Three Clonal Lineages of *Phytophthora cinnamomi* in Australia Revealed by Microsatellites

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ABSTRACT

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The genetic structure of populations of *Phytophthora cinnamomi*, a pathogen of an enormous variety of woody plants, was investigated using microsatellites. Three intensively sampled disease sites in southwest Australia were analyzed along with a large culture collection of Australian isolates and some isolates from elsewhere in the world. The mutation in the four microsatellite loci analyzed revealed spatial patterns at the disease sites that correlated with the age of the infestation. Only three clonal lineages were identified in Australian populations and these same clonal lineages were present in worldwide populations, where it is suggested that a limited number of clonal lineages have spread in most regions. No evidence for sexual reproduction between these clonal lineages in Australia has been found even though the pathogen has the opportunity. Instead, mitotic recombination is frequent within the clonal lineages. The implications of this are discussed.

Additional keywords: mitotic crossing over, oomycete.

Clonality is common in fungi and oomycetes because of the prominent role that asexual reproduction plays in their life histories (2). The use of molecular techniques for studying population genetic structure has confirmed many examples of this in fungal pathogens, for example, *Candida albicans* populations in humans (31) and worldwide populations of *Phytophthora infestans* (17), which are both diploid pathogens. In both these organisms, genetic variation that could not be attributed to sexual reproduction has been found and mitotic recombination suggested as a possible cause because of their diploid nature (17,38). However, conclusive evidence for mitotic recombination in natural populations of clonal fungi is inadequate (22), even though it has been observed in the laboratory (21), usually because of a lack of highly polymorphic genetic markers. Genetic variation generated by mitotic recombination may explain some phenotypic variation arising within clonal lineages of diploid fungi and oomycetes.

The diploid oomycete *Phytophthora cinnamomi* Rands is a root and collar rot pathogen of an enormous variety and number of mainly woody plants (37,39). In southern Australia, the dieback disease it causes has a devastating impact on native ecosystems, because many of the dominant plant species show no resistance. *P. cinnamomi* is a hermaphrodite that requires the interaction of isolates of opposite mating type (A1 and A2) to stimulate sexual reproduction although it readily reproduces asexually by mycelial growth and motile zoospores (32). It was most likely introduced into Australia following European settlement and into Europe early in the 19th century (4), with Southeast Asia suggested as the origin of the species from biological and historical perspectives (40).

Investigations on the genetic structure of *P. cinnamomi* populations around the world have relied on the use of isozyme polymorphisms as genetic markers. These investigations have revealed low genetic and genotypic diversity in isolates from most regions in the world. Only three multilocus genotypes were found in Australian isolates, two of A2 mating type and one of A1 mating type, with no evidence for sexual recombination (28,29). These same three multilocus genotypes were found around the world, with only three additional multilocus genotypes of A1 mating type present in global *P. cinnamomi* populations (30). This suggested clonal spread of *P. cinnamomi* through much of the world (30). South African isolates also show low genotypic diversity with isozymes (23) and comparisons of these isolates with Australian isolates by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA techniques indicate common genotypes and a lack of evidence for sexual recombination (24), which further supports the clonal spread hypothesis. However, Papua New Guinea shows high genotypic diversity, as seven additional multilocus genotypes of A1 mating type were found there, hence it is a possible place of origin of *P. cinnamomi* (3,29).

In recent years isozymes have been superseded by microsatellites as the marker of choice for population genetic studies because of their high degree of length polymorphism and ubiquity in eukaryote genomes. We have previously cloned and sequenced a number of microsatellite loci from *P. cinnamomi* (12). We have shown that they exhibit length polymorphism and studied their inheritance in the progeny of sexual crosses. These markers are particularly suitable for population genetic studies of this pathogen. This paper describes their use to test the hypothesis that *P. cinnamomi* is clonal in Australia and in doing so reveals the occurrence of frequent mitotic recombination within clonal lineages.

MATERIALS AND METHODS

Field sampling and fungal culture methods. The three infested sites chosen for intensive sampling were spread across the range of occurrence of *P. cinnamomi* in southwest Australia (Moore River, Buller Reserve, and Gull Rock) (Figs. 1 and 2A). The sites were dominated by *Banksia* woodlands with *B. attenu-*

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ata and *B. ilicifolia* common to all sites, *B. laricina* and *B. menziesii* at Moore River, *B. grandis* at Buller Reserve, and *B. coccinea*, *B. nutans*, and *B. quercifolia* at Gull Rock. These sites had *P. cinnamomi* disease fronts of 300 m to >1 km in length. All three sites had active infection fronts and susceptible species were dying. We chose these active sites because previous sampling from infested sites with deep sandy soil profiles that did not have susceptible species dying showed low rates of recovery of *P. cinnamomi* (<10%) and we wished to recover sufficient isolates for a meaningful genetic study. At each disease front we selected and identified up to 100 diseased plants, mainly *Banksia* spp., and mapped their approximate location. Maps of these sites were initially drawn by pacing distances and subsequently confirmed by reference to archived aerial photographs. From each plant, bark tissue samples were taken from the collar and soil was sampled (approximately 500 g) adjacent to the plant, hereafter referred to as paired plant tissue and soil samples. The implements used to take samples were carefully sterilized between samples. At each front, four diseased plants were selected randomly for more intensive sampling. In proximity to each of the four plants, 10 soil samples, instead of the usual one, were collected to add another level to the hierarchy of sampling.

P. cinnamomi was isolated from plant tissue by surface sterilizing the bark pieces with ethanol then placing slices onto petri dishes of NARPH (nystatin ampicillin rifampicin PCNB hymexazol) medium (20). *P. cinnamomi* was isolated from soil by

