Microsatellite and mating-type markers reveal unexpected patterns of genetic diversity in the pine root-infecting fungus *Grosmannia alacris*

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Abstract

Grosmannia alacris is a fungus commonly associated with root-infesting bark beetles occurring on *Pinus* spp. The fungus has been recorded in South Africa, the USA, France, Portugal and Spain and importantly, has been associated with pine root diseases in South Africa and the USA. Nothing is known regarding the population genetics or origin of *G. alacris*, although its association with root-infesting beetles native to Europe suggests that it is an invasive alien in South Africa. In this study, microsatellite markers together with newly developed mating type markers were used to characterize a total of 170 isolates of *G. alacris* from South Africa and the USA. The results showed that the genotypic diversity of the South African population of *G. alacris* was very high when compared to the USA populations. Two mating types were also present in South African isolates and the *MAT1-1*/*MAT1-2* ratio did not differ from 1:1 (χ^2 = 1.39, *P* = 0.24). This suggests that sexual reproduction most likely occurs in the fungus in South Africa, although a sexual state has never been seen in nature. In contrast, the large collection of USA isolates harbored only a single mating type. The results suggest that multiple introductions, followed by random mating, have influenced the population structure in South Africa. In contrast, limited introductions of probably a single mating type (*MAT1-2*) may best explain the clonality of USA populations.

Keywords: *Grosmannia alacris*, pine pathogen, population genetics, SSR markers

Introduction

Grosmannia alacris is a fungus commonly associated with root-infesting bark beetles occurring on *Pinus* spp. It was first described as *Verticicladiella alacris* associated with a pine root disease in South Africa (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980). The fungus was later reduced to synonymy with *Leptographium serpens* based on morphological similarities (Wingfield & Marasas, 1981) and together with other *Leptographium* spp. has more recently been treated in *Grosmannia* as *G. serpens* (Zipfel *et al.*, 2006). Based on molecular phylogeny, Duong *et al.* (2012a) split this species from *G. serpens* and revived the name as *G. alacris*. *Grosmannia alacris* as defined by Duong *et al.* (2012a) has been found in South Africa, USA, France, Portugal and Spain.

In South Africa, *G. alacris* occurs on the roots of *Pinus* spp. (including *P. elliottii*, *P. patula*, *P. pinaster* and *P. radiata*) and is vectored by the root-infesting bark beetles (Coleoptera: Scolytinae) *Hylastes angustatus* and *Hylurgus ligniperda*, which are of European origin (Zhou *et al.*, 2001; Duong *et al.*, 2012a). It is thus assumed that the fungus was introduced into South Africa with these insects. In the USA, *G. alacris* has been consistently isolated from *Hylastes salebrosus* and *Hylastes tenuis* as well as root tissue of *Pinus taeda* and *P. palustris* infested with these insects (Eckhardt *et al.*, 2007; Zanzot *et al.*, 2010). Isolates of *G. alacris* have also been collected from France, Portugal and Spain, however thorough studies have neither been conducted on the insect association nor the host range of this species in those countries (Jacobs & Wingfield, 2001; Duong *et al.*, 2012a).

Various studies have suggested an association of *G. alacris* with diseased pine trees or pine decline. The fungus was originally described in association with root disease of *Pinus pinaster* and *P. radiata* in South Africa with similar symptoms on both species (Wingfield &

Knox-Davies, 1980; Wingfield & Marasas, 1980). A subsequent study conducted by Zhou *et al.* (2002) showed that this fungus [as *L. serpens*] produced lesions in inoculated trees in South Africa. Although the fungus was capable of causing lesions on the stem of inoculated trees, the authors concluded that this species could not be considered as a primary pathogen of pine in South Africa. In the USA, *G. alacris* [as *L. serpens*] has consistently been isolated from root tissue of symptomatic trees and from sites with severe decline symptoms and high levels of mortality (Eckhardt *et al.*, 2007). Pathogenicity tests showed that *G. alacris* [as *L. serpens*] produced lesions on inoculated seedlings and mature *P. taeda*, and was a cause of seedling mortality (Eckhardt *et al.*, 2004b). Although none of these studies have shown *G. alacris* to be a primary pathogen of pines, a combination of abiotic stress with intensive feeding by beetles following by the colonization of the fungus could contribute to pine decline and mortality (Wingfield *et al.*, 1988; Manion & Lachance, 1992; Eckhardt *et al.*, 2007).

Grosmannia alacris has the widest distribution of all species in the recently described *G. serpens* complex (Duong *et al.*, 2012a). It has been associated with pine decline in various studies as well as in fungal-insect association studies in the USA and South Africa (Zhou *et al.*, 2001; Eckhardt *et al.*, 2004a; Eckhardt *et al.*, 2007; Matusick & Eckhardt, 2010b; Matusick & Eckhardt, 2010a; Zanzot *et al.*, 2010; Duong *et al.*, 2012a). However, nothing is known regarding the centre of origin nor the genetic diversity of the fungus in these countries. The aim of this study was to characterize the genetic diversity of *G. alacris* populations from South Africa and the USA using microsatellite markers. Isolates from these populations were also compared based on mating type and thus in terms of their capacity to undergo sexual reproduction.

