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Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers

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Abstract. *Pinus* spp. have a number of fungal endophytes of which the latent pathogen *Diplodia pinea* is a well-known example. In this study, 12 simple sequence repeat (SSR) markers were used to consider the origin of the southern hemisphere isolates and to evaluate genetic diversity and gene flow between populations of *D. pinea*. Three populations were isolated from *Pinus* sp. within the native range of the fungus in the northern hemisphere and three populations were isolated from *P. radiata* in the southern hemisphere. Populations of *D. pinea* exhibited low allelic diversity and appear to be clonal, not only regionally, but also across continents. The origin of the southern hemisphere populations could not be determined, as the same alleles are found in all populations. There was no evidence for genetic drift or fixation of alleles in local populations, and some genotypes were found across continents, suggesting both a long asexual history and considerable movement of this pathogen probably assisted by human activities.

Additional keywords: genotypic diversity, fungal endophyte, *Pinus*, microsatellite marker, population genetics.

Introduction

Diplodia pinea [= *Sphaeropsis sapinea*] is a well-known pathogen causing a shoot or tip blight of numerous pine species and some other conifers (Punithalingam and Waterson 1970). Pines were introduced into southern hemisphere countries shortly after European colonisation, where they were commonly used in early afforestation programs (Burgess and Wingfield 2001). Early plantations were relatively free of pests and diseases. However, rapid expansion of afforestation was soon followed by increased incidence of such problems (Wingfield 1999). *D. pinea* was recorded in South Africa in 1909, where it was the first fungal pathogen found in pine plantations (Lundquist 1987). This fungus has subsequently become well known in the southern hemisphere, and areas prone to hail, drought and waterlogged soils were found to be unsuitable for pine species that were susceptible to *D. pinea* (Empire Forestry Association 1934; Laughton 1937; Thomson 1969; Wright and Marks 1970; Gibson 1979; Burgess and Wingfield 2002).

D. pinea is now also known to be an endophyte in healthy trees and it has been isolated from asymptomatic needles, stems, buds, immature cones and the bracts of mature cones (Petrini and Fisher 1988; Stanosz *et al*. 1995; Smith *et al*. 1996; Burgess *et al*. 2001*a*; Flowers *et al*. 2001). *D. pinea* is not known as a seed endophyte, although it can infect seeds if mature cones are left on the ground (Fraedrich *et al*. 1994). The association between *D. pinea* and conifers (particularly *Pinus* spp.) suggests a common native range, predominantly in the northern hemisphere.

Classical taxonomic studies, together with molecular techniques, resulted in the description of four morphotypes of *D. pinea* (Palmer *et al*. 1987; Smith and Stanosz 1995; Hausner *et al*. 1999; Stanosz *et al*. 1999; de Wet *et al*. 2000; Zhou *et al*. 2001). Burgess *et al*. (2001*b*) developed and tested 11 polymorphic simple sequence repeat (SSR) markers on 40 isolates of *D. pinea,* representing the four different morphotypes. The ‘I’ morphotype isolates were found to be identical to *Botryosphaeria obtusa*. These markers clearly distinguished the remaining three morphotypes and, furthermore, showed the ‘C’ morphotype to be more closely related to the ‘A’ than to the ‘B’ morphotype. The ‘B’ morphotype was the most genetically diverse and the isolates in this group could be further divided, based on their geographic origin. The large variation between the

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A and B morphotypes suggested cryptic speciation and led to a subsequent study using multiple gene genealogies and microsatellite sequence data to compare the different morphotypes. The results supported the description of the 'B' morphotype of *D. pinea* as a new species, now known as *Diplodia scrobiculata* de Wet, Slippers & Wingfield (de Wet *et al*. 2003). All isolates of *D. pinea* collected from *Pinus* spp. outside their natural range belong to the 'A' morphotype or *D. pinea sensu stricto* (Wang *et al*. 1985; Stanosz *et al*. 1999; de Wet *et al*. 2000; Smith *et al*. 2000; Burgess *et al*. 2001*b*; Zhou *et al*. 2001) and it is this species that forms the basis of the present study.

