Genetic variability in the canker pathogen fungus, *Gremmeniella abietina*. 2. Fine-scale investigation of the population genetic structure

Xiao-Ru Wang, Richard A. Ennos, Alfred E. Szmidt, and Per Hansson

Abstract: Genetic variation at 32 polymorphic random amplified polymorphic DNA loci was analysed in the ascomycete canker pathogen fungus *Gremmeniella abietina* (Lagerb.) Morelet collected from one plantation of *Pinus contorta* Dougl. ex Loud. in northern Sweden. The genetic variability maintained in the *G. abietina* population was high. Many different multilocus genotypes were found on each tree and in each sampling site within the plantation. The clonal fraction of the population was small, and identical genotypes were found either on the same tree or branch or on trees in the same sampling site. The finding of very few widely distributed clones suggests that the effective dispersal of asexual spores is limited to a few metres and resulted in small clusters of clones in local sites. Analysis of molecular variance revealed that 45.3% of the total variation was attributable to differences among isolates within trees, 22.5% to trees within sites and 32.3% to sampling sites differences. Allele frequencies at most of the loci varied significantly among the sampling sites and average total genetic diversity over the 32 loci was 0.27 indicating high population subdivision. The factors that could have contributed to the observed population structure were discussed.

Key words: *Gremmeniella abietina*, genotype and clone distribution, population subdivision, RAPD.

Résumé : Les auteurs ont analysé la variation génétique de 32 lieux RAPD polymorphes chez le champignon pathogène ascomycète *Gremminiella abietina* (Lagerb.) Morelet causant un chancre, et récoltés dans une plantation de *Pinus contorta* Dougl. ex Loud. dans le nord de la Suède. La variabilité génétique maintenue dans la population du *G. abietina* est élevée. Ils ont trouvé plusieurs génotypes à lieux multiples différents sur chaque arbre et sur chaque site d'échantillonnage dans la plantation. La fraction clonale de la population est faible et des génotypes identiques n'ont été trouvés soit sur le même arbre ou branche ou sur des arbres du même site d'échantillonnage. La constatation qu'il y a très peu de clones largement distribués suggère que la dispersion réelle des spores asexuées est limitée à quelques mètres et conduits à de petits foyers de clones sur des sites localisés. L'analyse de la variation moléculaire montre que 45,3% de la variation totale est attribuable aux différences entre les isolats du même arbre, 22,5% à des arbres de même site et 32,3% à des sites d'échantillonnage distincts. La fréquence des allèles sur la plupart des lieux varie significativement parmi les sites d'échantillonnage et le F_{ST} moyen pour les 32 lieux est de 0,27 indiquant une forte sub-division de la population. Les auteurs discutent les facteurs qui pourraient influencer la structure de population observée.

Mots clés : *Gremminiella abietina*, distribution des génotype et clone, sub-division de la population, RAPD.
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Introduction

Analysis of population spatial patterns in pathogens is necessary for identification of the source of infection, predicting the spread of a disease across locations, and studying local extinction and recolonization (Real and McElhany 1996). Many pathogenic fungi go through alternating cycles of sexual and asexual reproduction in nature. The degree of sexual versus asexual reproduction and the dispersal of sexual and asexual spores have profound effects on the spatial structure of fungal populations. In pathogens where asexual reproduction is prevalent and dissemination of asexual spores is limited to short distances, clusters of clones form in the field (McDonald and Martinez 1990; Anderson and Kohn 1995; Goodwin et al. 1995).

The development of DNA-based markers has provided powerful tools for population genetic studies in fungal populations. With large number of genetic markers clonal individuals can be identified without ambiguity, and the distribution of genotypes or clones can be mapped in the field. Diverse population genetic structures have been reported for different pathogen fungi ranging from highly random mating to almost strictly clonal (Smith et al. 1992; Kohli et al. 1995; Chen and McDonald 1996). In addition, pathogen populations can be highly subdivided on a local level or at large geographical scales (Gordon et al. 1992; Boeger et al. 1993; Milgroom and Lipari 1995). For example, in populations of *Mycosphaerella graminicola* (McDonald and Martinez 1990), the majority of genetic variation was distributed in areas as small

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as a few square metres, and the field population was highly subdivided into a mosaic of unrelated genotypes or clones. On the other hand, populations of *Crumenulopsis sororia* (Ennos and Swales 1991) were found to be less differentiated on a larger geographic scale. The spatial distribution of clonal genotypes can also differ dramatically among fungal species. For example, clones of *Sclerotinia sclerotiorum* (Anderson and Kohn 1995; Kohli et al. 1995) were found dispersed and spatially mixed in the field. On the other hand, clones of *Armillaria* species were found in discrete patches with very localized distribution in the field (Smith et al. 1992; Anderson and Kohn 1995). Therefore, to reveal the genotypic distribution pattern in a fungal population, the spatial scale used for sampling must be properly adjusted according to the reproductive biology of the fungus. For example, if asexual spores are dispersed only over short distances (e.g., centimetres or metres), the clonal structure of a population may not be adequately represented if sampling is conducted at a macroscale (Chen and McDonald 1996).