Materials and methods

Fungal isolates

Isolates of *G. alacris* from South Africa (*N* = 46) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. These included isolates collected from Jessievale and Tweefontein, Mpumalanga, South Africa (*N* = 37), and other isolates collected from four locations within a 50 km radius from Cape Town, Western Cape, South Africa (*N* = 9). Isolates from the USA (*N* = 124) were collected from three locations including Fort Benning, Georgia (*N* = 40), Holly Springs Ranger District, Mississippi (*N* = 26) and Oakmulgee Ranger District, Alabama (*N* = 58). Fungal isolations were made on 2 % malt extract agar (MEA) supplemented with cycloheximide (200 mg/L). The isolate (CMW1136) used to clone the mating type gene was obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. In all cases, isolates used in this study were purified from single germinating conidia or single hyphal tips. All fungal isolates have been identified as *G. alacris* by sequencing the partial beta-butulin gene (Duong *et al.*, 2012a) before use in the population analyses.

DNA extraction and ISSR amplification and allele determination of SSR loci

Single conidial or single hyphal tip cultures were grown on YM broth (2% malt extract and 0.2% yeast extract) for 3-5 days. The mycelium was harvested by centrifugation and lyophilized in 1.5 mL Eppendorf tubes. The tubes were submerged in liquid nitrogen and mycelium was ground into a fine powder using a pipette tip. DNA was extracted from the powder using PrepMan™ Ultra reagent (Applied Biosystems). About 10 mg of lyophilized mycelial powder was added to 1.5 mL Eppendorf tubes, to which 100 µl PrepMan™ Ultra reagent was added and mixed. The tubes were heated at 96 °C for 5 minutes, followed by centrifugation at 10.000 g for 10 minutes. DNA solution obtained was diluted five times with

10 mM Tris-HCl, pH 8.0 and stored at -20 °C until further use. Two microliters of diluted DNA solution were used as template in a SSR-PCR reaction. SSR-PCR was performed using 16 microsatellite markers developed and described in detail by Duong *et al.* (2012b). SSR-PCR reaction components, PCR cycles and allele assignment were as described in Duong *et al.* (2012b).

Gene and genotypic diversity

Allele frequency at each locus was calculated using the program POPGENE (Yeh *et al.*, 1999). Nei's genetic diversity (Nei, 1973) was calculated for each population using the same program. Genotypic diversity (*G*) was estimated following the equation $G = \frac{1}{\sum Pi^2}$ where Pi is the observed frequency of the i^{th} genotype (Stoddart & Taylor, 1988). Maximum percentage genotype diversity $G^* = (G/N) \times 100$, where N = number of isolates, was calculated to avoid sample size bias. Chi-square (χ^2) tests for differences in allele frequency were performed for each locus across all populations. The obtained χ^2 values were compared using the contingency table at $P = 0.001$ to determine the significance of the allele frequency differences at each locus. Where the observed values were higher than those on the contingency table, the gene diversities across loci were considered significantly different to one another.

Population structure and differentiation

The computer program STRUCTURE 2.3 was used to infer and assign population structure of all isolates (Pritchard *et al.*, 2000). The Monte Carlo Markov Chain (MCMC) was run with 100 000 replicates with initial burn-in of 20 000 for K ranging from 1 to 15 at 20 iterations for each cycle. Clusters were assigned with cluster identity above 75%.

Population differentiation was calculated between populations using the program Multilocus (Agapow & Burt, 2000) with an estimate of Wright's Fst as $\theta = (Q - 1)/(1 - q)$ where Q is the

probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Weir, 1997). When $\theta = 0$, allele frequencies are the same between two populations, inferring that two populations are identical. In contrast, when $\theta = 1$, there are no alleles shared between two populations and, therefore, the two populations are completely isolated. The statistical significance of θ was determined by comparing the observed value to that of 1000 randomized datasets. The null-hypothesis was that there is no difference between two populations. Gene flow (M) between populations was also calculated following the formula $M = [(1/\theta) - 1]/2$ (Cockerham & Weir, 1993).

