Genotypic Diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra

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ABSTRACT

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Sphaeropsis sapinea is the most important pathogen of *Pinus* spp. in South Africa. The fungus, which reproduces only asexually, occurs on exotic *Pinus* spp. In this study, the diversity of the *S. sapinea* population in South Africa was compared with a population from Northern Sumatra. The populations for both countries were obtained from exotic *Pinus patula* plantations. The phenotypic diversity of these populations was assessed using vegetative compatibility tests. The percent maximum genotypic diversity, based on Stoddart and Taylor's index, for the South African population was 30.5% compared with 1.5% for the Northern Sumatran population. Based on the number of phenotypes, the South African *S. sapinea* population was significantly more diverse ($P = 0.05$) than that of the Northern Sumatran population. The results indicate that the population of *S. sapinea* in South Africa has, in all likelihood, arisen as a result of introductions of the fungus on pine seeds imported from various parts of the world during the last century.

Additional keywords: *Diplodia pinea*, vegetative compatibility groups

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton in Sutton (=*Diplodia pinea* (Desmaz.) J. Kickx fil.) is the most important pine pathogen in South Africa, causing serious annual losses due to dieback after hail (14–18). *S. sapinea* occurs on pines, which are exotic in the country, suggesting that it may be an introduced pathogen. The pathogen has been present in South Africa since the turn of the century and was first reported in 1909 from the Eastern Cape Province (5). Since that time, numerous outbreaks of dieback after hail have been reported, mostly from the summer rainfall areas of the Mpumalanga Province, where *Pinus patula* is grown extensively. This fungus is also reported to cause diseases of pines elsewhere in the world (10), although the extent of the disease appears to be less severe than it is in South Africa (15).

Recently, studies have shown that *S. sapinea* is present as latent infections in third-year, mature seed cones of *P. patula* and *Pinus radiata* (11). Latent infections have also been reported from the stems of pine seedlings in the United States (12). In South Africa, *S. sapinea* colonizes cones extensively and has been recovered from the cone pith tissue, seeds, seed wings, and ovuliferous scales. It is most abundant in the cone pith tissue (11).

The fungus is widespread in South Africa and occurs wherever pines are cultivated, including Mpumalanga Province, Northern Province, KwaZulu Natal, Eastern Cape Province, and Western Cape Province. *S. sapinea* has recently been recorded from Northern Sumatra and Indonesia, where it causes tip dieback on various *Pinus* spp., including the exotic *P. patula* and native *Pinus merkusii* (M. J. Wingfield, *unpublished*). Plantations of *P. patula* are commonly established adjacent to natural stands of *P. merkusii* in Northern Sumatra.

No teleomorph has been associated with *S. sapinea*. All indications are that only asexual reproduction occurs, and this would lead to clonal lineages within a population (6). By measuring the level of genotypic diversity within a population, it is possible to gain an indication of whether sexual or asexual reproduction occurs (6). Levels of genotypic diversity can be obtained as multilocus genotypes derived from molecular markers or by determining vegetative compatibility groups (VCGs) (4). The use of VCGs to determine the variability within fungal populations has been widely and successfully used in recent years (1,4,8,9).

The objective of this study was to determine the diversity within the South African *S. sapinea* population on introduced pines and to compare this with the diversity of a population of the fungus collected from trees in Northern Sumatra, where young plantations of *P. patula* occurred in close proximity to a native population of pines. It was hypothesized that the population in South Africa would be relatively uniform, typical of an introduced pathogen, and that isolates from Northern Sumatra, where native pines occur, would be more diverse.

MATERIALS AND METHODS

Sampling in South Africa. *S. sapinea* isolates were obtained only from *P. patula* in the Sabie region of Mpumalanga Province, South Africa. A three-level hierarchical sampling strategy was followed. The sampling commenced in a commercial plantation of 15-year-old trees on the farm Klipkraal. An individual tree, approximately in the middle of the plantation, was selected, and 10 third-year mature seed cones were sampled from it. The second level of sampling comprised 50 randomly selected trees from the same plantation. From each of these 50 trees, one third-year mature seed cone was sampled. The third level of sampling comprised two plantations, both within a 50-km radius from the first sampling site (Klipkraal). On each of these plantations, Hebron and Renosterhoek, 50 third-year seed cones were collected from 50 approximately 15-year-old *P. patula* trees.