Forest and plantation health depend largely upon the resistance of the host population and the ability of the pathogen population to overcome this resistance. The evolutionary potential of the pathogen has a substantial impact on the durability of host resistance (McDonald 1997; McDonald and Linde 2002). A pathogen with a high evolutionary potential is more likely to breakdown host resistance than a pathogen with a low evolutionary potential. The evolutionary processes that lead to the formation of new genotypes include mutation, reproduction/mating, gene flow, population size and selection. The same evolutionary processes affect genetic structure (McDonald and Linde 2002). Genotype diversity can be resolved using selectively neutral molecular markers (McDonald 1997; Taylor *et al*. 1999*a*; Burgess *et al*. 2001*b*). The structure of a fungal population, as estimated using molecular markers, can be used to predict risk of that pathogen causing epidemics, with sexually reproducing fungi with high gene flow posing the greatest risk (McDonald and Linde 2002). Even for asexually reproducing fungi such as *D. pinea*, diverse pathogen populations resulting from high mutation rates, unrestricted gene flow or large population sizes, could all lead to high disease risk (McDonald and Linde 2002).

In the current study, the SSR markers developed by Burgess *et al*. (2001*b*) were used to evaluate three populations from within the native range of *D. pinea* in the northern hemisphere and three populations of the pathogen from exotic plantations in the southern hemisphere. The northern hemisphere populations were from native and planted trees in Central Europe and North America. Although *D. pinea* is an endophyte, it is seldom present in nursery stock, (Ganley *et al*. 2003) and, as such, genotypes isolated from planted trees should reflect the pathogen diversity of an area as effectively as native trees. The overall objective was to determine the diversity and origin of *D. pinea* in the southern hemisphere.

Methods

Fungal isolates

In an earlier study, *D. pinea* was isolated, using a hierarchical sampling strategy, from *P. radiata* cones collected in Australia, New Zealand and South Africa (Burgess *et al*. 2001*a*). The second level of hierachy consisted of collecting individual cones from individual trees within 100 m^2. Three of these southern hemisphere, introduced populations were used in the current study; 24 isolates from individual *P. radiata* trees at Lewana Plantation near Balingup, Western Australia (WA), 26 isolates collected from individual *P. radiata* trees at George in the Eastern Cape (RSA) and 29 isolates collected from individual *P. radiata* trees at the Matakana Island seed orchard in the Bay of Plenty, New Zealand (NZ).

Three populations were also obtained from within the native range of *D. pinea* in the northern hemisphere. These included 27 isolates from individual *P. sylvestris* trees on the campus of Michigan State University (MI), 29 isolates from various *Pinus* spp. around the Great Lakes region of eastern United States and Canada (GL) and a European (EUR) population consisting of 19 isolates from *P. sylvestris* and *P. nigra* growing in the Alps of Switzerland and 8 isolates from *P. sylvestris* in France.

Deoxyribonucleic acid extraction and SSR-PCR

Fungal cultures derived from single conidia were grown in malt-extract (ME) broth in Eppendorf tubes, after which the broth was removed and the mycelium freeze-dried. Deoxyribonucleic acid was extracted from the dried mycelium following the protocol of Raeder and Broda (1985). SSR-PCR was performed on all isolates with 12 fluorescently-labelled markers, specifically designed to amplify polymorphic regions in *D. pinea*. Eleven of these markers, SS1-11, were previously described (Burgess *et al*. 2001*b*). A twelfth marker, SS12 was also used. The primers (forwards 5′ GTG AAG GGT TCT GCC TGT GT and reverse 5′ GAC TGG GAG GGG AGC ATA TG) amplify a polymorphic region rich in GAT, AT and T repeats using an annealing temperature of 58°C (GenBank accession number AF418599). SSR-PCR was conducted as described previously (de Wet *et al*. 2003).

Fluorescently labelled SSR PCR products were separated on an ABI Prism 377 DNA sequencer as previously described (Burgess *et al*. 2001*b*). Allele size was estimated by comparing the mobility of the SSR products with that of the TAMRA internal size standard (Applied Biosystems) as determined by GeneScan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 2 (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility.