Gremmeniella abietina (Lagerb.) Morelet is a haploid, ascomycete fungus that infects several conifer species and causes shoot dieback and stem canker (Donaubauer 1972; Skilling 1972). In Sweden, the fungus has caused severe damage to plantations of the introduced *Pinus contorta* (Dougl.) (Karlman et al. 1994). *Gremmeniella abietina* can reproduce both sexually and asexually resulting in two types of fruiting bodies: apothecia and pycnidia. The abundance of apothecia varies in different environments (Roll-Hansen and Roll-Hansen 1973) and is generally high in northern Sweden (Karlman et al. 1994). Observations on physical dispersal of *G. abietina* spores are not conclusive. It has been observed that the release of ascospores and conidia from the fruiting bodies requires rain or very high humidity, and they are mainly dispersed by rain splash (Skilling et al. 1986; Laflamme and Archambault 1990). On the other hand, ascospores are also known to be dispersed in dry weather by wind (Skilling et al. 1986). Very little is known about the survival of spores after dissemination and how far they may be dispersed yet retain biological activity. Given these uncertainties, it is difficult to anticipate the scale on which spatial structuring of genetic diversity might be evident. Several previous authors have utilized DNA markers to assign taxonomic groupings or infer genetic relatedness, among geographically diverse collections of *G. abietina* isolates (Hamelin et al. 1993, 1996; Hansson et al. 1996; Hellgren and Högberg 1995), but almost all have ignored variation and genetic structure at the level of individual populations. Furthermore, in most cases, sample sizes were not adequate to measure within population variability.

In a previous study, a set of random amplified polymorphic DNA (RAPD) markers was developed for *G. abietina* (Wang 1997). These markers proved to be stable and revealed sufficient genetic variation in *G. abietina* to study local populations in detail. Compared with other types of DNA markers, RAPDs have the advantages of technical simplicity and the ability to provide large numbers of polymorphic loci. The dominant character of most RAPD markers prevents their use for genotype inference in diploid organisms (Szmidt et al. 1996). However, analysis of RAPD variation is particularly well suited for genetic studies of haploid organisms such as *G. abietina*, since there is no loss of genetic information caused by the dominant inheritance of RAPDs. The results of the previous study demonstrated that analysis of RAPD variation can yield information on mating system, clone identification, and gene and genotypic diversity in *G. abietina* (Wang 1997). However, the full potential of the technique in population structure studies can only be realized if applied to collections of isolates that have been sampled in a systematic manner. In the present report we focus on the fine-scale population structure of the fungus and determine the spatial patterns of genotype or clone distribution, as well as the hierarchical partitioning of the genetic variation in the sampled field.

Materials and methods

Field sampling and fungal isolates

A heavily infected *P. contorta* plantation in northern Sweden was selected for the experiment. A plot of 300 × 200 m was marked in the plantation. Five sampling sites located approximately at the corners and the centre of the plot were selected (Fig. 1). Within each sampling site two to five infected trees were identified. Trees were 1–5 m apart (Fig. 1). A total of 20 trees were selected. On trees A1 and A4, two and three branches were sampled, respectively. For the other 18 trees, one branch was sampled from each tree. Two to 18 pycnidia were collected from the sampled branch(es) on each tree. Pycnidia that were selected from the same branch were usually 3–4 cm apart. *Gremmeniella abietina* isolates were obtained from single pycnidia. The isolation procedure and culture conditions were described previously (Hansson et al. 1996; Wang 1997). A total of 126 single pycnidium isolates were made from the field collection distributed on 20 host trees (Fig. 1).