Sampling in Northern Sumatra. *S. sapinea* isolates were obtained only from *P. patula* in the Habinsaran region of Northern Sumatra. Shoots with dieback were collected from three plantations of approximately 5-year-old trees. These plantations were in the same region where native stands of *P. merkusii* occurred. All three plantations were within 20 km of one another. A single dead shoot was collected from each of 12 trees in Habinsaran plantation 1, seven dead shoots from each of five trees in Habinsaran plantation 2, and eight dead shoots from each of seven trees in Habinsaran plantation 3.

Isolations. The mature third-year seed cones were cut into halves under sterile conditions in the laboratory. Cone pith tissue pieces were placed on 2% MEA (malt extract agar, Biolab Diagnostics, Midrand, South Africa) in petri dishes, supplemented with 200-mg/liter chloram-

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phenicol to suppress bacterial growth, and incubated at 25°C. Fast growing, darkly pigmented colonies were transferred to water agar plates with sterile pine needles on which *S. sapinea* isolates formed pycnidia for confirmation of their identity. Shoots with dieback were placed on moist filter paper in petri dishes and incubated at 25°C. *S. sapinea* pycnidia formed on the surface of pine tissue from which single conidial isolates were obtained. Isolates, positively identified as *S. sapinea*, were transferred to MEA slants in culture vials and stored at 4°C. The hierarchical sampling of *S. sapinea* isolates from South Africa was comprised of 107 isolates from three sampling levels. A total of 83 isolates were obtained from shoots with dieback in Northern Sumatran plantations.

Vegetative compatibility tests. Vegetative compatibility tests with the South African isolates were first done within each sampling level of the hierarchy. Isolates within each sampling level were paired in all possible combinations on oatmeal agar (OMA). Oatmeal (60 g/liter) was steamed in a water bath at 70°C for 2 h with periodic stirring. It was then separated from the liquid by filtration through a double layer of cheesecloth, resulting in approximately 600 ml of oatmeal suspension. Agar (20 g, Biolab) was melted in 400 ml of distilled water and then added to oatmeal suspension and autoclaved. The medium was thoroughly mixed prior to dispensing into plastic assay dishes (20-cm square; Nunc Industries, Roskilde, Denmark).

Representative VCGs from each sampling level were then paired in all possible combinations. The same approach was used for the Northern Sumatran isolates of *S. sapinea*, where isolates were first paired within plantations and then as representative VCGs between plantations. Isolates were placed approximately 1 cm apart on the OMA plates in such a way as to pair all isolates with themselves and with all other isolates. Assay plates were incubated at 20°C in the dark for 4 to 6 days. The occurrence of different VCGs was determined on the basis of barrage line formation between incompatible isolates (2). *S. sapinea* isolates grew actively on OMA, and colonies made contact with each other within 2 to 4 days. Incompatible reactions resulted in dark mycelial barrage lines (Fig. 1) and were most obvious approximately 5 days after inoculation. After this time mycelial growth became very dense and the reactions were no longer visible. Compatible isolates (Fig. 1) merged without the formation of any barrage lines. All isolates are available in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Geographic differentiation. Isolates within each population of *S. sapinea* belonging to the same VCG were treated as individual phenotypes, since the genetic background was unknown. Stoddart and Taylor's (13) index of genotypic diversity was used for this haploid fungus to measure and statistically compare the diversity of the different phenotypes in the different populations. Thus, the null hypothesis that no significant difference in genotypic diversity exists between the South African and Northern Sumatran populations of *S. sapinea* was tested. The genotypic diversity ($G^{\wedge}$) of the sampled population was calculated for each population according to the method of Stoddart and Taylor (13). The percentage of maximum diversity was calculated by dividing genotypic diversity ($G^{\wedge}$) of the observed population by the sample size (N) (7). This facilitates the direct comparison of the South African and Northern Sumatran populations, despite their different sample sizes. Genotypic diversity for the two populations was tested for significant differences using the Students *t* test ($P = 0.05$) (13).