Linkage disequilibrium

The possibility of random mating within different populations was evaluated based on linkage disequilibrium analyses. The index of association (I_A) and rBarD ($\bar{r}D$) were calculated using the program Multilocus (Agapow & Burt, 2000). The observed value of I_A and $\bar{r}D$ were compared to that of 1000 time randomized datasets. The null-hypothesis was that the alleles are randomly associated and the population is expected to freely undergo recombination. If the observed I_A and $\bar{r}D$ were significantly different from those obtained from the randomized datasets ($P \leq 0.05$), the null-hypothesis was rejected. In contrast, where the observed I_A and $\bar{r}D$ were not significantly different from those obtained from the randomized datasets, the null-hypothesis of random association of alleles would be supported, showing that the populations undergo random mating.

Development of mating type markers for G. alacris

The mating type gene *MAT1-2-1* in *G. alacris* isolate CMW1136 was cloned using the method described in detail in Duong *et al.* (2013). Degenerate primers NcHMG1 and NcHMG2 (Arie *et al.*, 1997) were used to obtain a part of the HMG box of the *MAT1-2-1*

gene. The PCR reaction ingredients and cycling conditions were the same as those described in Duong *et al.* (2013). The PCR product was cloned into pGEM®-T (Promega) and transformed into *E. coli* JM109. Plasmids were extracted, confirmed by sequencing using T6 an SP7 primers. The plasmid containing HMG box sequence was used as a template to synthesize a DIG-labeled probe using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH) with NcHMG1 and NcHMG2 as primers. Southern hybridization of DNA samples, digested to completion with *Eco*RI, *Hin*dIII and *Bam*HI was conducted. Based on the results obtained from Southern analysis, the *Hind*III digested fragments with size ranges corresponding to that of the hybridized signal were recovered from agarose gel, ligated into the pBluescript II KS (+) phagemid vector (Stratagene) and transformed into *E. coli* JM109 to create a partial genomic library. Colony hybridization using the same probe as that used in Southern analysis of genomic DNA was conducted to screen for *MAT1-2-1* gene. Plasmids from positive clones were prepared and sequenced using primer "walking". Genes were predicted using FGENESH+ (Salamov & Solovyev, 2000) and identified as *MAT1-2-1* by BLAST against the NCBI database. The sequence of *MAT1-2-1* obtained was used to design *MAT1-2* specific primers, LA-Mat2F (5'- CGACGGTGAAGTATGTCATTGA -3') and LA-MAT2R (5'- TTCGGTCTCGTCGCCAGT -3'). For detection of *MAT1-1*, primers Oph-MAT1F1 (5'- ATGKCCRATGARGAYTGCT -3') and Oph-MAT1R2 (5'- GGCGKTKGCRTTGTAYTTGTA -3'), were developed based on sequences of *MAT1-1-3* genes from *Leptographium procerum* (GenBank number: KC883456), *L. profanum* (GenBank number: KC883458) and *O. novo-ulmi* sub. *novo-ulmi* (GenBank number: FJ858801).

The *MAT1-1* and *MAT1-2* primers were used in separate PCRs (with either *MAT1-1* or *MAT1-2* primers) as well as combined in a multiplex PCR (with both *MAT1-1* and *MAT1-2* primers) to test for the ability to identify the mating type of *G. alacris* isolates. The PCR mixture consisted of 2.5 µl 10 × PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM

of each primer, 1 U FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH), and 20 to 50 ng of genomic DNA. PCR cycles used were an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C annealing for 30 sec, and 72 °C extension for 60 sec, with a final extension at 72 °C for 8 min. PCR products were examined using agarose gel electrophoresis, stained with GelRed (Biotium) and examined under UV light.

Determination of mating type ratio in populations

DNA samples of all isolates from South Africa and the USA populations were used in multiplex PCRs (containing both *MAT1-1* and *MAT1-2* primers) to determine the mating type and calculate mating type ratio in the populations. PCR reaction ingredients and cycling conditions used were as noted above. The mating type of isolates was assigned based on the size of resulting PCR products. The PCR primers were designed such that the *MAT1-1* amplicon was 479 bp and the *MAT1-2* amplicon was 623 bp. The mating type ratio was noted as number of *MAT1-1* isolates per number of *MAT1-2* isolates for each population.

Results

Gene and genotypic diversity

All 16 primer pairs consistently amplified microsatellite loci from all isolates considered in this study. Six markers namely LSM-14, LSM15, LSM-22, LSM-25, LSM-39 and LSM-40 were monomorphic across all isolates and were excluded from further analyses. Ten markers were polymorphic and produced 35 alleles across all isolates from South Africa and the USA (Table 1). Of these, seven were polymorphic for the South African population, seven for Georgia, five for Alabama and all ten for the Mississippi populations of isolates. Only three alleles were shared in all populations of isolates.

The number of alleles per locus ranged from two to five across all 170 isolates. There were 20 alleles for the South African population, and 23, 20 and 16 for the Georgia, Mississippi and

Table 1. Allele frequencies and genotype diversity by population.