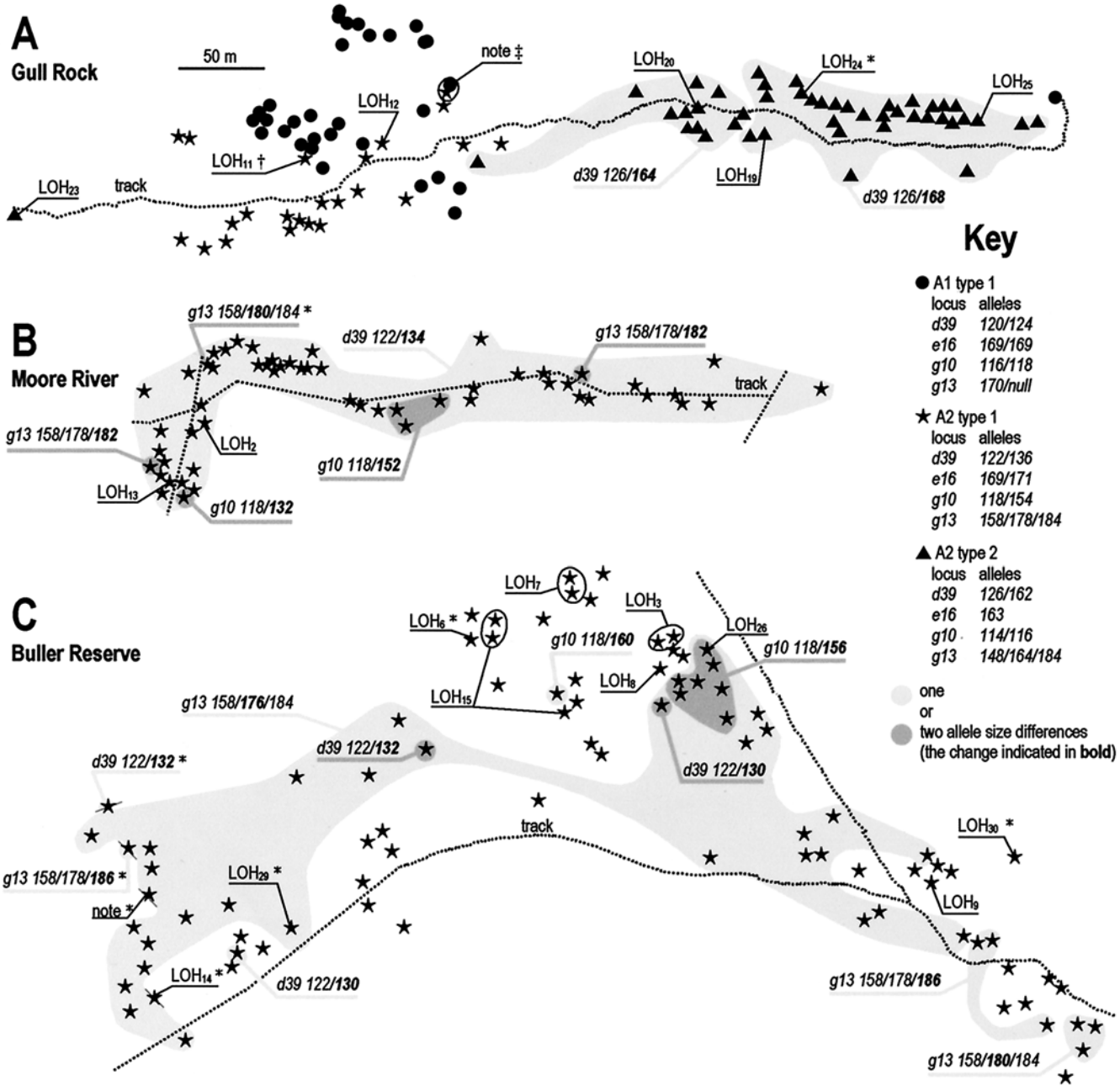

Fig. 1. Site maps indicating the genotypes of *Phytophthora cinnamomi* isolates from the intensively sampled disease sites **A,** Gull Rock, **B,** Moore River, and **C,** Buller Reserve, drawn to a common scale as indicated in **A**. Symbols indicate the position of soil and plant tissue samples from which an isolate was recovered. The loci and allele sizes of the three common microsatellite multilocus genotypes are given in the key. Shading indicates allele size changes but does not imply contiguous mycelium. Loss of heterozygosity (LOH) isolates are numbered to correspond with Table 1 where details of the allelic changes are given. *, isolates from the paired plant tissue and soil samples were members of the same genotype group but differed in allele size or LOH; †, LOH isolates were recovered from one of the set of 10 soil samples taken here, the other samples yielding isolates of the A1 type 1 genotype; and ‡, the soil isolate was A1 type 1 and the plant tissue isolate was A2 type 1.

baiting flooded soil samples for zoospores with *Eucalyptus sieberii* cotyledons as described by Marks and Kassaby (25) and placing infected cotyledons onto NARPH medium (20). Multiple isolations of *P. cinnamomi* were made from five tissue samples from each disease front and also from the four sets of 10 soil samples made at each disease front. These isolates were the second level of hierarchy of the sampling. *P. cinnamomi* that grew out of the plant tissue and cotyledons was transferred to 1.5% corn meal agar (CMA). If isolates were contaminated with bacteria or fungi, they were forced to grow through hard CMA (CMA plus agar to 2.5%), and then subcultured to CMA, which purified 99% of cases. All isolates were examined microscopically for morpho-