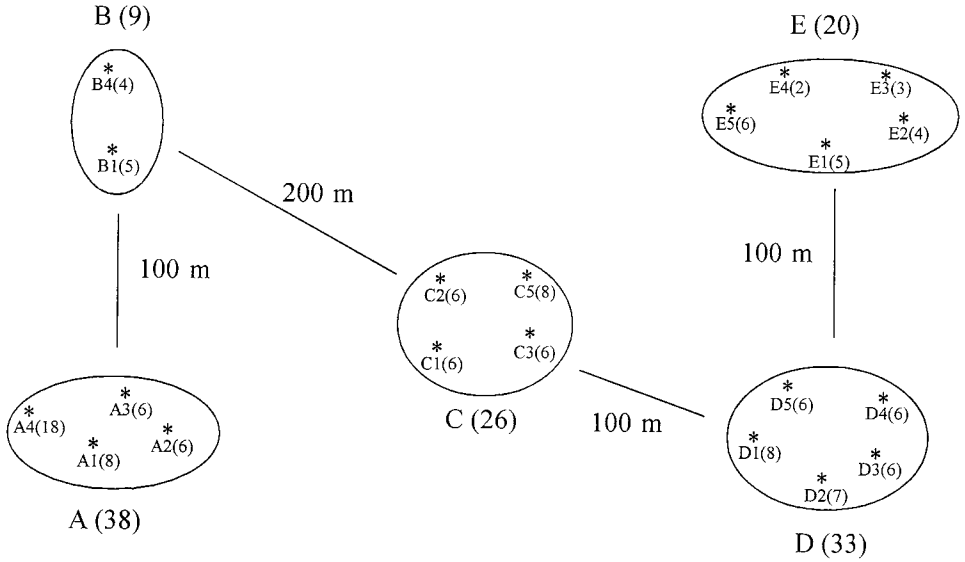
RAPD analysis

DNA from each isolate was extracted from actively growing mycelium by the method described previously (Hansson et al. 1996). Polymerase chain reaction (PCR) amplifications were carried out in a thermal cycler PTC 100 (MJ Research Inc. USA) programmed for an initial denaturation of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. The primer extension step at 72°C of the final cycle was extended to 5 min. The RAPD reaction mix consisted of 10 ng of fungal DNA, 150 μM of each dNTP (Pharmacia, Sweden), 0.2 μM primer, and 0.5 U of Taq DNA polymerase (Pharmacia, Sweden), in a total volume of 25 μL. Primers used in the experiment were 10-base random primers from Operon Technologies (Alameda, Calif.). Seven primers (OPA-04, OPA-10, OPA-12, OPA-13, OPA-20, OPX-12, and OPX-13) were used for RAPD variation analysis in the *G. abietina* isolates. The PCR products were resolved by electrophoresis on 1.4% agarose gels in 0.5 × TBE buffer. The gels were stained with ethidium bromide and RAPD profiles were visualized under UV light. The RAPD amplifications with each of the primers were repeated twice in the present study to identify the reproducible RAPD fragments. Only the strong and reproducible fragments within the size range of 500–2000 base pairs were scored.

Data scoring and gene diversity analysis

Amplified RAPD fragments were named according to the primer used and their molecular weight in base pairs. Each distinct and reproducible RAPD fragment was regarded as a putative locus. This assumption was supported by the lack of RAPD fragments in repulsion among haploid individuals in the population (see Results). Two alleles were scored for each locus: 1 for the fragment presence and 0 for its absence. Isolates from the same sampling site in the field were regarded as one group (Fig. 1). Thus, the 126 isolates

Fig. 1. Distribution of the five sampled groups of host trees in a *P. contorta* plantation. Values in parentheses indicate the number of *G. abietina* isolates from each tree and group. Asterisks show location of the sampled trees in each group. Distances between trees within a group ranged from 1 to 5 m.



were divided into five groups (A–E) by their locations in the field. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.99 in at least one group. In the data analysis, only the polymorphic loci were used. Loci were analysed individually and then combined to form a multilocus genotype, which is the combination of the alleles present at each locus for each isolate. Isolates with the same multilocus genotype were regarded as clones.

Allele frequencies at each locus were determined for each group of isolates and compared by a heterogeneity chi-square test. Nei's (1978) unbiased measure of gene diversity (h) was calculated for each locus and each group. The above computations were performed using release 1.7 of the BIOSYS-1 program (Swofford and Selander 1981). The input data file for BIOSYS-1 was prepared by diploidizing the haploid data to homozygotes at all loci. This input data manipulation does not affect the allele frequency estimates. However, the chi-square statistics from BIOSYS-1 were divided by two to correct for the fact that *G. abietina* is a haploid organism. The total gene diversity at each locus was further partitioned into differences within and among groups (F_{ST}) according to the method of Weir and Cockerham (1984). The probability tests (Fisher's exact test) for F_{ST} at each locus were performed using the Markov chain method (Guo and Thompson 1992; Raymond and Rousset 1995*a*). The parameter settings for the Markov chain analysis were 1000 dememorizations, 50 batches, and 1000 iterations per batch. Estimations of F_{ST} and the probability tests were performed using the GENEPOP version 2.0 computer program (Raymond and Rousset 1995*b*).

Genotype and clone identification and genotypic diversity

Each multilocus genotype detected in the isolates was given a consecutive number as genotype ID. To evaluate the ability of identifying genotypes and clones with the RAPD loci analysed, two parameters, P_{gen} and P_{se}, described by Parks and Werth (1993) were employed. P_{gen} is the probability that consecutively sampled samples originating from different sexual events would, by chance, share the same multilocus genotype. For haploid organism, the original equation presented by Parks and Werth (1993) was modified to the following:

$$[1] \quad P_{gen} = \Pi p_i$$

where p_i is the frequency in the population of allele at ith locus present in a genotype. The allele frequencies used in this computation were based on 126 isolates. The second parameter P_{se} is the probability that an isolate will be sampled somewhere in the population that, although it originates from sexual reproduction, shares the same multilocus genotype as another isolate in the same population. P_{se} is defined as

$$[2] \quad P_{se} = 1 - (1 - P_{gen})^G$$

where G is the number of distinct genotypes in the population (Parks and Werth 1993).

Genotypic diversity was quantified by a normalized Shannon's diversity index as described in Goodwin et al. (1992):

$$[3] \quad H_s = \frac{-\Sigma P_i \ln P_i}{\ln N}$$

where P_i is the frequency of ith multilocus genotype and N is the sample size. Values for H_s range from 0 to 1. The maximum possible value for H_s occurs when each individual in the sample has a different genotype.