Locus	Allele	South Africa	USA		
			Georgia	Mississippi	Alabama
LSM-02	1	0.739	0.400	0.654	0.069
	2	0.261	–	–	–
	3	–	0.600	0.346	0.931
LSM-11	1	0.978	0.400	0.654	–
	2	0.022	0.600	0.346	1.000
LSM-19	1	0.478	–	–	–
	2	0.283	0.400	0.654	–
	3	0.239	0.600	0.346	1.000
LSM-21	1	0.500	–	–	–
	2	0.500	1.000	0.539	1.000
	3	–	–	0.462	–
LSM-26	1	0.565	1.000	0.539	1.000
	2	–	–	0.462	–
	3	0.435	–	–	–
LSM-30	1	0.565	–	–	–
	2	–	0.400	0.615	–
	3	0.391	0.075	–	0.224
	4	0.044	0.525	0.385	0.776
LSM-31	1	1.000	1.000	0.539	1.000
	2	–	–	0.462	–
LSM-32	1	–	0.050	0.385	–
	2	–	0.350	0.269	–
	3	–	0.025	–	0.069
	4	0.587	0.500	–	0.931
	5	0.217	–	–	–
	6	–	0.075	0.346	–
	7	0.196	–	–	–
LSM-33	1	1.000	0.400	0.654	–
	2	–	0.550	0.231	0.638
	3	–	–	0.115	0.052
	4	–	0.050	–	0.310
LSM-37	1	–	0.025	–	0.086
	2	–	0.575	0.346	0.914
	3	–	0.075	0.385	–
	4	1.000	0.325	0.269	–
G		25.805	4.211	3.558	4.450
*G**		56.098	10.526	13.684	7.672
MLH		32	9	5	13

G = Genotype diversity (Stoddart & Taylor, 1988), *G** = percentage maximum diversity, *MLH* = Multilocus haplotype

Alabama populations respectively. There were seven unique alleles in the South African population and 15 alleles were unique for three populations from the USA. Most of the 28 alleles were shared between different populations from the USA, in which only the population from Mississippi had three unique alleles that were not found in any other population. Seven of the total of 35 alleles were shared between all four populations including those from the USA and South Africa (Table 1).

A total of 32 multilocus haplotypes (*MLH*) per 46 isolates were observed for the South African population, of which many *MLHs* were represented by only a single isolate. The number was much lower for populations from the USA where there were nine *MLHs* per 40 isolates for Georgia, five *MLH*s per 26 isolates for Mississippi and 13 *MLH*s per 58 isolates for Alabama. No *MLH* was shared between the South African and the USA populations. Common *MLH*s were found in isolates from Georgia and Alabama, Georgia and Mississippi, but not between the Alabama and Mississippi isolates. The genotype diversity was high for the South African population (G = 25.8) and much lower for the three populations from the USA (G = 4.2 for Georgia, G = 3.6 for Mississippi, and G = 4.5 for Alabama) (Table 1). The maximum percentage diversity (G^*) was highest for the South African population (56.1%), followed by Mississippi (13.7%), Georgia (10.5%) and Alabama (7.7%). Mean genetic diversity across all loci (H) ranged from 0.126 to 0.515 for all populations (Table 2). Chi-square values obtained from the clone-corrected population indicated significant differences (P = 0.001) for gene diversity at all loci across all populations (Table 2).

Table 2. Gene diversity (*H*) and chi-square value for differences in allele frequencies for 10 SSR loci across clone-corrected populations.

Locus	South Africa	USA			Chi-square (χ^2)	*df*
		Georgia	Mississippi	Alabama		
LSM-02	0.386	0.480	0.453	0.128	36.287*	6
LSM-11	0.043	0.480	0.453	0.000	41.474*	3
LSM-19	0.634	0.480	0.453	0.000	28.757*	6
LSM-21	0.500	0.000	0.497	0.000	39.861*	6
LSM-26	0.492	0.000	0.497	0.000	38.376*	6
LSM-30	0.526	0.559	0.473	0.348	48.509*	9
LSM-31	0.000	0.000	0.497	0.000	22.358*	3
LSM-32	0.570	0.619	0.660	0.128	66.749*	18
LSM-33	0.000	0.535	0.506	0.494	59.936*	9
LSM-37	0.000	0.558	0.660	0.158	58.553*	9
Mean	0.315	0.371	0.515	0.126		

* Indicate significant difference at $P = 0.001$, *df* = degree of freedom.

Population differentiation and gene flow

The results (θ) indicated a significant distance between the South African and USA populations (at $P < 0.001$). In contrast, the three populations from the USA were closer to one another, and Georgia and Mississippi, and Georgia and Alabama were not significantly different from each other (θ = 0.02 and 0.08 respectively). Within the USA, there was a moderate distance between the Mississippi and Alabama populations (θ = 0.3) (Table 3). Isolates from South Africa resided in a single group as suggested by Structure analysis (Fig. 1). The isolates from the USA fell into two groups, one of which included isolates from Georgia and Mississippi and another comprised of isolates from Georgia, Mississippi and Alabama (Fig. 1). There was very little evidence of gene flow (*M*) between the South African and USA populations (*M* value was between 0.4 and 0.57) (Table 3). In contrast, the levels of gene flow were much higher between populations from the USA, in which the highest value was observed between Georgia and Mississippi (*M* = 24.5) and the lowest was between Mississippi and Alabama (*M* = 1.17) (Table 3).