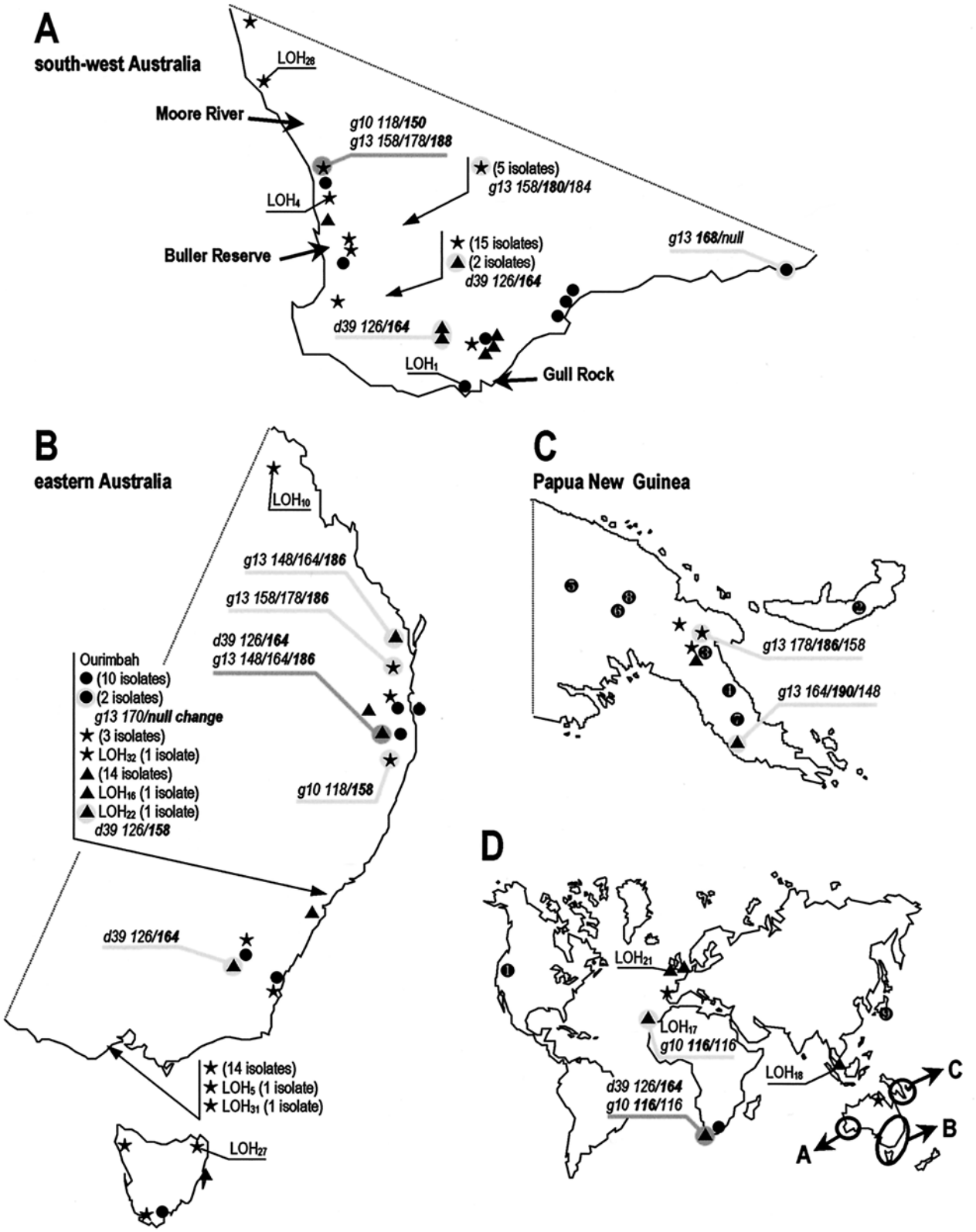

Fig. 2. Geographical maps showing the locations and genotypes of culture collection isolates from **A,** southwest Australia, **B,** eastern Australia, **C,** Papua New Guinea, and **D,** elsewhere in the world. Each symbol represents a single isolate, except where marked. Number 1 through 9 in black circles represent completely different A1 genotypes.

logical characters diagnostic for *P. cinnamomi* including sporangia, and then single hyphal tipped from water agar to CMA to ensure no isolates were mixtures of individuals. Isolates reached this stage in the culture process within 3 to 5 weeks after soil baiting or 1 to 3 weeks from bark, and within an additional 1 week all mycelium had been grown for DNA extraction as described previously (11). Mating type testing of field isolates was performed as described previously (29).

In addition to these hierarchically sampled field isolates of *P. cinnamomi*, culture collection isolates were used. These were from across Australia and Papua New Guinea, the majority from a well-studied culture collection (28,29), and some isolates from elsewhere in the world (Fig. 2).

Genetic analysis. DNA was extracted as described previously (13). Four dinucleotide repeat microsatellite loci were amplified using primer pairs d39, e16, g10, g13, and g13(4), the latter two pairs amplifying a single locus (*g13*) as described previously (12). At this locus, A2 mating type isolates have three alleles, some of which appear to contain a deletion in the primer binding site and so require an alternate primer to amplify them. Trisomy is suspected in these isolates. Isolates with unusual genotypes were analyzed a second time for confirmation; no ambiguities resulted. Three culture collection isolates, A15, A2421, and A2412, each being a different isozyme type (28,29), were used as size standards on all gels in addition to molecular weight markers. The microsatellite allele size of all isolates was carefully compared with these standard isolates, and where necessary, loci were reamplified and electrophoresed on a single gel to accurately reconfirm microsatellite size variation. In total, 790 isolates were analyzed, 647 from the three intensively sampled disease fronts, 116 from the Australian culture collection, 17 from the Papua New Guinea culture collection, and 10 from elsewhere in the world.

Statistical analysis. To test the mode of reproduction (clonal versus recombining), the data sets of multilocus genotypes from the different populations were subjected to randomization tests to compare the observed index of association (I_A) to that generated from 1,000 randomly generated data sets (8) using the computer program Multilocus version 1.2.2 (1) (provided free on the Internet by P.-M. Agapow and A. Burt). The same program was used to test for population differentiation by calculating θ, Weir's formulation of Wright's F_{ST} (36), for the observed and randomized data sets. Randomization of the data sets respected the linkage between loci *d39* and *g13* (12).

RESULTS

Genetic variation at disease sites. The key to understanding the genetic variation of the entire collection of *P. cinnamomi* isolates is seen in the mapped genetic variation of the intensely sampled disease sites. At the Gull Rock disease site (Fig. 1A), the isolates recovered belonged to one of three microsatellite multilocus genotype groups (hereafter referred to as genotype groups). These genotype groups were named A1 type 1, A2 type 1, and A2 type 2 to indicate their mating type and their relationship to previously described isozyme multilocus genotypes (29) described later. These genotype groups are hypothetical, but allow us to more easily describe the complexity of variation found among the populations of *P. cinnamomi*. All microsatellite multilocus genotypes found and the genotype groups to which they belong are listed in Table 1.

At the three intensively sampled sites, no isolates were found to have sexual recombinant genotypes that could have arisen through crosses between the A1 type 1 and A2 type 1 or A2 type 2 genotype groups. The *P. cinnamomi* isolates of different mating types have microsatellite alleles unique to the mating type at two or more loci; hence, sexual recombinant genotypes would be readily identified. We demonstrated this previously with sexual progeny of *P. cinnamomi* (12). Each genotype also had its own consistent growth morphology on agar, which all isolates of that genotype group shared. No morphological variation like that seen among sexual progeny (12) was found in our field isolates from Australia. The lack of sexual reproduction is in spite of the fact that A1 and A2 isolates were recovered from Gull Rock in very close proximity, for example, within 1 m in soil from a set of 10 soil sample isolates (Fig. 1A, †), and from a single dying plant, the soil isolate was A1 and the plant tissue isolate was A2 mating type (Fig. 1A, ‡). Apart from these two examples, all other sets of multiple isolates from either a set of 10 soil samples or from plant tissue samples had identical genotypes within that set with one exception at Moore River. The majority of paired soil and plant tissue isolates also had identical genotypes apart from those pairs from Gull Rock and Buller Reserve, in which the isolates had the same genotype group but with some variation in genotype (Fig. 1A and C, *).