Analysis of molecular variance (AMOVA)

Genetic distance, calculated as the Euclidean metric (Excoffier et al. 1992), was computed for all pairs of the 126 isolates. The Excoffier's metric distance between two isolates was defined as

$$[4] \quad E = n\left(1 - \frac{n_{xy}}{n}\right)$$

where n is the total number of polymorphic fragments and n_{xy} is the number of fragments shared by two isolates. This distance is equivalent to the number of fragments not shared between two isolates. The analysis of molecular variance (AMOVA) procedure (Excoffier et al. 1992) was used to estimate variance components for RAPD genotypes and for partitioning the variation among isolates within host trees, among host trees within groups, and among groups. Levels of significance for variance components were computed by the nonparametric permutational procedure using the WINAMOVA program (Excoffier et al. 1992), with 1000 permutations.

Table 1. Measures of genetic variability at 32 loci in five groups of *G. abietina* isolates collected from one plantation of *P. contorta*.

Group	Mean sample size per locus[a]	Mean no. of alleles per locus[a]	Polymorphic loci[b] No.	Polymorphic loci[b] %	Gene diversity[a,c]
A	38.0 (0.0)	1.8 (0.1)	25	78.1	0.303 (0.034)
B	9.0 (0.0)	1.6 (0.1)	19	59.4	0.255 (0.042)
C	26.0 (0.0)	1.7 (0.1)	22	68.8	0.256 (0.038)
D	33.0 (.00)	1.3 (0.1)	8	25.0	0.060 (0.025)
E	20.0 (0.0)	1.8 (0.1)	27	84.4	0.277 (0.032)
A + B + C + D +E	126.0 (0.0)	2.0 (0.1)	32	100	0.291 (0.031)

[a]Values are mean, with SE given in parentheses.
[b]0.99 criterion.
[c]Unbiased estimate (Nei 1978).

Results

Gene diversity and differentiation among groups

In the previous analysis, 32 polymorphic RAPD fragments were scored in *G. abietina* with the 7 selected primers (Wang 1997). The validity of assigning each of the 32 reproducible RAPD fragments as a putative locus was tested by searching for fragments produced by the same primer that are always in repulsion within a population of haploid individuals. Three to seven fragments were scored for different primers, which resulted in 3–21 pairwise fragment combinations per primer. The total number of possible pairwise fragment combinations for the 32 scored fragments was 64. Among the 64 combinations, only 4 pairs of RAPD fragments failed to show coexistence in any of the haploid individuals in the population. Three of these four combinations involved fragments occurring at very low frequencies (0.024–0.040). In these four cases, the probability for the occurrence of both fragments in an individual was 0.0009, 0.0032, 0.0054, and 0.117 respectively. With these low detection probabilities, it is difficult to assume that each pair of these RAPD fragments are truly mutually exclusive and are the products of the same locus but with different sizes. Thus, these fragments were also regarded as different putative loci.

In this study we analysed the genetic variability within and among five groups of *G. abietina* isolates collected at five sampling sites within a plantation at these loci. Among the 32 loci, 6 loci were polymorphic in all the groups. Within each group, the number of polymorphic loci varied from 8 to 27 (Table 1). Chi-square tests for heterogeneity among groups revealed that, except for one locus, allele frequencies in the 5 groups were significantly different among groups at all the other 31 loci ($P < 0.01$) (data not shown). Nei's unbiased measure of gene diversity in the whole field population was 0.29 and varied from 0.06 to 0.30 among groups (Table 1). Except for group D, which had the lowest percentage of polymorphic loci and gene diversity, all the other four groups had similar and high values for variability (Table 1). The partitioning of the total gene diversity within and among groups revealed significant differentiation among the groups. Average F_{ST} for the 5 groups over the 32 loci was 0.27. Single-locus F_{ST} estimates were reasonably consistent over loci and highly significant ($P < 0.001$) values were observed at 24 of the 32 loci (Table 2). When only one representative

Table 2. Summary of *F* statistics at 32 loci for the five groups of *G. abietina* isolates and the significance tests, based on the method of Weir and Cockerham (1984).

Locus	$F_{ST}(126)^a$	P^b	$F_{ST}(85)^c$	P^b
OPA20-1200	0.292 07	< 0.000 01	0.226 10	0.000 94
OPA20-1600	0.236 18	< 0.000 01	0.099 54	0.035 82
OPA20-1700	0.201 88	0.000 26	0.179 42	0.002 86
OPA10-600	0.370 38	< 0.000 01	0.320 86	< 0.000 01
OPA10-770	0.275 09	< 0.000 01	0.220 04	0.001 10
OPA10-800	0.377 06	< 0.000 01	0.226 87	0.000 30
OPA10-900	0.303 02	< 0.000 01	0.140 09	0.006 60
OPA10-1000	0.326 21	< 0.000 01	0.177 28	0.001 82
OPA10-1050	0.435 37	< 0.000 01	0.290 70	< 0.000 01
OPA10-1350	0.257 03	< 0.000 01	0.175 22	0.003 74
OPA4-1200	0.232 01	< 0.000 01	0.291 94	< 0.000 01
OPA4-1250	0.174 88	0.000 14	0.139 70	0.007 78
OPA4-1500	0.140 78	0.006 08	0.286 62	0.002 04
OPA13-650	0.127 83	0.001 62	0.069 00	0.076 80
OPA13-1050	0.129 19	0.001 20	0.048 94	0.133 72
OPA13-1150	0.188 35	0.000 12	0.173 30	0.004 42
OPA13-1200	0.181 52	0.000 78	0.161 28	0.009 60
OPA12-970	0.083 55	0.017 66	0.024 92	0.186 42
OPA12-1300	0.109 11	0.007 82	0.150 73	0.007 48
OPA12-1350	0.077 69	0.034 22	0.071 08	0.062 50
OPA12-1700	0.200 37	< 0.000 01	0.219 89	0.000 52
OPX13-570	0.050 87	0.062 36	0.022 70	0.213 48
OPX13-1450	0.240 35	0.000 04	0.209 96	0.000 76
OPX13-1580	0.098 02	0.010 58	0.045 43	0.132 86
OPX13-1700	0.348 96	< 0.000 01	0.165 87	0.003 70
OPX13-1900	0.419 59	< 0.000 01	0.349 26	< 0.000 01
OPX12-535	0.174 25	0.000 38	0.101 97	0.028 20
OPX12-550	0.375 49	< 0.000 01	0.347 35	< 0.000 01
OPX12-660	0.185 92	< 0.000 01	0.109 62	0.023 96
OPX12-830	0.350 44	0.000 36	0.327 11	0.000 46
OPX12-1550	0.431 15	< 0.000 01	0.253 05	0.000 12
OPX12-1600	0.236 34	0.000 24	0.161 28	0.009 74
Mean	0.273 13		0.190 50	

