Chapter 4

Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobromae* revealed using SSR markers

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Lasiodiplodia theobromae is a cosmopolitan fungus with a worldwide distribution in the tropics and sub-tropics, where it causes shoot blight and dieback of perennial trees and shrubs and imparts blue stain in timber. In this study, 8 SSR markers were used to evaluate the genetic diversity and gene flow between populations of *L. theobromae*. The relationships between isolates from different host were considered using 3 populations from different tree species in Venezuela and the relationships between isolates from different geographic origins included populations from Venezuela, South Africa and Mexico. A small number of predominant genotypes were encountered in the Venezuela and South African populations and thus genotypic diversity was low. There was no evidence of host specificity for isolates of *L theobromae* and there was very high gene flow between populations from different hosts. Geographic isolation existed between populations of the pathogen from different regions, with unique alleles fixed in the different populations. Gene flow was, however, less restricted between isolates from Mexico and the other populations, consistent with Mexico as a common source of seed in both Venezuela and South Africa. Genetic analysis suggested predominantly clonal reproduction with some genotypes widely distributed within a region. The broad host range of *L. theobromae* and the lack of evidence for host specialization, coupled with its endophytic nature and the common appearance of symptoms only after harvest, is likely to hinder disease management strategies.

INTRODUCTION

The fungal pathogen *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (= *Botryodiplodia theobromae* Pat.) represents the asexual state of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx. It has a worldwide distribution in tropical and subtropical regions and occurs on a very wide range of plants (Punithalingam, 1976). Hosts are mainly woody plants including fruit and tree crops such as mango (Sangchote, 1991), peach (Britton *et al.*, 1990), avocado (Darvas & Kotze, 1987) and *Eucalyptus* spp. (Apetorgbor *et al.*, 2004; Roux *et al.*, 2000, 2001; Sharma *et al.*, 1984). In Venezuela, *L. theobromae* causes shoot blight and dieback of *Pinus caribaea* var. *hondurensis*, *P. oocarpa*, *Azadirachta indica*, *Citrus aurantiifolia*, *C. sinensis*, and *Passiflora edulis* and is also an important agent of blue stain in lumber (Cedeño & Palacios-Pru, 1992; Cedeño *et al.*, 1995, 1996; Mohali, 1993; Mohali *et al.*, 2002). The greatest disease impact is encountered in eastern Venezuela where areas of *P. caribaea* have been established in plantations. *Lasiodiplodia theobromae* is common, causing distension and disruption of the cell walls, weakening the strength and toughness of the Caribbean pine wood, thus reducing its value by up to 50% (Cedeño *et al.*, 1996; Mohali, 1993).

Lasiodiplodia theobromae can colonise healthy plant tissue without exhibiting symptoms. Müllen (1987) for example isolated *L. theobromae* from stem cankers on dogwood (*Cornus florida*). Subsequent pathogenecity tests on dogwood stems with and without drought stress, showed that *L. theobromae* could be isolated from all inoculated plants, but cankers developed only on stressed plants (Müllen, 1987). Thus, *L. theobromae* can be considered as a latent pathogen capable of endophytic infection such as has been reported for the related fungi *Diplodia pinea* (Desm.) Kickx. on *Pinus* spp. (Burgess *et al.*, 2001a; Flowers *et al.*, 2003; Smith *et al.*, 1996) and *B. dothidea* (Fr.: Mough.) Ces. & De Not. on *Eucalyptus* spp. (Smith *et al.*, 1996).

DNA-based markers have been used to recognise and characterise populations, gene flow, and evidence of speciation in many fungal pathogens. SSR markers represent a class of co-dominant molecular markers consisting of tandem repeat loci, rich in polymorphisms with allele size determined by the addition or deletion of one or more repeats (Levinson & Gutman, 1987). SSR markers have recently been used to examine gene and genotype flow, reproductive mode and speciation in a number of fungi, including *Botryosphaeria* spp. and their anamorphs (Barnes *et al.*, 2001; Burgess *et al.*, 2001b, 2003, 2004a, b; Slippers *et al.*, 2004; Zhou *et al.*, 2002).

An earlier study using SSR markers developed for *L. theobromae*, suggested relationships among isolates were more closely linked to host than to geographic origin (Burgess *et al.*, 2003). That study was focussed largely on the development of appropriate markers to study populations of the pathogen and it included only 9 isolates. The aim of the present study was to consider the relationships between host and geographic origin of isolates of *L. theobromae* in greater detail and with a considerably more robust collection of isolates. The study initially emerged from an interest in the fungus in Venezuela, where it causes serious problems on forestry crops. Thus, relatively large populations of *L. theobromae* isolates were available from Venezuela and these could be compared with those available from South Africa and Mexico.