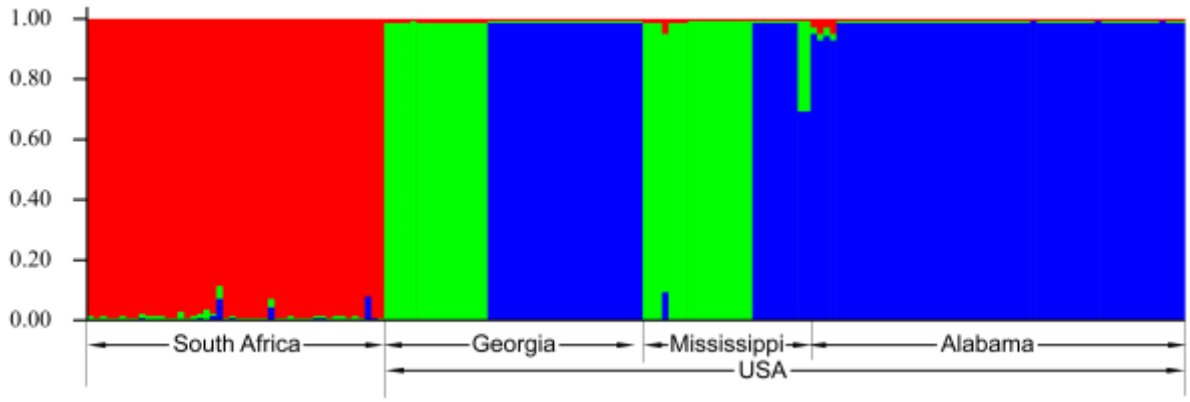

Figure 1. Affiliation of individual genotypes of *G. alacris* as suggested by Structure 2.3.4. Different colours within a vertical bar indicate population affiliation of individual genotypes. The sources of isolates are given underneath the bar.

Table 3. Population differentiations (θ) (above the diagonal) and gene flow (M) (below the diagonal) between populations.

	South Africa	Georgia	Mississippi	Alabama
South Africa	–	0.40*	0.41*	0.57*
Georgia	0.75	–	0.02^{NS}	0.08^{NS}
Mississippi	0.72	24.50	–	0.3*
Alabama	0.38	5.75	1.17	–

* Indicate significant difference at P < 0.001 and ^{NS} Indicate no significant difference (at $P < 0.05$).

Table 4. Observed I_A, $r\bar{}D$ values with corresponding significant level (P) and mating type ratio of each populations.

Population	I_A	$r\bar{}D$	P	*MAT1-1*/*MAT1-2* ratio
South Africa	0.014	0.002	0.373	19/27
Georgia	3.629	0.609	< 0.001	0/40
Mississippi	2.912	0.325	< 0.001	0/26
Alabama	-0.185	-0.046	0.936	0/58

Linkage disequilibrium

The observed I_A and $r\bar{}D$ values from each population with corresponding P values are presented in Table 4. The observed $r\bar{}D$ values for the South African and Alabama populations fell within the 1000 randomized dataset (Fig. 2 A and D) with P values of 0.373 and 0.936 respectively. The observed $r\bar{}D$ values for the Georgia and Mississippi populations fell beyond