[a]F_{ST} based on 126 isolates.
[b]F_{ST} exact test (Fisher's method).
[c]F_{ST} based on 85 genotypes.

Table 3. Eighty-five different multilocus genotypes of *G. abietina* encountered in the sampled population and their estimated probability of re-encounter.

Genotype ID	P_{gen}	P_{se}	Genotype ID	P_{gen}	P_{se}
1	3.63×10^{-8}	3.09×10^{-6}	44	1.48×10^{-10}	1.26×10^{-8}
2	1.82×10^{-8}	1.55×10^{-6}	45	3.85×10^{-10}	3.27×10^{-8}
3	6.11×10^{-8}	5.19×10^{-6}	46	7.36×10^{-9}	6.25×10^{-7}
4	5.67×10^{-9}	4.82×10^{-7}	47	6.41×10^{-5}	0.005 434
5	2.18×10^{-9}	1.86×10^{-7}	48	1.00×10^{-9}	8.54×10^{-8}
6	2.39×10^{-10}	2.03×10^{-8}	49	2.61×10^{-9}	2.22×10^{-7}
7	6.21×10^{-10}	5.28×10^{-8}	50	4.69×10^{-7}	3.98×10^{-5}
8	5.08×10^{-7}	4.32×10^{-5}	51	8.10×10^{-9}	6.89×10^{-7}
9	1.95×10^{-8}	1.66×10^{-6}	52	1.29×10^{-7}	1.10×10^{-5}
10	1.24×10^{-8}	1.06×10^{-6}	53	1.12×10^{-8}	9.53×10^{-7}
11	8.15×10^{-6}	0.000 692	54	6.42×10^{-8}	5.46×10^{-6}
12	4.57×10^{-5}	0.003 875	55	9.83×10^{-7}	8.36×10^{-5}
13	3.15×10^{-7}	2.68×10^{-5}	56	1.61×10^{-7}	1.37×10^{-5}
14	1.43×10^{-6}	0.000 122	57	0.000 101	0.008 522
15	1.48×10^{-7}	1.26×10^{-5}	58	2.20×10^{-6}	0.000 187
16	1.70×10^{-8}	1.44×10^{-6}	59	0.000 153	0.012 915
17	5.00×10^{-8}	4.25×10^{-6}	60	7.26×10^{-8}	6.17×10^{-6}
18	9.97×10^{-8}	8.47×10^{-6}	61	5.18×10^{-5}	0.004 393
19	3.20×10^{-7}	2.72×10^{-5}	62	6.62×10^{-5}	0.005 608
20	1.61×10^{-7}	1.36×10^{-5}	63	3.34×10^{-6}	0.000 284
21	5.32×10^{-7}	4.52×10^{-5}	64	0.000 104	0.008 797
22	2.78×10^{-6}	0.000 237	65	0.000 158	0.013 332
23	3.26×10^{-7}	2.77×10^{-5}	66	4.50×10^{-5}	0.003 816
24	5.37×10^{-7}	4.57×10^{-5}	67	2.86×10^{-6}	0.000 243
25	8.64×10^{-6}	0.000 734	68	1.44×10^{-7}	1.23×10^{-5}
26	3.48×10^{-6}	0.000 296	69	8.74×10^{-8}	7.43×10^{-6}
27	1.90×10^{-9}	1.62×10^{-7}	70	5.20×10^{-8}	4.42×10^{-6}
28	1.50×10^{-5}	0.001 276	71	1.21×10^{-10}	1.03×10^{-8}
29	5.78×10^{-6}	0.000 492	72	7.10×10^{-9}	6.03×10^{-7}
30	2.97×10^{-6}	0.000 252	73	4.25×10^{-7}	3.61×10^{-5}
31	7.70×10^{-6}	0.000 654	74	4.16×10^{-7}	3.54×10^{-5}
32	3.67×10^{-7}	3.12×10^{-5}	75	4.97×10^{-9}	4.23×10^{-7}
33	2.69×10^{-7}	2.29×10^{-5}	76	3.99×10^{-6}	0.000 339
34	3.79×10^{-7}	3.22×10^{-5}	77	5.85×10^{-9}	4.97×10^{-7}
35	1.46×10^{-7}	1.24×10^{-5}	78	1.19×10^{-9}	1.01×10^{-7}
36	3.52×10^{-7}	2.99×10^{-5}	79	5.98×10^{-8}	5.09×10^{-6}
37	2.42×10^{-6}	0.000 206	80	2.23×10^{-7}	1.90×10^{-5}
38	7.12×10^{-8}	6.05×10^{-6}	81	7.70×10^{-7}	6.54×10^{-5}
39	9.32×10^{-7}	7.92×10^{-5}	82	1.12×10^{-7}	9.48×10^{-6}
40	2.74×10^{-6}	0.000 233	83	4.51×10^{-10}	3.83×10^{-8}
41	4.27×10^{-7}	3.63×10^{-5}	84	1.48×10^{-8}	1.25×10^{-6}
42	3.94×10^{-6}	0.000 334	85	1.55×10^{-8}	1.31×10^{-6}
43	5.92×10^{-6}	0.000 503			