Within the three genotype groups seen among isolates from Gull Rock, two types of variation have been indicated on the map (Fig. 1A). The first is differences in the size of one microsatellite allele compared with the common size for that genotype group. At Gull Rock, two clusters of isolates of A2 type 2 differ at locus *d39*, with the more commonly found *162* allele changed to *164* or *168* in isolates from these clusters. Similar clustering of isolates showing the same microsatellite allele size changes are also seen at the Moore River and Buller Reserve sites (Fig. 1B and C). At both these sites only the A2 type 1 genotype group was recovered. At Moore River all the isolates have allele size changes *d39 122/134*, and layered upon this are clusters of isolates with additional changes of microsatellite allele size at different loci. One of the isolates derived from a set of 10 soil samples at Moore River had the allele size change *g13 158/180/184*, although the other isolates from the same soil sample had the common *g13 158/178/184* genotype. At the Buller Reserve site, a more complex pattern of microsatellite allele size changes can be seen. There are seven different primary size changes with one of these changes, *g13 158/176/184*, representing just under half of the isolates. Layered on that cluster are three additional changes at different loci than the primary allele size change.

Many of these microsatellite allele size changes must have arisen at the sites from which isolates were recovered to form the patterns of clustering and layering of the different microsatellite allele size changes seen on the site maps (Fig. 1). If each individual genotype was a new introduction to a site, then we would expect microsatellite allele size changes to be randomly scattered on the site maps, not layered and clustered as they appear. The changes are also only one or a few repeat lengths removed from the common allele size of the genotype group to which the isolate belongs. That is, differences are of 2, 4, or 6 base pairs, given that all these microsatellites are dinucleotide repeats. The one exception to a small change in allele size is at Moore River where one isolate shows a *g10 118/132* genotype compared with the common *g10 118/154* genotype.

The second type of variation seen within the genotype groups is in isolates with a loss of heterozygosity (LOH). These show the loss of one allele from a locus or loci that is heterozygous in the commonly found multilocus genotype of the group to which the isolate belongs. For example, the gels in Figure 3 show two separate examples of LOH from isolates of different genotype groups. The exact genotype change for LOH isolates indicated on the maps (Figs. 1 and 2) can be seen in Table 1, where the corresponding LOH numbering is used.

Like the microsatellite allele size changes, the LOH must have arisen at the disease fronts from which the LOH isolates were recovered. For example, at Gull Rock, LOH_{23} and LOH_{24} isolates were recovered 700 m apart and have the same genotype at locus *g13* (Table 1), but these losses occurred independently at this site because of the microsatellite allele size difference at locus *d39* between these isolates (Fig. 1A). For this reason, in the three

intensively sampled sites, an independent LOH event was defined as either a single isolate with an LOH genotype spatially distant from other isolates of identical LOH genotype or a number of isolates occurring in a cluster having the same LOH genotype. For the culture collection, an isolate showing an LOH genotype was scored as an independent LOH event. LOH isolates were recovered from both plant tissue samples and soil samples. For example, at Buller Reserve, 17 isolates showing identical LOH genotypes were recovered from one of a set of 10 soil samples as well as from the tissue sample of an adjacent plant (Fig. 1C, LOH_3). At Gull Rock, four LOH isolates were recovered from one set of 10 soil samples (Fig. 1A, LOH_{11}) and 20 LOH isolates were recovered from another set of 10 soil samples (Fig. 1A, LOH_{12}).

LOH was found in all four microsatellite loci examined and in isolates from all three genotype groups. Especially significant was the LOH seen in loci *d39* and *g13*, loci previously shown to be linked (12). In this linkage group illustrated by isolates in Figure 3A, the linked alleles are lost together, resulting in isolates that

TABLE 1. All microsatellite multilocus genotypes of *Phytophthora cinnamomi* recovered, the genotype group to which they are classified, and indications of the independent loss of heterozygosity (LOH) event that some genotypes represent

Collection genotype found in[a]	Genotype group[b]	Independent LOH event[c]	Multilocus genotypes[d]			
			d39	*e16*	*g10*	*g13*
GL/Aust./World	A1 type 1•	…	*120/124*	*169/169*	*116/118*	*170*/null
Aust.	A1 type 1	…	*120/124*	*169/169*	*116/118*	*168*/null
Aust.	A1 type 1	…	*120/124*	*169/169*	*116/118*	*170*/null change
Aust.	A1 type 1	LOH_1	*124*/*124*	*169/169*	*116/118*	*170*/*170*
BU/GL/Aust./PNG/World	A2 type 1★	…	*122/136*	*169/171*	*118/154*	*158/178/184*
BU/Aust./PNG	A2 type 1	…	*122/136*	*169/171*	*118/154*	*158/178/186*
BU/Aust.	A2 type 1	…	*122/136*	*169/171*	*118/154*	*158/180/184*
BU	A2 type 1	…	*122/130*	*169/171*	*118/154*	*158/176/184*
BU	A2 type 1	…	*122/130*	*169/171*	*118/154*	*158/178/184*
BU	A2 type 1	…	*122/132*	*169/171*	*118/154*	*158/176/184*
BU	A2 type 1	…	*122/132*	*169/171*	*118/154*	*158/178/184*
MR	A2 type 1	…	*122/134*	*169/171*	*118/132*	*158/178/184*
MR	A2 type 1	…	*122/134*	*169/171*	*118/152*	*158/178/184*
MR	A2 type 1	…	*122/134*	*169/171*	*118/154*	*158/178/182*
MR	A2 type 1	…	*122/134*	*169/171*	*118/154*	*158/180/184*
Aust.	A2 type 1	…	*122/136*	*169/171*	*118/150*	*158/178/188*
BU	A2 type 1	…	*122/136*	*169/171*	*118/154*	*158/176/184*
BU	A2 type 1	…	*122/136*	*169/171*	*118/156*	*158/176/184*
Aust.	A2 type 1	…	*122/136*	*169/171*	*118/158*	*158/178/184*
BU	A2 type 1	…	*122/136*	*169/171*	*118/160*	*158/178/184*
Aust.	A2 type 1	LOH_5	*122*/*122*	*169/171*	*118/118*	*158*/*184*/*184*
MR/BU/Aust.	A2 type 1	$LOH_{2,3,4}$	*122*/*122*	*169/171*	*118/154*	*158*/*184*/*184*
BU	A2 type 1	$LOH_{14,15}$	*122/136*	*169/171*	*118/154*	*—/178/184*
Aust.	A2 type 1	$LOH_{27,28}$	*122/136*	*169/—*	*118/154*	*158/178/184*
BU	A2 type 1	LOH_{26}	*122/136*	*169/—*	*118/156*	*158/176/184*
BU	A2 type 1	LOH_{29}	*122/136*	*—/171*	*118/154*	*158/176/184*
BU/Aust.	A2 type 1	$LOH_{30,31,32}$[e]	*122/136*	*—/171*	*118/154*	*158/178/184*
MR	A2 type 1	LOH_{13}	*134*/*134*	*169/171*	*118/154*	*158/178*/*178*
BU/GL	A2 type 1	$LOH_{6,7,8,9,11,12}$	*136*/*136*	*169/171*	*118/154*	*158/178*/*178*
Aust.	A2 type 1	LOH_{10}[e]	*136*/*136*	*—/171*	*118/154*	*158/178*/*178*
Aust./PNG/World	A2 type 2▲	…	*126/162*	*163*	*114/116*	*148/164/184*
GL/Aust.	A2 type 2	…	*126/164*	*163*	*114/116*	*148/164/184*
Aust.	A2 type 2	…	*126/162*	*163*	*114/116*	*148/164/186*
PNG	A2 type 2	…	*126/162*	*163*	*114/116*	*148/164/190*
Aust.	A2 type 2	…	*126/164*	*163*	*114/116*	*148/164/186*
World	A2 type 2	…	*126/164*	*163*	*116/116*	*148/164/184*
GL	A2 type 2	…	*126/168*	*163*	*114/116*	*148/164/184*
GL/Aust./World	A2 type 2	$LOH_{16,18,19}$	*126*/*126*	*163*	*114/116*	*164*/*164/184*
World	A2 type 2	LOH_{17}	*126*/*126*	*163*	*116/116*	*164*/*164/184*
GL	A2 type 2	LOH_{20}	*164*/*164*	*163*	*114/116*	*148/164/184*
World	A2 type 2	LOH_{21}	*162*/*162*	*163*	*114/116*	*148*/*148*/*184*
Aust.	A2 type 2	LOH_{22}	*126/158*	*163*	*114/116*	*148/164/—*
GL	A2 type 2	LOH_{23}	*126/162*	*163*	*114/116*	*148/164/—*
GL	A2 type 2	$LOH_{24,25}$	*126/168*	*163*	*114/116*	*148/164/—*
World	❶	…	*126*	*169*	*114/118/120*	*174/190*
PNG	❷	…	*126/144*	*161/169*	*114/116/118*	*154/184*
PNG	❸	…	*126/136*	*161/167/169*	*114/116/118*	*154/178*
PNG	❹	…	*130/146*	*161/167/169*	*116/118*	*154/172*
PNG	❺	…	*126/134/136*	*161/167/169*	*114/116/118*	*152/180*
PNG	❻	…	*126/128/144*	*161/169*	*116/118*	*172/184*
PNG	❼	…	*124/132*	*161/167*	*114/116/118*	*152/180*
PNG	❽	…	*132/144*	*161/169*	*114/116/118*	*154/172/180*
World	❾	…	*122/140*	*167/171*	*116/126*	*158*