Gene and genotypic diversity

For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele at each of the seven polymorphic loci (e.g. AABDCGD). The frequency of each allele at each locus for complete and clone corrected populations was calculated, and allele diversity determined, using the program POPGENE (Yeh *et al*. 1999) and the equation $H = 1 - \sum x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973). Chi-square tests for differences in allele frequencies were calculated for each locus across clone corrected populations (Workman and Niswander 1970). The Bonferroni correction was applied to significance levels of all χ^2 tests (Weir 1997). The central European population contained too few genotypes to conduct χ^2 tests.

Each genotype was assigned a number and the genotypic diversity (G) was estimated (Stoddart and Taylor 1988) using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the i^{th} phenotype. To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N \times 100$ where N is the population size (Chen *et al*. 1994).

Population differentiation

Population differentiation, theta (θ) was calculated between clone corrected populations in Multilocus (Agapow and Burt 2000) for the data matrix of seven multistate characters using an estimate of Wright's F_{ST}, as $\theta = Q - q/1 - q$ where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Burt *et al*. 1997;

Table 1. Allele size (bp) and frequency at 12 loci (SS1-12) for *Diplodia pinea* populations collected from north-eastern America (GL), Michigan (MI), Europe (EUR), New Zealand (NZ), Western Australia (WA) and South Africa (RSA)

Locus	Allele	GL	MI	EUR	NZ	WA	RSA
SS1	326	1.000	1.000	1.000	1.000	1.000	1.000
SS2	198	0.931	1.000	1.000	0.965	0.875	1.000
	200	0.069	–	–	0.035	0.125	–
SS3	182	1.000	1.000	1.000	1.000	1.000	1.000
SS4	406	1.000	1.000	1.000	1.000	1.000	1.000
SS5	452	–	–	–	0.207	0.125	–
	453	0.310	0.111	0.929	0.621	0.875	0.692
	454	0.345	0.296	0.071	0.172	–	0.307
	455	0.345	0.407	–	–	–	–
	456	–	0.185	–	–	–	–
SS6	237	1.000	1.000	1.000	1.000	1.000	1.000
SS7	383	0.345	–	–	–	–	–
	384	0.655	1.000	1.000	1.000	1.000	1.000
SS8	287	1.000	1.000	1.000	1.000	1.000	1.000
SS9	250	–	0.111	–	0.035	0.333	0.077
	252	0.931	0.704	1.000	0.517	–	0.500
	254	–	0.185	–	–	–	–
	256	0.069	–	–	0.448	0.667	0.423
SS10	313	1.000	0.926	1.000	0.965	0.958	0.807
	315	–	0.074	–	0.035	0.042	0.192
SS11	173	–	0.074	0.107	0.035	0.042	0.192
	174	0.448	0.593	0.892	0.621	0.583	0.346
	175	0.035	0.296	–	0.345	0.375	0.461
	176	0.138	–	–	–	–	–
	177	0.310	–	–	–	–	–
	178	0.069	–	–	–	–	–
	179	–	0.037	–	–	–	–
SS12	475	0.069	–	–	0.241	0.791	0.577
	476	–	–	–	0.276	0.167	–
	477	0.310	0.111	1.000	0.207	–	0.077
	478	0.517	0.703	–	0.276	–	0.231
	479	0.103	0.185	–	–	0.042	0.115
N^A		29	27	28	29	24	26
No. alleles		24	23	14	23	20	21
No. unique alleles		4	3	0	0	0	0
Polymorphic loci		6	5	2	6	6	5
H^B		0.223	0.192	0.027	0.206	0.152	0.218
H (clone corrected)		0.236	0.229	0.074	0.226	0.190	0.225

[A] N = Number of isolates.
[B] H = Gene diversity of the population (Nei 1973).

Weir 1997). If two populations have the same allele frequencies, $\theta = 0$, and this is interpreted as the populations having no differentiation, thus being identical. If two populations share no alleles then $\theta = 1$, and this is interpreted as the populations being completely isolated from each other. The statistical significance of θ was determined by comparing the observed value with that of 1000 randomised datasets in which individuals were randomised among populations. A significant P value ($P < 0.05$) means that the null hypothesis of no population differentiation can be rejected.