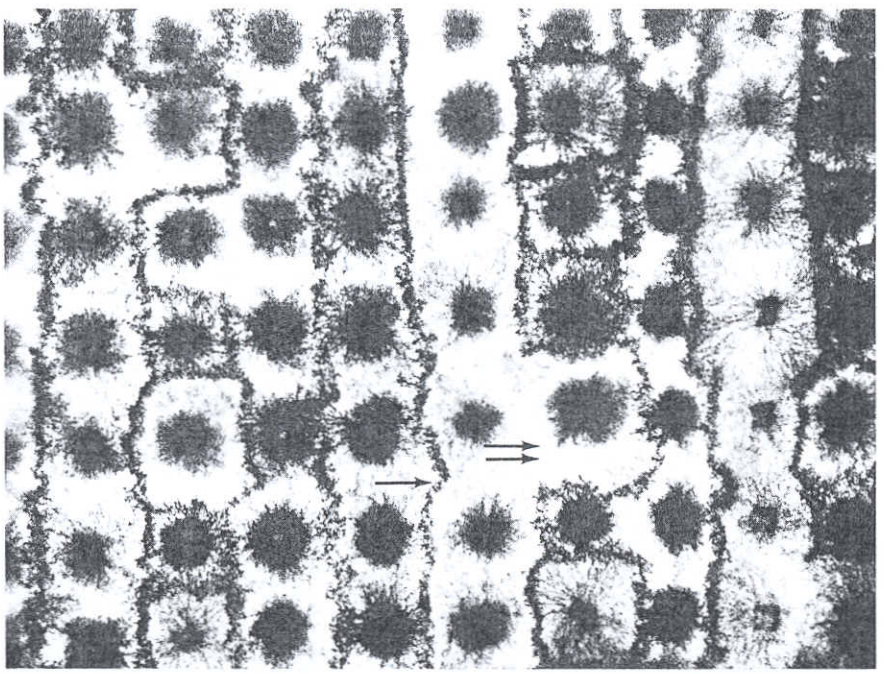

Fig. 1. Dark barrage lines (arrow) formed between incompatible isolates of *Sphaeropsis sapinea* belonging to different vegetative compatibility groups (VCGs) and confluent mycelium (double arrows) between compatible isolates belonging to the same VCG.

RESULTS

South African population. A total of 62 VCGs were found among the 107 isolates from the three plantations (Table 1). The 44 isolates of *S. sapinea* obtained from the Klipkraal plantation were represented by 16 VCGs (largest observed VCG = 11 isolates). The 31 isolates obtained from the Hebron plantation accounted for 23 VCGs (largest observed VCG = 3 isolates), and the 32 isolates from the Renosterhoek plantation also accounted for 23 VCGs (largest observed VCG = 4 isolates). Although these three plantations were within 50 km of one another, no common VCGs were found to occur between plantations.

Northern Sumatran population. Four VCGs were found among the 83 isolates from three plantations (Table 1). The 11 isolates obtained from Habinsaran plantation 1 were represented by two VCGs (largest observed VCG = 6 isolates). The 31 isolates from Habinsaran plantation 2 accounted for two VCGs (largest observed VCG = 30 isolates), and the 41 isolates obtained for Habinsaran plantation 3 also accounted for two VCGs (largest observed VCG = 39 isolates). In the Habinsaran plantations 2 and 3, where several isolates originated from the same tree, it was found that isolates from the same tree belonged to the same VCG, with the exception of one isolate in plantation 2 and two isolates in plantation 3. A large common VCG that included 75 isolates was found to occur among the three plantations.

Geographic differentiation. The percentage of maximum genotypic diversity for the South African sampled population of *S. sapinea* was 30.5% compared with the 1.5% of the Northern Sumatran sampled population (Table 1). The South African population was significantly more diverse ($P = 0.05$) than the Northern Sumatran one (South Africa: $G^{\wedge} = 32.6$, STD = 18.6; Northern Sumatra: $G^{\wedge} = 1.2$, STD = 26.5). The null hypothesis, that no significant difference in diversity exists between the South African and Northern Sumatran populations of *S. sapinea*, was consequently rejected. A high degree of clonality was evident in the Northern Sumatran population, where the dominant

VCG comprised 90% of the total sample and was present in all three plantations sampled. In contrast, the dominant South African VCG comprised only 10% of the total sample and was present in only one plantation.