[a] GL, Gull Rock; Aust., Australian; BU, Buller Reserve; PNG, Papua New Guinea; and MR, Moore River.
[b] Each symbol corresponds to those used in Figures 1 and 2 and indicates the multilocus genotype that is the most widespread and hypothetically is the genotype from which all other variants in the same group are derived.
[c] LOH numbers correspond to figures and text.
[d] A dash indicates loss of an allele or homozygosity with the remaining allele(s). Genotype changes due to LOH are underlined.
[e] LOH_{32} is isolate A2421 and LOH_{10} is isolate A2120, used in a study of sexual progeny (12).

appear to be homozygous at these loci. Similarly, in all but one (LOH_{20}) of the 19 LOH events involving the alleles of the *d39-g13* linkage group, the linked alleles are lost together (Table 1). One such isolate, A2120 (Table 1, LOH_{10}), was fortuitously used in the production of sexual progeny, which confirmed that the *d39* and *g13* loci were homozygous for the *136* and *178* alleles, respectively (12). Additional evidence for LOH isolates homozygosity is the increase in intensity of the remaining allele at the locus due to greater amplification by polymerase chain reaction, which may indicate the presence of two copies (Fig. 3). LOH was found in only one linkage group at a time in most cases. Only LOH_5 and LOH_{10} from the Australian culture collection (Fig. 2B) had LOH in two linkage groups (Table 1). Consecutive LOH could have formed these isolates.

Genetic variation in culture collection isolates. The same three microsatellite genotype groups are seen in all culture collection isolates from Australia and in a large proportion of the isolates from elsewhere in the world (Fig. 2). The variation found within each genotype group among these isolates is very similar to that found within each intensively sampled site. Both microsatellite allele size changes and LOH are present in no greater amount (quality or frequency) than that observed in any of the three intensively sampled sites.

The Australian culture collections include multiple isolates from four sites (Fig. 2A and B). In isolates from two of these sites, variation within the genotype groups was very similar to that observed among isolates from the three intensively sampled sites in southwest Australia. Of note is the Ourimbah site in eastern Australia from which isolates of all three genotype groups were recovered from a single soil sample with no evidence of sexual recombination in the genotypes.

Also at Ourimbah is an A1 type 1 isolate showing a microsatellite allele size change in the null allele of locus *g13*. Although called a null allele, it is visible (but not assigned a size) using an alternative primer pair for the *g13* locus. This allele can be seen in Figure 3B and missing in the isolate LOH_1 (Fig. 3B; Table 1).

Isolates from elsewhere in the world could also be classified as members of the genotype groups found in Australian isolates. This includes isolates from Papua New Guinea, only of A2 mating type (Fig. 2C), and isolates from Malaysia, South Africa, Canary Islands, and the United Kingdom (Fig. 2D). Again, these isolates show no more variation from the common microsatellite genotype than do isolates of the three intensively sampled sites. The A2 type 2 isolates from South Africa and the Canary Islands show the microsatellite change *g10 116/116*, which may be indistinguishable from LOH at this locus. We have written it as a microsatellite change because the common genotype is so close in size, namely *g10 114/116*.

Isolates from Papua New Guinea, Japan, and the United States (Fig. 2C and D), all of A1 mating type, could not be assigned to any of the three genotype groups we define (Table 1). The microsatellite allele size changes and LOH events could not account for the much greater variation seen among these isolates as it did for isolates within the three genotype groups. Although we believe the allelic interpretation of these isolates to be correct, we cannot be as certain as for the three genotype groups because sexual inheritance of the microsatellite bands has not been studied. The A1 isolate from the United States, A117, was found to have an identical microsatellite profile to isolate A17 from Australia. Prior to our analysis of this culture collection, we were informed that isolate A17 (29) was most probably a wrongly labeled A117 isolate (M. Dudzinski, *personal communication*), and the analysis supports this belief. Given this, our analysis confirms only three genotype groups of *P. cinnamomi* in Australia.