Results

Segregation of SSR alleles

The SSR markers produced 32 alleles across the 12 loci examined (Table 1). Isolates of *D. pinea* were monomorphic at six (GL, WA and NZ), seven (MI and RSA) and ten (EUR) loci (Table 1). Loci SS1, SS3, SS4, SS6 and SS8 were monomorphic in all isolates and were excluded from all further analyses. Of the 32 alleles, ten (31%) were present in all six populations and a further 19% were present in at least five of the six populations. There were 24 alleles in the GL population, 23 alleles in both the MI and NZ populations, 21 alleles in the RSA population, 20 alleles in the WA population and 14 alleles in the EUR population (Table 1). Seven alleles were unique to specific populations of *D. pinea* (Table 1). At locus SS9, allele 254 was unique to the MI population of *D. pinea,* as was allele 456 at locus SS5 and allele 179 at locus SS11. At locus SS7, allele 383 was unique to the GL

population of *D. pinea*, as were alleles 176, 177 and 178 at locus SS11.

Both the presence of monomorphic loci and unique alleles influenced measures of gene diversity. The mean gene diversity for all 12 loci across all populations of *D. pinea* was 0.226. With the exception of the Central European population, the gene diversities of the other five populations were relatively similar, ranging from 0.152 to 0.218. In contrast, the EUR population with ten monomorphic loci had an extremely low gene diversity of 0.027.

Genotypic diversity

The genotypic diversity ($\hat{G}$) as estimated from the SSR profiles of isolates varied between populations of *D. pinea* (Table 2). The lowest diversity (5%) was found in the native Central European population of *D. pinea*. The next lowest (21%) value was for an introduced Australian population and moderate diversities of around 30% emerged for the other populations of *D. pinea*. Many of the genotypes of *D. pinea* were shared across the populations (Table 2). For example, genotype MS1 was shared by the majority of isolates representing the Central European population as well as with one isolate from the Michigan, two isolates from the Great Lakes population and five isolates from New Zealand. Three isolates from New Zealand, nine isolates from Australia and one isolate from South Africa represented genotype MS42.

Population differentiation

For the seven polymorphic SSR loci, χ^2 tests indicate significant differences ($P < 0.05$) in gene diversity between the clone corrected populations of *D. pinea* at only five of the loci, SS5, SS7, SS9, SS11 and SS12 (Table 3). Gene diversity did not differ significantly at any loci among the three southern hemisphere populations or between the two American populations (Table 3). Comparison of the northern and southern hemisphere populations resulted in significant ($P < 0.05$) χ^2 values at two loci (Table 4).

Results of the χ^2 tests showed little variation in gene frequencies among the populations of *D. pinea.* This observation can be confirmed by comparing pairs of populations. Theta values indicate that the only significant population differentiation was between the Australian population and the two North American populations and between the South African population and the GL population (Table 5). The Central European population consisted of only three genotypes that differed from each other by one allele at one locus. Because there were so few alleles in this population, θ values are moderately high when comparing this with some of the other populations (e.g. the Australian population). However, although these values are high, they are not significant ($P < 0.05$) and there was no differentiation between the central European and other populations (Table 5).

Table 2. ***Diplodia pinea*** **genotypes as estimated from multilocus profiles generated from the seven polymorphic SSR loci. Genotypes were distributed among populations collected from north-eastern America (GL), Michigan (MI), Europe (EUR), New Zealand (NZ), Western Australia (WA) and South Africa (RSA)**

G	GL	MI	EUR	NZ	WA	RSA
MS1	2	1	23	5		
MS2		1				2
MS3		1				
MS4						1
MS5				1		
MS6			3			
MS7					1	
MS8	2			1		2
MS9				2	2	
MS10					3	2
MS11	3		2			
MS12						3
MS13	1			1		3
MS14					2	
MS15	1					
MS16				3		
MS17	4					
MS18	5					
MS19	1					
MS20		1				
MS21	1					
MS22	4					
MS23		7				
MS24		3				
MS25						2
MS26				1		
MS27		2				
MS28		3				
MS29	3	4		2		
MS30		2				
MS31		2				
MS32				2		
MS33						2
MS34	2					
MS35					1	
MS36				4	1	
MS37						6
MS38				1	1	
MS39					3	
MS40				3		
MS41					1	2
MS42				3	9	1
N^A	29	27	28	29	24	26
N(g)[B]	12	11	3	13	10	11
G[C]	7.38	9.15	1.45	9.86	5.13	9.58
$\hat{G}$[D] (%)	27.3	31.6	5.2	34.0	21.4	36.8