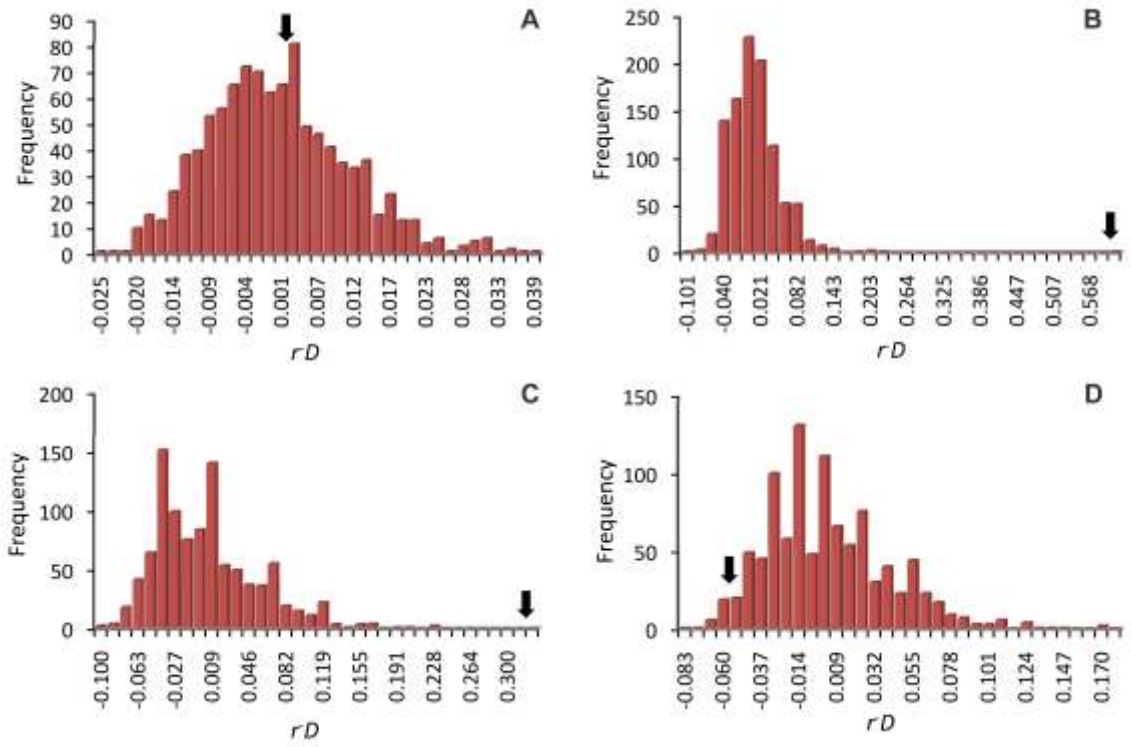

Figure 2. Histograms constructed from $\bar{r}D$ values obtained from 1000 time randomized datasets of South African (A) and USA populations from Georgia (B), Mississippi (C) and Alabama (D). Arrows indicated the observed $\bar{r}D$ values of each population.

the ranges obtained from the 1000 randomized datasets ($P < 0.001$) (Fig. 2 B and C). Similar results were obtained for the I_A values. The null-hypothesis, that alleles are randomly associated and population structure is consistent with past recombination through outcrossing, were supported for the South African and Alabama populations and rejected for the Georgia and Mississippi populations.

Development of mating type markers for G. alacris

PCR using NcHMG1 and NcHMG2 primers and DNA from *G. alacris* isolate CMW1136 as a template resulted in a product of about 300 bps in size. Cloning of this PCR product into pGEM®-T and sequencing the recombinant vector using T6 and SP7 primers confirmed that it was the HMG domain of the *MAT1-2-1* gene. BLAST analysis showed that HMG domain in

G. alacris shared a high level of homology with those in other closely related ophiostomatoid fungi such as *L. procerum*, *L. profanum*, and *G. clavigera*. Southern analysis of the *Hind*III digested DNA sample produced a hybridized signal from a fragment of about 2.5 kb in size. A partial genomic library was successfully constructed from *Hind*III digested fragments of corresponding size (around 2.5 kb) and screening of this library by colony hybridization resulted in one positive clone out of about 700 colonies. The cloned insert [2.47 kb] was sequenced (GenBank number: KJ528492) and FGENESH+ was used for annotation. Additional analyses using BLAST against the NCBI database confirmed the presence of a complete *MAT1-2-1* gene (CDS 948 bp in size, 828 bp as mRNA). The *MAT1-2* specific primers (LA-Mat2F and LA-MAT2R) were designed from sequence of *MAT1-2-1* and consistently amplified a 623-bp portion of *MAT1-2-1* gene from *MAT1-2* isolates of *G. alacris* (Fig. 3). The *MAT1-1* primers (Oph-MAT1F1 and Oph-MAT1R2) were used to amplify a 479-bp portion of *MAT1-1-3* from *MAT1-1* isolates (Fig. 3). Both *MAT1-1* and *MAT1-2*