Statistical analysis. The randomization tests using the index of association indicated significant linkage disequilibrium in the multilocus genotypes found at the Gull Rock population, where all three of the genotype groups are represented (Fig. 4). Tests on the multilocus genotypes from Australia and elsewhere in the world (excluding A1 isolates that were not members of the A1 type 1 group from the analysis) also gave significant results ($P < 0.001$) indicating linkage disequilibrium. Again, these populations contain representatives of the three genotype groups. Conversely, tests on the Moore River and Buller Reserve populations, which have only one genotype group represented, were not significant ($P = 1$ and 0.96, respectively).

The comparison of θ of the observed data sets with θ of randomized data sets indicates no population differentiation between the Gull Rock, Australian, and world populations ($P = 0.17$, clone corrected data and excluding A1 isolates that were not members of

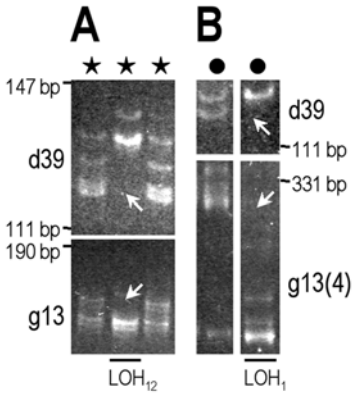

Fig. 3. Gel photographs of microsatellite loci that illustrate loss of heterozygosity (LOH) in two isolates with arrows indicating the position of lost alleles. **A,** Isolates from Gull Rock, one showing loss of the linked alleles *d39 122* and *g13 184*. Another 19 isolates from this set of isolates from a single soil sample showed this LOH (Fig. 1A, LOH_{12}). **B,** A1 culture collection isolates, one showing loss of the linked alleles *d39 120* and *g13* null (Fig. 2A, LOH_1). The null allele at this locus is clearly visible with the primer pair g13(4), but its inheritance in all the cross progeny could not always be scored accurately because of interference with other shadow bands (12), hence it was not routinely scored in the field and culture collection isolates.

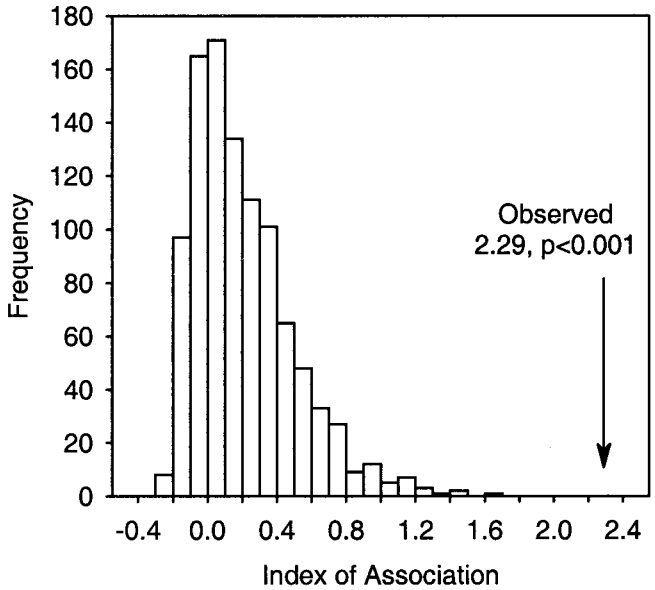

Fig. 4. Randomization test comparing the observed Gull Rock data set's index of association (I_A) with the I_A of 1,000 randomized data sets (8). The observed I_A (arrowed) falls well outside the distribution for the randomized data sets (histograms) indicating a significant correlation of alleles across loci ($P < 0.001$). The data set was clone corrected (only one example of each multilocus genotype found at Gull Rock was included) to ensure a significant result was not simply due to the repeated sampling of predominant genotypes that occurs with an epidemic population structure (26).

the A1 type 1 group from the analysis). Including any or all of the Moore River, Buller Reserve, or Papua New Guinea populations in this analysis caused a significant result indicating population differentiation, again because Moore River and Buller Reserve have only one genotype group represented and Papua New Guinea has significantly different A1 isolates.

DISCUSSION

We believe that the three microsatellite multilocus genotype groups represent clonal lineages of *P. cinnamomi* and that they have a worldwide distribution. The microsatellite allele size changes found within these clonal lineages are due to mutation of the microsatellites and may provide a useful ecological tool for studying *P. cinnamomi*. Furthermore, we suggest that the LOH seen frequently in isolates of these clonal lineages is due to a form of mitotic recombination, mitotic crossing over.

Clonal lineages of *P. cinnamomi*. There are several lines of evidence to support the hypothesis that the three genotype groups represent clonal lineages. First, the changes within a genotype group from isolates Australia-wide and from non-Australian isolates can be explained by the variation seen in a single intensively sampled disease front. None of this variation is due to sexual recombination and there is no evidence of sexual recombination even when the opposite mating types are found in close proximity. There was a similar absence of sexual recombinant genotypes in the Ourimbah *P. cinnamomi* populations when isozymes were used as genetic markers (28), which was confirmed by our microsatellite analysis.

Second, the randomization tests we performed indicated the presence of linkage disequilibrium in populations with representatives of the three genotype groups, as would be expected if there was clonal reproduction. However, with such tests of the mode of reproduction, the analysis should be biased toward recombination if hypervariable loci, like microsatellites (34), are used. Yet with our data, for areas where the three genotype groups are present in a population, the tests strongly support the hypothesis of clonal reproduction. In the two populations in which only one genotype group is present, the tests are not significant because the microsatellite mutation and LOH (hypervariable loci) occurs upon a common underlying multilocus genotype to give the impression of recombination with this test. Adding a single representative of the other genotype groups to the Moore River or Buller Reserve data sets gave significant test results, which further supports the interpretation that it is the genotype groups that are responsible for the significant linkage disequilibrium in the data sets, and hence are the clonal entities.

The lack of population differentiation among the Gull Rock, Australian, and world populations (excluding A1 isolates that were not members of the A1 type 1 group) is also significant in showing that the variation in one disease site can explain the variation seen in potentially much larger and more widespread populations of *P. cinnamomi*. Admittedly, many of the culture collection isolates are not true population samples, but their lack of differentiation certainly supports the hypothesis that the genotype groups are clonal lineages.