[A] N = Number of isolates.
[B] N(g) = Number of genotypes.
[C] G = Genotypic diversity (Stoddart and Taylor 1988).
[D] $\hat{G}$ = Percent maximum diversity.

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the seven polymorphic SSR loci across clone corrected populations of *Diplodia pinea*, from (A) all populations, excluding central Europe, (B) the three southern-hemisphere populations (NZ, WA and RSA), and (C) the two North American populations (GL and MI)

Locus	Gene diversity (H)					(A)		(B)		(C)	
	GL	MI	NZ	WA	RSA	χ^2	df	χ^2	df	χ^2	df
SS2	0.28	0.00	0.14	0.32	0.00	4.6	4	2.6	2	2.0	1
SS5	0.65	0.72	0.59	0.18	0.46	32.3*[A]	16	6.2	4	2.7	3
SS7	0.37	0.00	0.00	0.00	0.00	11.9*	4	-	-	3.2	1
SS9	0.15	0.51	0.56	0.48	0.56	26.4*	12	2.0	4	5.9	3
SS10	0.00	0.53	0.14	0.18	0.40	4.6	4	2.1	2	1.7	3
SS11	0.68	0.68	0.56	0.58	0.66	30.1*	24	2.2	4	2.4	1
SS12	0.69	0.53	0.74	0.48	0.62	32.7*	16	10.0	8	9.1	6
N	12	11	13	10	11						
Mean	0.41	0.39	0.39	0.32	0.38						

[A]Indicates significant χ^2 values ($P < 0.05$).

Table 4. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the seven polymorphic SSR loci between clone corrected populations of *Diplodia pinea* from the two hemispheres. The northern hemisphere population includes the two American populations and central Europe

Locus	Gene diversity (H)		χ^2	df
	NH	SH		
SS2	0.28	0.00	0.0	1
SS5	0.65	0.72	14.8*[A]	4
SS7	0.37	0.00	3.6	1
SS9	0.15	0.51	11.1*	3
SS10	0.00	0.53	0.5	1
SS11	0.68	0.68	9.3	6
SS12	0.69	0.53	14.6*	4
N	22	25		
Mean	0.44	0.41		

[A]Indicates significant χ^2 values ($P < 0.05$).

Table 5. Pairwise comparisons of population differentiation (theta) among *Diplodia pinea* populations collected from north-eastern America (GL), Michigan (MI), Europe (EUR), New Zealand (NZ), Western Australia (WA) and South Africa (RSA). Values obtained are for clone corrected populations

	GL	MI	EUR	NZ	WA
MI	0.024				
EUR	0.036	0.116			
NZ	0.062	0.056	0.085		
WA	0.211***[A]	0.239***	0.268	0.011	
RSA	0.104**	0.074	0.127	−0.030	0.024

[A]For theta values, asterisks represent level of significance (*** $P < 0.001$, ** $P < 0.01$), no asterisks indicate no significant differentiation between populations.

Discussion

In this study, SSR markers were used to consider the gene and genotypic diversity and population differentiation among six populations of *D. pinea*. Populations of *D. pinea* exhibited moderate to very low gene diversities, with little population differentiation. In addition, many multilocus genotypes are shared between the different populations. All the populations were closely related, but the North American populations were more similar to each other than they were to the southern hemisphere populations. There was low population differentiation among the two North American populations and also among the three southern hemisphere populations. Between the two regions, there was evidence of population differentiation and thus restricted gene flow. Due to the limited diversity of this pathogen in the collections we examined and the lack of unique alleles, the origin of the southern hemisphere populations could not be determined.