of each genotype was included, the average F_{ST} for the five groups became 0.19 (Table 2). When group D was eliminated from the whole data set, the average F_{ST} for the other four groups decreased to 0.13.

The ability to identify genotypes and clones

With the 32 loci analysed, a total of 85 different multilocus genotypes were detected in the sampled 126 isolates. Among the 85 different genotypes, 66 genotypes were detected only once (unique genotypes) and 19 were detected more than once. Estimation of the probability that identical genotype can be produced by chance through different sexual events (P_{gen}) proved to be extremely low (Table 3). Most of the genotypes had their P_{geb} at the magnitude of less than 10^{-5}. The highest P_{geb} was 0.000 158 (Table 3, genotype ID 65). Similar low probability was found for P_{se} (Table 3), the probability of re-encounter of a genotype in a random mating population. Of all the 85 genotypes, only 2 genotypes (genotype ID 59 and 65) had $0.01 < P_{se}$ 0.01 < 0.05. All the other genotypes had their P_{se} at negligible levels (Table 3).

Genotypic diversity and genotype distribution

A summary of the genotype composition on each tree and genotypic diversity measures for each group is presented in

Table 4. Genotype composition on each tree and genotypic diversity (H_s) for each group.

Host tree	No. of isolates	No. of different genotypes	No. of unique genotypes[a]	No. of nonunique genotypes[b]	Genotypic diversity (H_s)
Group A					
A1	8	4	2	2	
A2	6	5	4	1	
A3	6	5	4	1	
A4	18	14	11	3	
Total	38	27	21	6	0.86
Group B					
B1	5	5	5	0	
B4	4	4	4	0	
Total	9	9	9	0	1.00
Group C					
C1	6	4	3	1	
C2	6	5	4	1	
C3	6	5	4	1	
C5	8	6	4	2	
Total	26	20	15	5	0.90
Group D					
D1	8	2	0	2	
D2	7	3	1	2	
D3	6	2	0	2	
D4	6	4	2	2	
D5	6	2	0	2	
Total	33	10	3	7	0.56
Group E					
E1	5	5	5	0	
E2	4	4	4	0	
E3	3	3	3	0	
E4	2	2	2	0	
E5	6	5	4	1	
Total	20	19	18	1	0.98
A + B + C + D + E	126	85	66	19	0.87

[a]Genotypes that were detected only once in the sampled population.
[b]Genotypes that were detected more than once in the sampled population.

Table 4. Large variation in genotype composition was found among host trees. On six trees (B1, B4, E1, E2, E3, and E4), every isolate represented a unique genotype that was detected only once in the whole field population (Table 4). The opposite situation was found on trees D1, D3, and D5, on which no unique genotypes were found, and each isolate represented a member of a different genotype. When summarized for each group, except for group D, the majority (75–95%) of the genotypes present within each group were unique. In contrast, most (30 of 33) of the isolates from group D represented 7 genotypes, and only 3 isolates were unique genotypes (Table 4). After correction for the sample size of each group, the genotypic diversity (H_s) varied from 0.56 to 1.00 among groups. The lowest H_s was found in group D and all the other four groups had very high H_s values (Table 4). The average genotypic diversity in the whole field population was 0.87.

The distribution of the 19 genotypes that were detected more than once is presented in Table 5. The majority of these genotypes had very low frequency, with only two or three members present in the population. Only one genotype was found 12 times in the whole collection. Thirteen of the 19 (68.4%) genotypes had their members on the same tree (branch). When multiple branches were sampled for trees A1 and A4, the members of identical genotypes were all located on the same branch. Only 6 of the 19 genotypes had their members distributed on two trees in the same group that were only a few metres apart (Table 5, Fig. 1). None of the genotypes were distributed on more than two trees, and no genotype was shared among different groups. Even the most frequent genotype, detected 12 times in the field population, was found only on two adjacent trees (Table 5).