DISCUSSION

This is the first study to consider diversity and geographic differentiation in the important pine pathogen *S. sapinea*. Contrary to expectation, the results showed that the introduced population of *S. sapinea* in South Africa is very diverse (62 VCGs), while the opposite is true for the population from Northern Sumatra (4 VCGs). The results from this study clearly indicate that the pathogen has a broad diversity base in South Africa.

By determining the occurrence and frequency of distinct VCGs in populations of *S. sapinea* from South Africa and Northern Sumatra, and assuming that each VCG represented a particular genotype, we were able to compare the genotypic diversity of these populations in a statistically meaningful way. Although the inferences that can be made from determining genotypic as opposed to gene diversity are limited, we were able to make various assumptions concerning the population structures of the South African and Northern Sumatran populations of *S. sapinea*.

Genotypic diversity is based on the number and frequency of genotypes occurring in a population (13). The genotypic diversity of an observed sample ($\hat{G}$) is a robust method of testing genotypic diversity in asexually reproducing fungi (13). This method has low type I errors but lacks the ability to distinguish highly repeated genotypes (large clones) from genotypes that occur rarely within a population, as is the case in the Northern Sumatran population. As a result, highly repeated genotypes greatly reduce the genotypic diversity of the observed sample and cause high type II errors (13). In this case, however, large type II errors are more the result of the clonal population structure (Northern Sumatran population) than the lack of power of the test (13).

The discrepancy in expected VCG diversity within the sampled populations of *S. sapinea* from South Africa and Northern Sumatra is interesting, and we have put forward a number of hypotheses that need further investigation. The diverse population of *S. sapinea* present in South Africa is, in our opinion, largely due to the fact that this fungus is an endophytic latent colonizer of mature seed cone tissue, including seed, of *P. patula* (11). The diverse nature of the fungus from South African *P. patula* is consistent with the hypothesized occurrence of multiple introductions into the country over a long period of time. This would most likely have occurred because the South African Forestry Industry has imported pine seed for many decades from many parts of the world where pines are grown and found naturally. The broad genetic base existing in South Africa also suggests that this fungus may have an undiscovered teleomorph or that the sexual stage was lost fairly recently. Laughton (3) mentioned the presence of rarely formed perithecia; unfortunately the anamorph connection was never made, rendering the report of dubious value.

There are various possible hypotheses to explain the low diversity within the *S. sapinea* population from Northern Sumatran *P. patula*. Native *P. merkusii* occurs in Northern Sumatra, with the exotic *P. patula* only established fairly recently. The low diversity of *S. sapinea* occurring on this exotic pine may thus be a result of a limited number of introduced VCGs on seed from a common origin. Alternatively, the *S. sapinea* population occurring on the native *P. merkusii* may not yet have moved onto *P. patula*. In light of the fact that *S. sapinea* causes tip dieback of both *P. merkusii* and *P. patula*, it can be argued that, from the indigenous population of *S. sapinea* occurring on *P. merkusii*, only one or more selectively fit VCGs may have moved onto the recently introduced *P. patula*. The selective pressure in such a situation may be pathogenicity, enabling a dominant VCG or VCGs to predominate in the population sampled from *P. patula*. In order to distinguish between the above-mentioned two hypotheses, a detailed sampling and population study is needed from native *P. merkusii* in Northern Sumatra.

Table 1. *Sphaeropsis sapinea* vegetative compatibility groups (VCGs) from *Pinus patula* seed cones from three plantations in South African and shoots with dieback from three plantations in North Sumatra

Location	Sample size	No. of VCGs	No. of isolates
Klipkraal (South Africa)	44	1	11
		1	7
		3	4
		1	3
		1	2
		9	1
Hebron (South Africa)	31	2	3
		4	2
		17	1
Renosterhoek (South Africa)	32	1	4
		2	3
		2	2
		18	1
Total	107	62	
Genotypic diversity ($\hat{G}$)	32.6		
Standard deviation (STD)	18.6		
Maximum percentage of genotypic diversity ($\hat{G}$/N%)	30.5%		
Habinsaran 1 (North Sumatra)	11	1	6
		1	5
Habinsaran 2 (North Sumatra)	31	1	30
		1	1
Habinsaran 3 (North Sumatra)	41	1	39
		1	2
Total	83	4	
Genotypic diversity ($\hat{G}$)	1.2		
Standard deviation (STD)	26.5		
Maximum percentage of genotypic diversity ($\hat{G}$/N%)	1.5%		

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