MATERIALS AND METHODS

Fungal isolates

Three *L. theobromae* sub-populations (total 84 isolates) were randomly collected in 2003 from *P. caribaea* var. *hondurensis*, *E. urophylla* and *Acacia mangium* at three locations in Venezuela (Table 1). The isolates were made from asymptomatic plant tissue as well as from

trees exhibiting blue stain, die-back and from entirely dead trees. In addition, two populations of *L. theobromae* were used for comparative purposes. These included 70 isolates randomly collected from blue-stained *P. elliotti* lumber in South Africa and 23 isolates obtained from *P. pseudostrobus* seed cones collected near San Cristobal, Mexico (Table 1). Each of these isolates was selected to originate from a different tree, growing in the same area.

For primary isolations, the plant tissue samples were surface sterilised, rinsed and placed on 2 % malt extract agar (MEA) at 25 °C. To induce sporulation, isolates were transferred onto water agar (WA) supplemented with sterilized pine needles and incubated for 3-6 weeks at 25 °C under near-ultraviolet and cool-white fluorescent light. Isolates were derived from single conidia and maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction and SSR-PCR

Fungal cultures were grown on half strength Potato Dextrose agar (DIFCO, Becton Dickinson, MD, USA) in Petri dishes. Mycelium was scraped from the surface of 7 day-old cultures and freeze-dried. DNA was extracted from the dried mycelium following the protocol of Barnes *et al.* (2001). SSR-PCR was performed on all isolates with 8 fluorescent labelled markers, specifically designed to amplify polymorphic regions in *L. theobromae* as described previously (Burgess *et al.*, 2003).

Labelled SSR PCR products were separated on an ABI Prism 377 DNA sequencer and allele size was estimated by comparing the mobility of the SSR products to that of the TAMRA internal size standard (Applied Biosystems, Perkin Elmer Corp.) as determined by GeneScan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 2 (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility.

Gene and genotypic diversity

For each of the loci, individual alleles were assigned a different letter. For each isolate, a data matrix of 8 multistate characters (one for each locus) was compiled (eg. AABDCGDD). The frequency of each allele at each locus for entire and clone corrected populations was calculated, and allele diversity determined using the program POPGENE (Yeh *et al.*, 1999) and the equation $H = 1-\sum x_k^2$, where x_k is the frequency of the kth allele (Nei, 1973). Chi-square tests for differences in allele frequencies at each locus were performed for clone corrected populations (Chen & McDonald, 1996).

Genotypic diversity (G) was estimated using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the ith phenotype (Stoddart & Taylor, 1988). To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N * 100$ where N is the population size.

Population differentiation

Population differentiation (G_{ST}) as measured by theta (Weir, 1996) was calculated between all pairs of clone corrected populations in Multilocus v. 1.3 (Agapow & Burt, 2001). The statistical significance were determined by comparing the observed G_{ST} value to that of 1000 randomized datasets in which individuals were randomized among populations. The number of migrants (M) that must be exchanged between populations for each generation, to give the observed G_{ST} value, was calculated using the equation $M=((1/\theta)-1)/2$ (Cockerham & Weir 1993).

Mode of reproduction

Index of association (I_A) was used to measure multilocus linkage disequilibrium for each clone-corrected population (Maynard Smith *et al.*, 1993). The tests were performed on a data

matrix of 8 multistate characters using the program Multilocus. The distribution under the null hypothesis of recombination was estimated by 1000 randomly recombining data sets and compared with the observed data.

RESULTS

Segregation of SSR alleles

The SSR markers produced 63 alleles across the 8 loci examined (Table 2). There were 47 alleles among the populations from Venezuela, 28 alleles in the Mexican population and 34 alleles in the South African population (Table 2). Of the 63 alleles, 17 (27%) were present in all regions and a further 12 (19%) were present in two of the three populations. Thirty-four alleles (54%) were unique to specific populations of *L. theobromae* (Table 2). There were unique alleles in the Venezuelan population at 7 loci (21 alleles in total), in the Mexican population at 3 loci (3 alleles in total) and in the South African population at 6 loci (10 alleles in total) (Table 2).