Variance components of RAPD genotypes by AMOVA

From the Euclidean distance matrix between all pairs of the 126 isolates, AMOVA partitioning of variance components revealed highly significant ($P < 0.001$) differences at all levels (Table 6). Of the total genetic variation, 32.27% was attributable to differences among groups, 22.45% to differences among host trees within groups, and 45.28% to differ-

Table 5. Distribution of the 19 genotypes that were detected more than once in the sampled population.

Genotype ID	No. of times detected	Found on host trees
1	2	A4, branch 1
6	2	A4, branch 2
11	3	A4, branch 3
15	4	A1, branch 1
17	2	A1, branch 2
19	4	A2, A3
37	3	C1
44	2	C2
46	2	C3
51	2	C5
56	2	C5
57	5	D3, D4
58	3	D3, D4
59	3	D4, D5
62	3	D5
63	2	D5
64	12	D1, D2
65	2	D1, D2
84	2	E5

ences among individual isolates within host trees (Table 6). AMOVA analysis was also performed on a data set excluding group D. The remaining data set consisted of 93 isolates from 15 trees in 4 groups. Results from this computation revealed a much lower among-group variance component (5.43%) and higher isolates within tree (65.23%) and among trees within group (29.34%) variance components (Table 6). When only one representative of each genotype was included, AMOVA analysis revealed that 13.75% of the total genetic variation was attributable to differences among groups, 20.88% to differences among host trees within groups, and 65.36% to differences among genotypes within host trees (Table 6).

Discussion

Genetic variability and population subdivision

The present study revealed a high degree of genetic variation in *G. abietina* isolates collected from one plantation of *P. contorta*. Gene and genotypic diversity over 32 loci was high for all groups except for group D. As discussed previously (Wang 1997), this high genetic variability in *G. abietina* distributed within a small area was probably the result of a high degree of sexual reproduction (Karlman et al. 1994) and a diverse founder population. Gametic disequilibrium tests in the previous study revealed a low level of association among loci and suggested a high degree of random mating in the sampled *G. abietina* population (Wang 1997).

Partitioning of the total gene diversity within and among groups revealed significant differentiation among the five groups (F_{ST} = 0.27). Chi-square tests showed that significant differences in allele frequencies existed among the five groups for 31 of the 32 loci analysed. AMOVA analysis based on the Euclidean distance metric between all pairs of the 126 isolates further demonstrated that differences among the 5 groups were highly significant, and 32.27% of the total genetic variation was attributable to group differences, which is comparable with the F_{ST} value. Taken together, these data suggest that the sampled *G. abietina* population was subdivided. The F_{ST} observed in this set of material is much higher than that reported for another canker pathogen, *Crumenulopsis sororia*, collected over a larger geographic scale (Ennos and Swales 1991). However, compared with other studies on microgeographic variation, much higher G_{ST} (F_{ST}) values were reported for *M. graminicola* collected from a field plot of only 40 × 40 m (McDonald and Martinez 1990).

As discussed by other authors several factors can contribute to the subdivision in pathogen populations (McDonald and McDermott 1993; Anderson and Kohn 1995). One of these is selection for different alleles and genotypes in different locations (Brown and Wolfe 1990; Ennos and McConnell 1995). However, this is very unlikely to be the cause for our samples, since all the sampling sites are in close proximity in one field and environmental heterogeneity is not evident. Thus, differential selection is unlikely to act on such a small scale on the putatively neutral markers employed in the present study. The low variation in F_{ST} among loci does not give any indication of selection acting on any particular locus. Thus, if selection was involved it would have to act at all loci and to result in very similar degree of subdivision. This seems very unlikely.

Founder effects and limited gene flow among locations in a field are the other factors that can contribute to the population subdivision. During the initial infection, different locations in the field may have been by chance colonized by different genotypes causing random differences in allele frequencies, especially in small groups. When coupled with limited gene exchange among locations, population subdivision would be observed (Gordon et al. 1992). However, as discussed by Slatkin (1987, 1993), clear evidence for the influence of these two factors can be difficult because, in most cases, current levels of gene flow cannot be distinguished from historical effects. Interpretations are subjective because it is not possible to determine with certainty whether gene flow is rare or whether populations are approaching equilibrium from a recent colonization (Milgroom and Lipari 1995). In fact, the establishment of the fungus in the investigated *P. contorta* plantation was recent, three or four generations (Wang 1997). Therefore, the observed population subdivision could be due to founder effects, restricted gene flow among groups, or both. Even in the presence of gene flow, a certain degree of population subdivision may still be observed in a newly established population that has not yet reached equilibrium. Nonetheless, we suggest that before the time of sampling gene flow had not been strong enough to eliminate the random differences in allele frequencies among groups that had been introduced by the founder events.

A high degree of asexual reproduction and clonal propagation of a limited number of the founder genotypes would also increase population subdivision (Burdon and Roelfs 1985). This is well illustrated in the present study by the effect of group D on the overall estimation of population subdivision. In contrast to other groups, group D was dominated by a few clones and showed the lowest gene and genotypic

Table 6. Analysis of molecular variance (AMOVA) for three sets of data: (I) 126 isolates of *G. abietina* collected from 20 host trees in five groups; (II) excluding group D, i.e., 93 isolates from 15 host trees in four groups; (III) 85 genotypes distributed on 20 host trees in five groups.