Populations of *D. pinea* shared many alleles and there was no evidence of migration from populations with different alleles. Of the 32 alleles amplified by the SSR markers, only four were unique to the North American populations. The lack of allelic diversity in the southern hemisphere populations, which are probably the result of multiple introductions from across the native range of *D. pinea* both in Europe and North America (Smith *et al*. 2000; Burgess *et al*. 2001*a*), emphasises the extremely low genetic diversity of this fungus in the regions so far examined. The Central European population of *D. pinea* was clonal with only three genotypes among 28 isolates. This is particularly interesting as the majority of these isolates were collected from undisturbed forests and plantations across Switzerland and France. Although this collection is not representative of the whole of Europe, the extremely low diversity across hundreds of square kilometres and very diverse terrain compared with the higher diversity in 100 m^2 in an exotic plantation is of great interest.

In undisturbed environments, population differentiation is related to the distance between populations. The closer two populations are to each other, the more likely they are to have similar allelic frequencies (Slatkin 1987; Linde *et al*.

2002). Our results show this to be true for the two North American populations of *D. pinea.* However, there was also no significant population differentiation between the three introduced populations of the fungus in the southern hemisphere. This implies that there has either been considerable exchange among these regions, even though they are separated by oceans, or alternatively, that the origin of the founder genotypes could have been similar for all three southern hemisphere populations. Both of these processes have probably contributed to the observed gene diversities.

All three introduced populations were collected from *P. radiata* in regions with well-established plantation forestry (Burgess and Wingfield 2001, 2002). Introductions of *P. radiata* and other *Pinus* spp. to these regions for plantation development were most likely from similar areas (Burgess and Wingfield 2001). In addition, plant genetic material (seeds and cuttings) for breeding has been exchanged between Australia, South Africa and New Zealand (Burgess and Wingfield 2001). Because *D. pinea* is an endophyte, genotypes of the pathogen would most likely have been distributed with this material, particularly the cuttings (Stanosz *et al*. 1995; Smith *et al*. 1996; Burgess *et al*. 2001*a*; Flowers *et al*. 2001). A single source of a given genotype in forest nurseries in France and Switzerland could perhaps explain the predominance of that genotypes in plantations throughout the region. This would not, however, explain the spread of the same genotype to the native forests of Switzerland.

The similarity of *D. pinea* genotypes and populations across continents suggests that the fungus has had a long asexual history. Clones generally accumulate unique alleles in geographically isolated populations (Taylor *et al*. 1999*b*). As mentioned above, there is no evidence of genetic drift in any of the *D. pinea* populations, suggesting a recent origin of this fungus. However, the close association this endophyte has with pines suggests a long association. Selection for the most ecologically fit genotypes could potentially explain the predominance of certain genotypes and alleles in populations of *D. pinea*. However, because of the high degree of similarity between populations, the selection pressure would have had to be similar in all the regions examined. The external environment, topography, climate and microclimate vary greatly within, and between, regions where isolates were collected. However, if the selection pressure on the fungus is related to its success and stability as an endophyte, then this pressure would be constant regardless of the external environment. Thus a constant selection pressure coupled with prolonged asexual reproduction could explain the low level of genetic diversity in *D. pinea.* Of particular interest is the native Central European population of the fungus, with its extremely low allelic diversity over a wide area of varied terrain. This observation suggests that only a small number of genotypes represent genetically fit endophytes.

In this study, *D. pinea* was found to have both a low gene and genotypic diversity with little variation across continents. *D. pinea* is obviously asexual and there is little evidence of genetic drift. The success of some genotypes as endophytes could possibly explain the low genetic diversity. One of the most important measures used to reduce the impact of disease caused by *D. pinea* is to select tolerant trees for commercial planting. Given the low diversity of this latent pathogen, it is highly likely that tree resistance would be relatively durable (McDonald and Linde 2002).

D. pinea has a world-wide distribution and causes significant losses, particularly of exotic *Pinus* sp. The co-dominant SSR markers used in this study allow for greater resolution of isolate and population diversity and gene flow than has been previously possible with dominant markers such as RAPDs and ISSRs. In the current study of a limited number of populations, *D. pinea* appears to be clonal, not only regionally, but also across continents. Thus, opportunities for breeding and selection of disease-tolerant planting stock should be effective.

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