Gene and genotype diversity

The mean gene diversity (H) for all 8 loci across all populations of *L. theobromae* was 0.665 for clone corrected populations. The gene diversity among hosts from Venezuela was 0.63 for *Pinus*, 0.67 for *Eucalyptus* and 0.51 for *Acacia* (Table 3). The distribution among geographic regions was 0.70 for Venezuela (VEN), 0.54 for Mexico (MEX) and 0.49 for South Africa (RSA) (Table 5). Values for RSA and MEX were lower than the total mean gene diversity, indicating greater between populations than within population diversity. Diversity for VEN was similar to the total diversity indicating that all observed diversity is reflected in VEN population.

The genotypic diversity for the Venezuelan sub-populations was moderate to low (Table 2) as each of these populations had a single dominant genotype (data not shown). Genotypic diversity for the combined VEN population was also low, again due to the predominance of a single genotype (Table 2). Genotypic diversity in RSA was also low with only 23 genotypes among 70 isolates. Diversity in Mexico was higher because, although there were fewer alleles, a single dominant genotype was not observed (Table 2).

Population differentiation and gene flow

Contingency χ^2 test indicated no significant differences (P>0.05) in allele frequencies at any loci for the Venezuelan populations of *L. theobromae* from *Pinus*, *Eucalyptus* and *Acacia* (Table 3). This is reflected in the lack of population differentiation and very high gene flow between the different populations (Table 4). Therefore, all three Venezuelan populations were pooled.

Results of the Chi-square test indicate significant differences (P<0.05) in allele frequency between the populations from the three different countries at 6 of the 8 loci (Table 5). Gene flow (number of migrants) between countries was restricted, especially between RSA and VEN (Table 6). Although 0 values indicate significant population differentiation, gene flow was less restricted between MEX and RSA and MEX and VEN, than between RSA and VEN.

Mode of reproduction

The index of association (I_A) of the observed data differed significantly from the values obtained for the recombined data set for all the individual *L. theobromae* populations (Fig. 1A-C).

DISCUSSION

In this study, we have considered for the first time, the population structure of the common, generally tropical pathogen *L. theobromae*. In terms of geographic distribution, this is a relatively poorly understood fungus. Whilst it was first described in South America (Patouillard & De Lagerheim, 1892), its very wide host range and geographic distribution suggests it has been actively moved between countries and its true origin in unknown. Populations of isolates considered in this study were specifically from forest tree crops and the results should be interpreted within the context of the relatively narrow focus of the study.

One of the interesting results of this study was the high gene flow between populations from the three host types considered. These hosts are from three very different families, including confers and hardwood trees and results show clearly that host of origin of isolates plays no role in partitioning of the pathogen genotypes. The study also included isolates from three geographically isolated countries and there was a barrier to gene flow between them.

Many species of *Botryosphaeria*, including *L. theobromae*, are known to have a cosmopolitan distribution with wide host range (Arx, 1987; Barr, 1972; Punithalingam, 1976). Thus, the association of *L. theobromae* with 3 different hosts in Venezuela was not unexpected. However, the lack of host specificity is surprising, with the same genotypes found on all three host species. In the study of Burgess, *et al.* (2003), only 9 isolates of *L. theobromae* were considered, however, those from *Eucalyptus* spp. and *Pinus* spp. grouped separately and host specificity was suggested. All three host species in Venezuela are non-native in that country and the lack of specificity might be associated with this fact. If it is assumed that *L. theobromae* is also non-native in Venezuela, there may have been limited introductions and selection pressure, coupled with the lack of niche competition, could have

forced the same genotypes onto the different hosts. This has been observed for a mycorrhizal fungus, *Pisolithus*, in the non-native environment (Dell *et al.*, 2002). *Pisolithus* spp. exhibit host specificity but, for example, a pine-specific isolate will develop superficial mycorrhizae on *Eucalyptus* spp. in the absence of *Eucalyptus*-specific isolates (Dell *et al.*, 2002). In order to determine whether a similar situation exists with *L. theobromae* in Venezuela, pathogenicity trials using the same fungal genotypes on different host species would be required.

While there appears to be no host specificity for *L. theobromae*, at least on the plants considered in this study, there was a clear restriction to gene flow between geographically isolated regions. The lowest level of gene flow was between populations from Venezuela and South Africa. However, while still somewhat limited, there was evidence of some gene flow between the population from Mexico and those from both Venezuela and South Africa. Across all loci only 3 alleles were unique to Mexico, compared with 21 unique alleles in Venezuela and 10 for South Africa. Mexico is a common source of *Pinus* seed in many sub-tropical countries maintaining plantations of non-native *Pinus* spp. (Burgess & Wingfield, 2001). *Lasiodiplodia theobromae* is well known to occur on *Pinus* seed (Cilliers, 1993) and Mexican isolates used in this study were also from seed collected in a native pine stand. It thus seems likely that this fungus has been distributed with seed to many sub-tropical pine growing regions including South Africa and Venezuela.

