Highly transferable microsatellite markers for the genera *Lasiodiplodia* and *Neofusicoccum*

Jan H. Nagel[1]

Elsie M. Cruywagen[1,2]

Joseph Machua[1]

Michael J. Wingfield[1]

Bernard Slippers[1]*

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0001 South Africa

[2] Agricultural Research Council, Vegetable and Ornamental Plants, Crop Protection Division, Roodeplaat, Pretoria, 0001, South Africa

*Corresponding Author.

Email: bernard.slippers@fabi.up.ac.za

Telephone: +27 (12) 420-3937 or 3938

FAX: +27 (12) 420-3960

Highlights

- 72 and 32 SSR markers were developed for Lasiodiplodia and Neofusicoccum.
- Most of the markers were transferable across species in these genera.
- A subset of markers were shown to be polymorphic within sample populations.
- Transferable SSR markers broadly enable molecular ecology studies in these genera.

Abstract:

Population genetic studies play an integral role in understanding the ecology and management of fungal plant pathogens. Such studies for species of *Botryosphaeriaceae* are hampered by a lack of available markers. Genomic sequences are available for multiple species in this family and they provide excellent resources for the development of population genetic markers. Here we describe highly transferable microsatellite or simple sequence repeat (SSR) markers for species in *Lasiodiplodia* and *Neofusicoccum*; two important and globally distributed members of the *Botryosphaeriaceae.* These were developed by extracting SSR-containing sequences from available genomes. Seventy-seven markers were developed for *Lasiodiplodia* and 32 markers were developed for *Neofusicoccum*. Most of these markers were transferable between species within a genus. Twelve markers tested for fragment length polymorphism in 20 isolates of *L. mahajangana* identified between two and nine alleles and gene diversities between 0.18 and 0.83. Eleven markers indicated between two and five alleles for 20 isolates of *N. parvum* and gene diversities between 0.26 and 0.57. The large number and high transferability of the developed markers will facilitate population studies of a wide range of *Lasiodiplodia* and *Neofusicoccum* species associated with tree diseases globally.

Keywords: *Botryosphaeriaceae*, plant pathogens, population genetics, genome mining, microsatellites, transferability

1. Introduction

Many species in the fungal family *Botryosphaeriaceae* are well-known pathogens of economically important, commercially propagated woody plant crops. These include grapevine (Urbez-Torres 2011), pome and stone fruits (Slippers *et al.* 2007), and plantation trees such as *Eucalyptus* spp., *Pinus* spp. and *Acacia mangium* (Mohali *et al.* 2007; Rodas *et al.* 2009; Alves *et al.* 2013). They are also found on trees in their native habitats (Marincowitz *et al.* 2008; Pavlic *et al.* 2008; Jami *et al.* 2014). *Botryosphaeriaceae* infections can result in various disease symptoms, including cankers, dieback, collar rot, damping-off of seedlings, blue-stain and may even result in the death of the host plant (Slippers & Wingfield 2007). Many of these species have very wide host ranges and have spread globally, due to trade in plants and plant products. Notable examples include *Botryosphaeria dothidea* (Marsberg *et al.* 2016), *Neofusicoccum parvum* and *Lasiodiplodia theobromae* (Slippers & Wingfield 2007; Farr & Rossman 2018). With the growing number of invasive plant pathogens (Wingfield *et al.* 2010; Wingfield *et al.* 2017), it is becoming increasingly important to be able to study the population dynamics of these organisms. But for the *Botryosphaeriaceae*, robust genetic markers are not currently available.

Microsatellite or simple sequence repeat (SSR) markers are widely used in population genetic studies. These have previously been developed for some species of *Botryosphaeriaceae*. Over the last two decades SSR markers have been developed for *Diplodia sapinea* (Burgess *et al.* 2001; Bihon *et al.* 2011), *D. scrobiculata* (previously *Diplodia* (=*Sphaeropsis*) *sapinea* morphotype B) (Burgess *et al.* 2001), *Lasiodiplodia theobromae* (Burgess *et al.* 2003; Cardoso & Wilkinson 2008), *Neofusicoccum parvum* (Slippers *et al.* 2004), *Macrophomina phaseolina* (Baird *et al.* 2009; Baird *et al.* 2010) and *Botryosphaeria dothidea* (Manawasinghe *et al.* 2018). Some of these markers have been tested to determine whether they are transferable to other species in the *Botryosphaeriaceae* (Slippers *et al.* 2004; Baird *et al.* 2010). However, there are few examples of cross-species and cross-genus transferable microsatellite markers and for

those that are available, the range of species for which they are transferable has not been well characterized.

The application of SSR markers to characterize populations of different *Botryosphaeriaceae* species has yielded significant insights into the biology of these fungi. For example, these include studies that indicate various levels of diversity that related to origins and patterns of introduction, both low (Zlatković *et al*. 2019) and high (Manawasinghe *et al.* 2018; Begoude Boyogueno *et al*. 2012; Burgess *et al.* 2006; Pavlic-Zupanc *et al.* 2015). Comparisons between populations occurring on different host plants have indicated that most *Botryosphaeriaceae* species do not exhibit population subdivision based on host preference (Begoude Boyogueno *et al*. 2012; Mehl *et al.* 2017; Mohali *et al*, 2005). Analysis of multilocus genotypes have also indicated that some populations are clonal (Bihon et al. 2011; Zlatković *et al*. 2019), while others are apparently outcrossing, even though sexual structures are not known (Bihon et al. 2012).