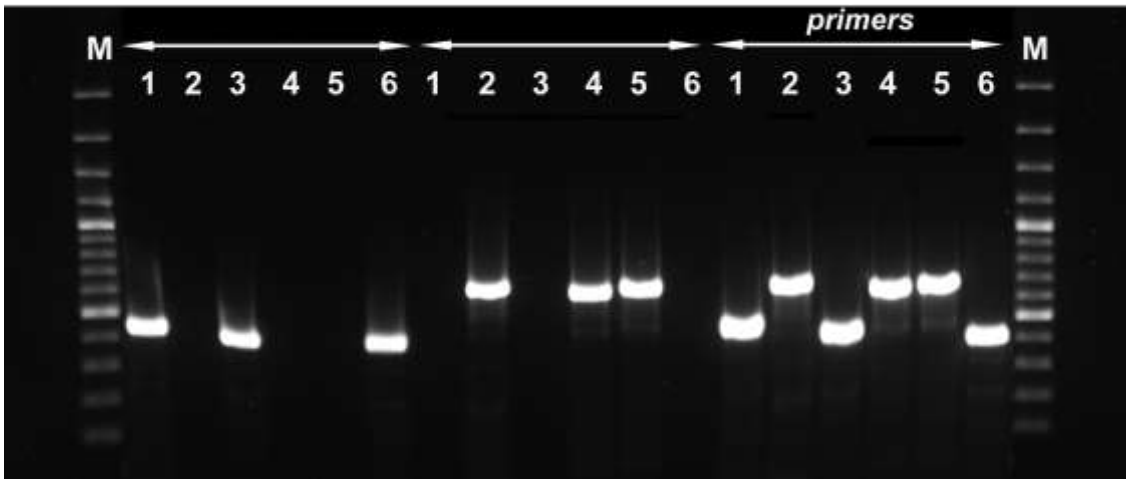

Figure 3. Agarose gel [1.5%] showing the amplicons obtained from PCR and multiplex PCR assays using mating type primers on *G. alacris* isolates: CMW7701 (1), CMW7702 (2), CMW7703 (3), CMW7705 (4), CMW7706 (5), CMW7707 (6). The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). The 500bp and 1000bp marker is more concentrated than the other bands.

primers could be used in a single multiplex PCR to amplify portions of *MAT1-2-1* gene and *MAT1-1-3* gene from *MAT1-2* and *MAT1-1* isolates respectively (Fig. 3). The amplified fragments were easily differentiated based on their size differences (Fig. 3).

Determination of mating type ratio in populations

By using the developed multiplex PCR assay, the mating type of all isolates from South Africa and USA were determined (Fig. 4). There were 19 *MAT1-1* and 27 *MAT1-2* isolates in the total of 46 South African isolates and Chi-square (χ^2) test showed that this did not differ from an expected 1:1 ratio of a random mating population ($\chi^2 = 1.39$, $P = 0.24$). All isolates from the USA were of the *MAT1-2* type (Table 4).

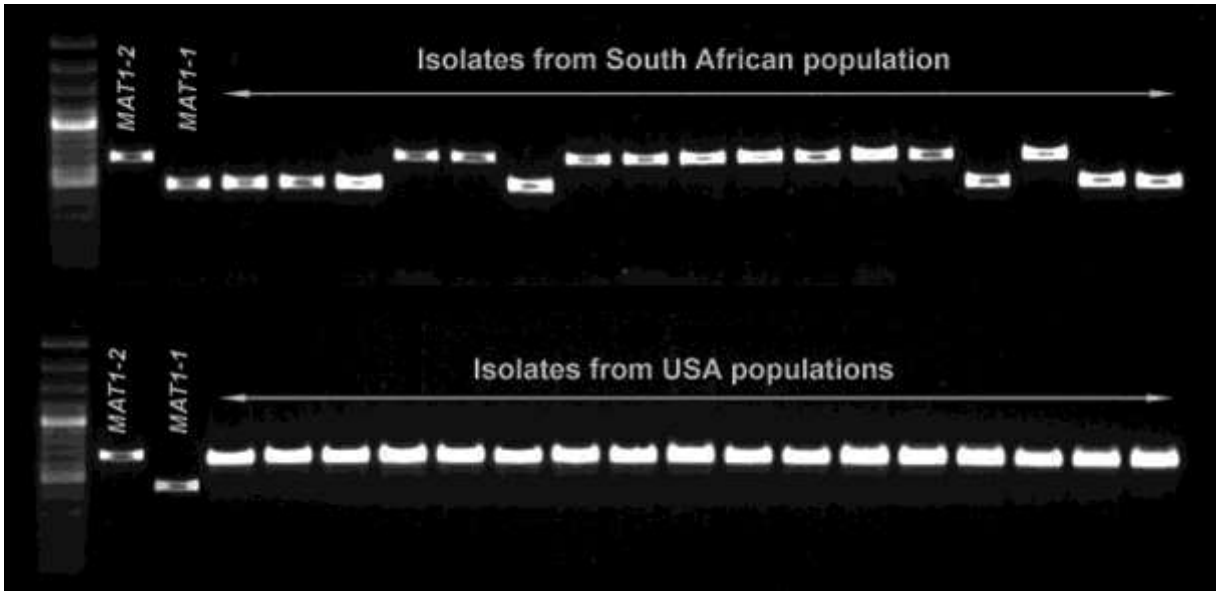

Figure 4. Agarose gel [1.5%] showing the amplicons obtained from a multiplex PCR assay to determine mating type of some representative isolates from South African and USA populations. The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). The 500bp and 1000bp marker is more concentrated than the other bands.