This observed linkage of alleles between different loci in all populations, suggests a predominantly clonal mode of reproduction for the fungus. This 'clonal' mode of reproduction can be either due to asexual reproduction or homothallic sexual reproduction (selfing) (Coppin *et al.*, 1997; Turgeon, 1998). However, pseudothecia (sexual structures) of *L. theobromae* are seldom seen in nature. Despite repeated collections, we have failed to connect isolates of *L. theobromae* from *Acacia*, *Pinus* and *Eucalyptus* to sexual structures on

these hosts. On these hosts and at the sites studied, the fungus appears to exist in a predominantly asexual form and we were not surprised to find association of alleles at unlinked loci and a clonal genetic structure. Similarly, Burgess *et al.* (2004b) found no evidence of recombination among populations of the related pine endophyte *Diplodia pinea*, and the same genotypes were found across continents. *Diplodia pinea* is the predominant pine endophyte in temperate regions (Burgess *et al.*, 2001a; Burgess & Wingfield, 2001, 2002) and this niche appears to be replaced by *Lasiodiplodia theobromae* in tropical and sub-tropical regions (Burgess & Wingfield, 2002). *L. theobromae* appears to be similar to *D. pinea* with single genotypes found over large distances.

Generally, fungi undergoing sexual reproduction exhibit greater genotypic diversity than those reproducing asexually (Milgroom, 1996). In our study, low genotypic diversity was observed in populations from Venezuela and South Africa, arising from the predominance of a single genotype. In both cases the area from which the samples were collected was greater than 100 km^2, indicating genotype flow across a region. Although the limited genetic diversity suggests this, the scope of this study was insufficient to be able to say that *L. theobromae* has been introduced into Venezuela and South Africa. The isolates from Mexico originated from native trees in an undisturbed area. The higher genetic diversity among isolates suggests that this population might be native. Confirmation of this fact would require larger numbers of isolates collected in a more structured fashion from a wider diversity of sites.

Lasiodiplodia theobromae is an important pathogen on many tree crops, tempting speculation of host specific groups as is, for example, found with the root pathogen *Fusarium oxysporum* (Gordon & Martyn, 1997). Our study has shown no evidence for host specificity, and demonstrated very high gene flow between populations of isolates from different hosts. Reproduction was predominantly clonal with some genotypes widely distributed with a

region. This was observed for a purported native population (Mexico) and probable introduced populations (South Africa, Venezuela). The broad host range of *L. theobromae* and lack of host specialisation, coupled with its endophytic nature and the appearance of symptoms such a blue stain only after harvest, are likely to hinder efforts to manage this pathogen.

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Table 1. Source of *Lasiodiplodia theobromae* isolates from Venezuela, Mexico and South Africa.

Country	Location	Cultivar	Origin of seed	No. of isolates	Collector
Venezuela	Falcon state	*P. caribaea* var *hondurensis*	Guatemala	30	S. Mohali
	Portuguesa and Cojedes state	*E. urophylla*	Brasil	29	S. Mohali
	Portuguesa and Cojedes state	*A. mangium*	Indonesia	25	S. Mohali
Mexico	San Cristóbal	*Pinus pseudostrobus*	Mexico	23	M. Wingfield
South Africa	Kwa Zulu Natal and Mpumulunga	*Pinus elliotti*	unknown	70	W. de Beer

Table 2. Allele size (bp) and frequency at 8 loci (LAS1-8) for *Lasiodiplodia theobromae* populations collected from Venezuela (VEN), Mexico (MEX) and South Africa (RSA).