Our clonality hypothesis is also supported by modeling of the probabilities of microsatellite band sharing between individuals in predominantly asexually reproducing populations (6,7). Brookfield (6) showed that band sharing out of 10 DNA fingerprint bands could be used to identify clones with greater than 95% probability (when level of mutability of the bands is assumed equal to that found in mini- or microsatellite bands, 0.002 mutations per band per generation). Brown (7) investigated these equations further to show that only six microsatellite bands are required to confirm that two isolates are members of the same clone. These conclusions are valid if the conditions of population size, N, and mutation rate per band per generation, μ, satisfy $2N\mu > 1$ (N = 1,000 and μ = 0.002 in the example given) (7). A lower frequency of sexual reproduction than that used by Brown (7) in his modeling, as would be appropriate for the case of *P. cinnamomi*, would lower the degree of band sharing between individuals of the same clone. However, these equations represent the equilibrium state where some clones would have existed for many generations and mutations would have had considerable time to accumulate. The populations of *P. cinnamomi* would not be in equilibrium given its recent introduction to Australia and probably most continents elsewhere in the world. Therefore, even if the frequency of sexual reproduction is lower, clonality could still be concluded on the basis of microsatellite band sharing. The four microsatellite loci characterized here, with six to nine microsatellite bands (depending on the genotype group), are therefore sufficient to demonstrate clonality.

These three genotype groups that we believe represent clonal lineages directly corresponded with the isozyme types of Old et al. (29). Seventy-nine isolates of the culture collection that we analyzed were previously analyzed with isozymes so we named the genotypes similarly (A1 type 1, A2 type 1, and A2 type 2). In addition to the 763 Australian isolates analyzed with microsatellites, a further 382 Australian isolates from diverse sampling sites belong to the three isozyme types of Old et al. (28,29) and are presumably members of the same three clonal lineages.

The clonal lineages of *P. cinnamomi* found in Australia are also found elsewhere in the world. If the isozyme groupings of

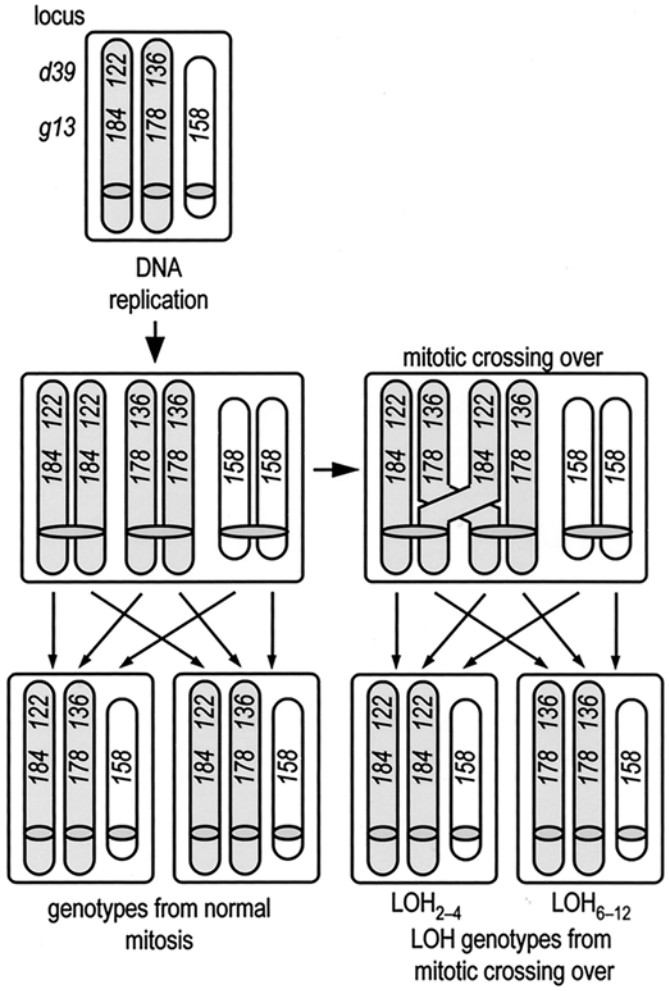

Fig. 5. A model of mitotic crossing over compared with normal mitosis in the *d39-g13* linkage group of clonal lineage A2 type 1. The loss of heterozygosity (LOH) events illustrated by this model correspond to figures, table, and text.

Oudemans and Coffey (30) correlate with clonal lineages, as do those of Old et al. (28,29), then the majority of *P. cinnamomi* isolated worldwide belong to only a few clonal lineages. Even more significant is that all A2 isolates analyzed by Oudemans and Coffey (30) belong to the two A2 clonal lineages described here: cinn4 equates to A2 type 1 and cinn5 equates to A2 type 2.

The A1 isolates from Papua New Guinea analyzed here are clearly not related clonally. The microsatellite data are consistent with their classification as seven different isozyme types (29) rather than only two isozyme types (30) and also consistent with high genotypic diversity shown with RFLP (24). These isolates have very different microsatellite genotypes and could only be clonally related if they diverged in ancient times. Of the 11 A1 isozyme types isolated worldwide (29,30), seven are from Papua New Guinea, which clearly supports the suggestion (3,29) that Papua New Guinea may be within a center of origin of *P. cinnamomi*.

Microsatellite mutation. The changes in microsatellite allele sizes within the three genotype groups are consistent with the microsatellite mutation process because both the more frequent stepwise changes and less frequent larger size changes have been seen in other genetic studies (16). In our case, because *P. cinnamomi* is a soilborne organism, the changes in allele size form a clustering pattern that is seen when the genotypes are mapped. These patterns are consistent with the ecology of *P. cinnamomi*, that is, its slow spread through soil either as mycelial growth or by zoospore motility in surface water (19).

There are other aspects of ecology for which the mutation in these microsatellite markers may prove useful. The complexity of changes mapped at the three sites should increase with the age of the infestation. From both anecdotal evidence of human activity and from historical aerial photography, we know that the infestation at the Moore River site dates from the 1960s, the Gull Rock site is a little older, and the Buller Reserve site is the oldest of all (first half of the 20th century). This anecdotal evidence is consistent with the patterns of complexity seen in the genotype maps of these three sites.

As well as indicating the relative age of a site infestation, the microsatellite markers may reveal the origins and vectors of isolates within a clonal lineage. This process would require more markers to be developed and perhaps the application of Bayesian statistical methods as is used in assigning parentage or population source in animal studies using microsatellites (15,27). An application could be to determine the effectiveness of hygiene measures used to stop the spread of *P. cinnamomi* into uninfested areas by monitoring the source of isolates causing an infestation. We have applied these markers to one such application to determine if trees in an experimental trial were dying from the *P. cinnamomi* used to inoculate them or from an advancing disease front that overwhelmed the experimental plot following an abnormal rainfall event. The inoculum isolate and the disease front isolate were of the same clonal lineage but could be distinguished by allele size differences, resulting in the definitive answer that the trees were dying from the disease front isolate (A. Lucas, *personal communication*).