Discussion

Microsatellite markers applied in this study to populations of *G. alacris* from South Africa provided the surprising result that the fungus is genetically diverse in this country. This result was unexpected given that the fungus is vectored by a non-native invasive insect in South Africa (Zhou *et al.*, 2001), and is presumably also not native. Mating type analyses also showed that South African isolates include representatives of both the *MAT1-1* and the *MAT1-2* types. The fungus is thus heterothallic and possibly undergoing sexual recombination. In contrast, a population of *G. alacris* from the USA was essentially clonal and represented by a single mating type. This suggests that the fungus was most likely introduced into that country from an as yet, unknown source.

The South African population of *G. alacris* had a very high level of genetic and genotypic diversity (G^* = 56.1%), which suggests that random mating most likely have occurred within this population. The test of random association of alleles supported this hypothesis with the observed I_A and r^-D values falling within the randomized datasets. Moreover, the mating type distribution of 19 *MAT1-1* and 27 *MAT1-2* isolates and a chi-square statistic showed no significant difference from the expected 1:1 ratio of a randomly mating population (χ^2 = 1.39, P = 0.24). This suggests strongly that random mating could account for the high genetic and genotypic diversity observed in the South African population, as has been found in studies on other fungi (Linde *et al.*, 2003; Groenewald *et al.*, 2006).

Grosmannia alacris is believed to have been introduced into South Africa. This view is based on the fact that it is intimately associated with non-native bark beetles that infest *Pinus* spp. that are also non-native and were first introduced into the country in the early 1700's (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980; Zhou *et al.*, 2001). The high level of genetic diversity in *G. alacris* revealed in this study is surprising for an introduced organism, which might more likely be reflected by a clonal population associated with a

founder effect (Brasier, 1996; Milgroom *et al.*, 2008). The broad genetic diversity could, however, be explained by the presence of two mating types that would facilitate random mating and an expansion of genetic diversity. While a sexual state has never been seen for *G. alacris* in nature, ascomata of the fungus have previously been produced in laboratory tests (Duong *et al.*, 2012a). More intensive inspection of galleries of *Hylastes angustatus*, the primary vector of *G. alacris* in South Africa, may reveal the presence of sexual structures of the fungus.

The mating type ratio tests showed that only *MAT1-2* gene was present in all of the isolates from the USA. Unlike the situation in South Africa, this implies that the fungus can reproduce only asexually in the United States. This explained the low level of genotypic diversity observed in all populations from the USA. Although linkage disequilibrium tests suggested non-random association of alleles in populations from Georgia and Mississippi, the alleles were randomly associated in the Alabama population. However, with the detection of only one mating type, the possibility for sexual reproduction as the source of random recombination of alleles observed in the Alabama population would most likely not be possible. It seems probable that this random combination of alleles reflects sexually derived genotypes that were initially introduced into this population.

It is possible that the *MAT1-1* mating type of *G. alacris*, not found in this study, is present at a very low frequency in the USA and thus not represented in the isolates sampled. Under such conditions, sexual reproduction could occur in the USA at a very low frequency. This scenario would explain the results of linkage disequilibrium tests for the Alabama population. However, considering the fairly large number of isolates sampled in the USA and the fact that they were collected relatively broadly distributed in the sampling area, this is very unlikely to be the case.

The lack of shared *MLH*s together with a very low level gene flow observed between the South African population and the USA populations of *G. alacris* suggests a long period of separation between these two sources of isolates. This was also well supported with the structure analysis that showed South African isolates residing in a single group, separate from those from the USA. The results of the population differentiation analyses (θ) also provide strong statistical support for our conclusion that the South African population is well separated from the USA population. The most tangible explanation here is that the South African and USA populations are most likely linked to different sources and times of introduction. In contrast, the number of shared *MLH*s as well as gene flow was high between populations from the USA. Given the low genotypic diversity observed, these populations are most likely linked to a single or a small number of introductions.

Duong *et al.* (2012a) suggested an European origin of *G. alacris* but also stated the possibility of the fungus being native to the USA. This was based on the fact that in the USA, the fungus is associated with native insects infesting native pine species. The results of the present study are consistent with introduction of *G. alacris* into the USA, in which case, it has established a novel association with native root-infesting bark beetles. Such novel associations between fungi associated with introduced bark beetles and native insects have been described for the Dutch Elm disease fungi (Strobel & Lanier, 1981; Anderson & Holliday, 2003) and more recently for at least two pine-infesting bark beetles (Jacobs *et al.*, 2004; Lu *et al.*, 2009).

Unlike the South African situation, it is not possible to identify an insect with which *G. alacris* might have entered the United States. However, at least one non-native pine root infesting bark beetle is known in the area in which *G. alacris* is found (Thompson, 2011). This *Hylastes* species (*H. opacus*) would be a good candidate for further study of its fungal associates, both in the USA as well as in its area of origin.

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