Source of variation	Data set	df	SS deviation	MS deviation	Variance component[a]	P^b
Among groups	I	4	245.999	61.500	2.035 (32.27)	<0.001
	II	3	59.295	19.765	0.287 (5.43)	0.019
	III	4	82.929	20.732	0.727 (13.75)	<0.001
Among trees within groups	I	15	172.445	11.496	1.416 (22.45)	<0.001
	II	11	138.918	12.629	1.549 (29.34)	<0.001
	III	15	117.691	7.846	1.104 (20.88)	<0.001
Among isolates within trees	I	106	302.731	2.856	2.856 (45.28)	<0.001
	II	78	268.669	3.444	3.444 (65.23)	<0.001
	III	65	224.698	3.457	3.457 (65.36)	<0.001

[a]Percentage of the total variance is given in parentheses.

[b]Probability of having a more extreme variance component than the observed value by chance, computed by nonparametric procedure from 1000 random permutations.

diversity. When this group was excluded, both F_{ST} and AMOVA partitioning among groups decreased dramatically, from 0.27 to 0.13 for F_{ST} and from 32.27 to 5.43% by AMOVA. Thus, the group D with substantial clonal structure contributed largely to the observed significant population subdivision in *G. abietina*. Although the cause of the observed population subdivision in *G. abietina* cannot be determined unequivocally from the above analysis, it seems reasonable to conclude that the recent establishment of the fungus in the plantation and limited gene flow among locations are the main factors responsible for the observed population subdivision. Increased asexual reproduction in some places in the field may have further contributed to the differences among groups.

Spatial distribution of genotypes and clones

To analyse the spatial patterns of genotypic distribution, it is essential that sexually reproduced genotypes and asexually produced clones can be identified without ambiguity. With the 32 loci analysed in the present study, the probability of a particular 32-locus genotype being produced by chance through different sexual events is extremely low. In addition, the probability of a genotype being re-encountered in a sexually reproducing population, i.e., the probability to mistake two true sexual individuals as clones, is also extremely low. Therefore, the different multilocus genotypes detected in the sampled population can be regarded as true individual genotypes and identical genotypes found on the same tree or adjacent trees can be safely regarded as clones.

In organisms with a mixed mode of reproduction, the observed population structure represents the outcome of both sexual and asexual reproduction as well as the dispersal of sexual and asexual spores. As a canker pathogen, *G. abietina* has the potential for a significant level of asexual reproduction (Hellgren and Barklund 1992). Canker caused by *G. abietina* survives for several years and generations are overlapping (Skilling 1972; Kurkela and Norokorpi 1979). Thus, more clones would be expected to occur repeatedly if asexual reproduction was common in the sampled field (Milgroom et al. 1992). However, among the 85 different genotypes, only 19 were detected more than once in the population. In addition, except for 1 genotype, most of the other 18 genotypes had only 2 or 3 members. Therefore, the clonal fraction in the sampled population is much smaller than the sexual component. When the genotype distribution was examined for different groups over the sampled field, it appeared that the distribution of clones was not uniform over the field. In group D, more pronounced clonal structure was found indicating that asexual reproduction can become significant in some places and microsite variation in the degree of sexual versus asexual reproduction may occur. Physical mapping of these 19 genotypes revealed very limited clonal distribution. The members of identical genotypes were found either on the same branch or a maximum of two trees away within the same group. No clones were distributed on more than two trees. The finding of few widely distributed clones provided genetic evidence for clonal dispersal via rainsplash dissemination of the conidiospores being restricted to only a few metres and resulting in localized small clusters of clones in the field.

In contrast to the nonrandom and restricted clonal distribution, isolates from the same tree or branch usually represented different genotypes. Similar findings were reported for *M. graminicola* (McDonald and Martinez 1990) and *Stagonospora nodorum* (McDonald et al. 1994) in which different genotypes and (or) haplotypes were found in isolates on the same leaf of the host plants. In both cases, airborne sexual ascospores were thought to be the main source of initial infection and responsible for the observed multiple fungal genotypes on the same leaf. In the present study, when the clonal fraction was excluded and only the sexually reproduced genotypes were analysed for spatial differentiation, AMOVA partitioning revealed substantial genotypes within tree variance component (65.36% of the total). The AMOVA analysis was based on genetic distances between multilocus genotypes. Therefore, in the presence of assortative mating, genotypes from the same branch or tree and closely located trees would tend to show greater similarity, which in turn would decrease the variance components for within tree and among trees within group. The finding of significant variation among genotypes within trees suggests that the distribution of sexual individuals in the field was more random

compared with the distribution of clones. However, both F_{ST} (0.19) and the AMOVA partitioning among groups (13.75%) for the sexual genotypes still gave statistical significant estimates, which may indicate that a certain degree of family structure existed in the sexual fraction of the sampled population. The accurate estimation of the degree of family structure present in the investigated *G. abietina* population cannot be elucidated from the present analysis. Samples used in this study were collected from only five sites in one plantation. Sampling over a larger area could provide more information about the spatial structure in *G. abietina* populations. In addition, continued examination of changes in the population over time and additional analysis of populations from different environments with different degree of sexual or asexual reproduction will shed new light on our understanding of the development of family and clonal structure in pathogen populations.

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