Frequent mitotic crossing over. Mitotic crossing over is the most likely explanation for the LOH found in isolates within the three clonal lineages of *P. cinnamomi*. A model of how such a cross-over event would occur in the *d39-g13* linkage group is shown in Figure 5. At this locus, in 18 of the 19 separate LOH events, the linked alleles were lost together which supports the hypothesis that a more substantial method of mitotic recombination (crossing over) has occurred rather than a more limited recombination event like gene conversion. With gene conversion, smaller sections of the genome would be rendered homozygous, as seen in *P. sojae* following sexual reproduction (10), rather than sections in this linkage group which are at least 8 to 21 centimorgans in length (12). The one exception to the linked alleles being lost together, LOH_{20}, could be explained by a cross-over occurring between the *d39* and *g13* loci, with the *d39* locus distal to the centromere, resulting in LOH at locus *d39* only.

It is unlikely that sexual reproduction by selfing could explain the LOH seen. Selfing in a diploid could theoretically lead to LOH. However, in all but two LOH isolates, the unlinked loci remained heterozygous. With selfing, one would expect these loci to be homozygous on average half of the time. In addition, many of the LOH events have occurred in sites where both mating types are not detected with very intensive sampling, such as Moore River and Buller Reserve. For selfing to occur at those sites, external agents like *Trichoderma* spp. would be required to stimulate sexual reproduction (4). However, LOH also occurs in the A1 mating type, which is not stimulated into sexual reproduction by external agents. Finally, none of the 201 oospore progeny that germinated in the laboratory resulted from selfing (12). The few putative selfed progeny of A2 isolates that germinated in the laboratory grew slowly, differed morphologically from their parents, and did not survive more than one subculture (I. C. Tommerup, *unpublished data*). The LOH isolates from the field were morphologically indistinguishable from common or progenitor isolates of the same clonal lineage and were isolated from soil and dying plants, hence they were ecologically fit and pathogenic.

Mitotic crossing over is a frequent phenomenon in *P. cinnamomi*. The LOH isolates resulting from it have been identified in the three clonal lineages from numerous locations across Australia and elsewhere in the world. The pattern of appearance of the LOH isolates in both the intensively sampled sites and culture collection isolates suggests that mitotic recombination occurs numerous times, rather than a few times with those isolates being subsequently spread. Mitotic crossing over is not an aberration of culture. *P. cinnamomi* isolates with LOH genotypes were recovered multiple times (e.g., LOH_{12}) and from paired samples of tissue and soil that require quite different isolation techniques. Also, the time between isolation of *P. cinnamomi* and culture of mycelium for DNA extraction was relatively short, 2 to 6 weeks, at least for the isolates from the three disease fronts.

The LOH seen at locus *e16* in isolates A2120 LOH_{10} and A2421 LOH_{32} (Table 1), both of which were used in the production of sexual progeny, cannot be explained by mitotic crossing over. This locus in these isolates is hemizygous or heterozygous for a null allele, not homozygous as in the *d39-g13* linkage group (12). The majority of isolates of the A2 type 1 clonal lineage are heterozygous (*169/171*), but isolates were found showing LOH at locus *e16* of both possible genotypes (Table 1). If these isolates were also hemizygous, whole or partial chromosome loss at mitosis could give rise to these isolates. The alternative explanation is the independent formation of *e16* null alleles due to mutation in the priming site of the microsatellite locus. Chromosome loss at mitosis appears possible given nondisjunction of chromosomes at meiosis resulting in some progeny inheriting fewer than their diploid complement of chromosomes as suggested by the analysis of sexual progeny (12) and meiotic nondisjunction seen in other *Phytophthora* spp. (9). However, microsatellite mutation at this locus could also explain the LOH seen in A2 type 1 isolates (though not in isolates A2120 and A2421). A one-step mutation would render locus *e16* homozygous and indistinguishable from the hemizygous condition. A similar situation is seen in the *g10* locus of the A2 type 2 isolates from South Africa and the Canary Islands (Fig. 2D). The homozygous *g10* locus in these isolates could have resulted from either mitotic crossing over or microsatellite mutation.

The consequences of clonality and mitotic recombination. The hypothesis for three clonal lineages of *P. cinnamomi* in Australia and the presence of these lineages elsewhere in the world is not without precedent in the genus *Phytophthora*. Clonality was demonstrated in *P. infestans*, but in that case, the introduction of new isolates of both mating types resulted in a

sexually reproducing population that displaced the original clonal lineage in Europe (33). In contrast, both mating types of *P. cinnamomi* coexist in Australia with apparently no sexual interaction. In the field, sexual reproduction may be unfavorable, oospores may fail to germinate or the progeny fail to survive. These three explanations for the lack of sexual reproduction could be environmental rather than genetic factors. However, from an evolutionary perspective, sexual reproduction may be disadvantageous in these clonal lineages. The breaking up of coadapted genes within these clonal lineages by meiosis may not be favorable because of the low selection pressure exerted by the highly susceptible vegetation. In a study of evolving yeast populations, experimental evidence supported the hypothesis that sexual reproduction is useful for removing deleterious genes in stable environments, but if the organism is adapting to new environments, there is no fitness advantage in sexual reproduction (41). Hence, clonality is an advantage for introduced pathogens. If frequent mitotic recombination is effective in purging the genome of deleterious recessive mutations as may be its function (32), then there also may be no fitness advantage in sexual reproduction in stable environments. However, we should not discount the likelihood that clonality in Australian and worldwide populations of *P. cinnamomi* is a result of genetic bottlenecks in the historical spread of the pathogen from its place of origin. In its place of origin, *P. cinnamomi* would likely be genetically diverse and sexually active as was *P. infestans* (35).

Clonality in a diploid organism like *P. cinnamomi* does not necessarily mean genetic uniformity. The variation in phenotypic traits found within the clonal lineages of *P. cinnamomi* like pathogenicity (14) may have a genetic cause that arose by mitotic crossing over. This could occur if genes affecting pathogenicity have dosage effects so that homozygotes are more pathogenic than heterozygotes. Just as deleterious mutations can be purged by mitotic recombination, advantageous mutations can become homozygous. So mitotic crossing over can give rise to new genetic variation, though not with the same order of magnitude as sexual reproduction which can recombine mutations arising in different lineages.

What is the true significance of mitotic recombination in *P. cinnamomi*? It remains to be seen how significant it is in the disease causing potential of this pathogen. It certainly was important to recognize mitotic recombination for our identification of clones of *P. cinnamomi* and the absence of sexual reproduction. In *C. albicans*, cryptic sex was given as an explanation for lower levels of linkage disequilibrium than expected in clonal populations, mainly due to reduced heterozygosity in parts of the genome (18). Mitotic recombination was subsequently recognized as an explanation for the observations (38). Mitotic recombination may have some evolutionary advantage such as purging deleterious mutations or providing limited genetic variation without the genome-wide disruption of meiosis.

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