Locus	Allele	VEN	MEX	RSA
LAS1	352	0.643	0.391	-
	355	-	-	0.014
	358	-	0.131	-
	360	-	0.217	0.071
	361	0.274	0.217	0.886
	364	0.012	-	-
	367	0.036	-	-
	369	-	-	0.014
	370	0.036	0.044	0.014
LAS2	312	0.060	-	-
	313	0.095	-	-
	314	-	0.044	-
	316	0.560	0.522	0.872
	317	0.274	0.391	0.114
	320	0.012	0.043	0.014
LAS3	326	-	-	0.014
	329	0.012	-	-
	330	0.155	-	-
	334	0.036	-	-
	336	0.262	0.348	0.871
	343	-	-	0.029
	348	-	-	0.029
	352	0.012	0.609	0.043
	354	0.466	-	-
	355	0.036	-	-
	null	-	0.043	0.014
LAS4	248	-	-	0.029
	251	0.012	0.044	0.043
	254	0.095	-	0.014
	255	0.993	0.956	0.900
	258	-	-	0.014
LAS5	383	0.024	0.522	-
	385	0.500	-	-
	387	0.143	0.435	-
	388	0.060	-	0.771
	389	0.202	0.043	0.200
	400	0.071	-	0.029
LAS6	454	-	-	0.029
	459	0.036	-	-
	463	0.262	0.435	0.828
	465	0.024	0.043	0.029
	468	0.476	0.478	0.100
	488	0.071	-	-
	490	0.048	-	-
	492	0.060	-	-
	496	0.024	-	-
	504	-	0.044	0.014
LAS7	180	0.012	-	-
	182	0.024	-	-
	183	0.643	0.522	-
	192	0.274	0.478	0.986
	195	0.036	-	-
	199	-	-	0.014
	201	0.012	-	-
LAS8	372	-	-	0.029
	376	0.012	0.087	0.428
	377	0.083	-	0.114
	380	0.012	0.261	0.400
	381	0.012	0.043	-
	382	0.190	-	-
	384	0.012	-	-
	385	0.679	0.565	0.029
	392	-	0.044	-
N		84	23	70
No. Alleles		47	28	34
No. Unique Alleles		21	3	10
H		0.543	0.513	0.274
N(g)		24	11	23

| G | 4.76 | 5.05 | 5.09 |
| Ĝ | 5.66% | 21.94% | 7.27% |

N = number of isolates
N(g) = number of genotypes
H = gene diversity of the population (Nei 1973)
G = Genotypic diversity (Stoddart and Taylor, 1988)
Ĝ = G/N% = percent maximum diversity
null = primers failed to amplify a product probably indication a mutation in the primer binding site.

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* from Venezuela collected from *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium*. No χ^2 values were significant.

	Gene diversity (H)				
Locus	Pinus	Eucalyptus	Acacia	χ^2	df
LAS1	0.62	0.58	0.57	10.9	8
LAS2	0.54	0.72	0.49	13.1	8
LAS3	0.80	0.66	0.49	20.4	12
LAS4	0.34	0.42	0.24	3.0	4
LAS5	0.80	0.74	0.69	7.8	10
LAS6	0.78	0.84	0.61	15.5	14
LAS7	0.62	0.74	0.49	12.1	10
LAS8	0.56	0.70	0.49	14.2	12
N (g)	10	10	7		
MEAN	0.63	0.67	0.51		

Table 4. Pairwise comparisons of population differentiation. G_{ST}, (above the diagonal) and number of migrants, M, (below the diagonal) among clone corrected populations of *Lasiodiplodia theobromae* from Venezuela collected from *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium*. There was no significant differentiation between populations.

	Pine	Eucalypt	Acacia
Pine	-	0.020	0.005
Eucalypt	24.5	-	0.065
Acacia	99.5	7.19	-

Table 5. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 8 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* from Venezuela (VEN) Mexico (MEX) and South Africa (RSA). For χ^2 values stars indicate level of significance (*** P<0.001, **P<0.01, *P<0.05), no stars indicate no significant differentiation between populations.

Locus	Gene diversity (H)			χ^2	df
	VEN	MEX	RSA		
LAS1	0.68	0.76	0.43	45.7***	16
LAS2	0.71	0.61	0.48	19.7*	10
LAS3	0.79	0.58	0.59	43.2**	20
LAS4	0.39	0.16	0.49	10.6	8
LAS5	0.82	0.51	0.57	27.6**	10
LAS6	0.73	0.55	0.58	23.6	18
LAS7	0.69	0.40	0.08	26.3**	12
LAS8	0.67	0.74	0.75	39.0***	16
N (g)	24	11	23		
MEAN	0.70	0.54	0.49		

Table 6. Pairwise comparisons of population differentiation, G_{ST}, (above the diagonal) and number of migrants, M, (below the diagonal) among clone corrected populations of *Lasiodiplodia theobromae* from Venezuela (VEN) Mexico (MEX) and South Africa (RSA). For G_{ST} values, stars represent level of significance (*** P<0.001, **P<0.01, *P<0.05), no stars indicate no significant differentiation between populations.

	VEN	MEX	RSA
VEN	-	0.077*	0.152***
MEX	5.99	-	0.087**
RSA	2.79	5.24	-

Fig. 1. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomized datasets. (A) Venezuela, (B) Mexico and (C) South Africa. Results were compared with the observed dataset (arrows).