–Celebes origin is necessary to gain a deeper understanding to the centre of origin for *P. cinnamomi*.

There are several reasons why it is important to know the centre of origin for plant pathogenic fungi. Ample opportunities exist to select for disease resistance in the geographical area known as the centre of origin for a particular pathogen. Such information, also provides further knowledge on quarantine issues, so that spread of a pathogen from the centre of origin should be avoided to restrict distribution of genotypes. The origin of *P. infestans* has been shown to be a single geographical area in central Mexico (Goodwin et al., 1994; Tooley et al., 1985). *P. infestans* has a very narrow host range and its host occurs only in a geographically restricted area in the highlands of central Mexico. In contrast *P. cinnamomi* has a host range of almost 1000 plant species (Zentmyer, 1980). It would, therefore, be expected that *P. cinnamomi* originated within a larger geographical area, or an area representing a rather diverse flora. The New Guinea–Malaysia–Celebes region would probably be an example of such a geographical area. Detailed genetic studies on large populations from this proposed centre of origin are needed to gain insight into the centre of origin of this important plant pathogen (Milgroom and Fry, 1997).

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