Cross-species transferable SSR markers are immensely valuable resources for population genetic studies. Development of SSR markers is expensive and time-consuming because it requires the sequencing and analysis of SSR-containing regions, as well as designing and testing of primers. There is consequently great benefit when these markers can be applied to multiple related species. The transferability of markers is determined by the sequence conservation of primer binding sites across a range of species and as such, is strongly influenced by the phylogenetic distance between the source and target species (Rossetto 2001; Barbara *et al.* 2007). SSR marker transferability has been demonstrated in many groups of fungi (Slippers *et al.* 2004; Wadud *et al.* 2006; Cristancho & Escobar 2008; Benichou *et al.* 2009; Baird *et al.* 2010; Leyva-Madrigal *et al.* 2014), but this has not been considered for various important species of the *Botryosphaeriaceae*.

The number of genomes sequenced for species of *Botryosphaeriaceae* has increased rapidly in recent years (Islam *et al.* 2012; Blanco-Ulate *et al.* 2013; van der Nest *et al.* 2014; Morales-Cruz *et al.* 2015; Wingfield *et al.* 2015; Marsberg *et al.* 2016; Yan *et al.* 2017). These available genomes are creating an opportunity for the development of transferable microsatellite markers. Genomes from multiple species in a genus allows greater power to select conserved regions for the design markers, that would be expected to make them transferable to other species in that genus, for which the genomes have not yet been sequenced. In the *Botryosphaeriaceae,* there are a number of sequenced genomes for some prominent genera (Slippers *et al.* 2017), i.e *Botryosphaeria* (2 genomes), *Diplodia* (6 genomes), *Lasiodiplodia* (1 genome), and *Neofusicoccum* (2 genomes). Additional, incomplete genomes of species of *Lasiodiplodia* (3 genomes) and *Neofusicoccum* (5 genomes) are also used in this study.

The aim of this study was to develop SSR markers for *Lasiodiplodia* and *Neofusicoccum* by mining their available genomes. We specifically aim to develop markers that are transferable between species within each of these two genera. Lastly, we demonstrate the polymorphism and utility of these primers in test populations.

2. Materials and Methods

2.1. Genome sequences, fungal material and phylogenetics

Genome sequences of species of *Lasiodiplodia* and *Neofusicoccum* were retrieved from online public databases or from unpublished genome assemblies (Table 1). Isolates, used to test the developed primers, are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Supplementary Table 2 and 3). Mycelium was scraped from the surface of cultures grown on malt extract agar (MEA; 2% Biolab malt extract, 2% Difco agar). DNA was extracted from these samples using a CTAB based extraction protocol (Möller *et al.* 1992).

Table 1. Genomes used during the development of microsatellite markers

Species	Reference collection number*	Genome accession	Genome reference
Lasiodiplodia gonubiensis	**CBS115812**	**RHKH00000000**	
L. pseudotheobromae	**CBS116459**	**RHKG00000000**	
L. theobromae	**CBS164.96**	**RHKF00000000**	
Neofusicoccum cordaticola	**CBS123638**	**RHKD00000000**	
N. kwambonambiense	**CBS123642**	**RKSS00000000**	
N. parvum	UCRNP2	AORE00000000	(Blanco-Ulate et al., 2013)
	UCD646So	PRJNA321421	(Massonnet et al., 2016)
	CMW9080	**RHJX00000000**	
	CBS123649	**RHJY00000000**	
N. ribis	**CBS115475**	**RHJZ00000000**	

* Entries in boldface represents isolates where microsatellite containing sequences were obtained from draft genomes

In order to compare SSR content and the number of markers transferable to selected species (described below) against their evolutionary relationship, a phylogenetic tree was constructed. Sequences of the internal transcribed spacer (ITS) region of well-known species of *Lasiodiplodia* and *Neofusicoccum* were retrieved from GenBank (Supplementary Table 1). Sequences were aligned using MAFFT (Katoh & Standley 2013). Maximum parsimony phylogenetic trees were constructed using PAUP (Swofford 2001) and 1000 bootstrap replicates were performed to assess branch support. Phylogenetic trees were rooted using *Phyllosticta citriasiana* CBS120486.

2.2. SSR marker primer development

Multiple sets of SSR marker primer pairs were developed for *Lasiodiplodia* and *Neofusicoccum*. In each case, SSR-containing sequences were identified and extracted from genomic sequences using SciRoKo (Kofler *et al.* 2007). Primers were designed using Primer3 (Untergasser *et al.* 2012) to yield amplicons between 100 and 500 bp in length and these were screened *in silico* with CLC Main Workbench 7.71 using the “Find binding sites and create fragments tool”.

2.2.1. *Lasiodiplodia* primers

Three sets of primers were developed for *Lasiodiplodia*. The first set was designed to enable amplification across as many *Lasiodiplodia* spp. as possible. SSR-containing sequences were extracted from the genome of *L. pseudotheobromae* and used in primer design. These primer pairs were tested *in silico* to determine their potential usage in *L. gonubiensis*, *L. pseudotheobromae* and *L. theobromae*. Primer pairs predicted to amplify in all three species were further investigated for synthesis.

The second set of *Lasiodiplodia* primers were designed to amplify a narrower range of species than the first, i.e. *Lasiodiplodia* spp. closely related to *L. pseudotheobromae* and *L. theobromae*. SSR-containing sequences were extracted from genomic sequences of *L.*

pseudotheobromae and *L. theobromae* and fragments containing trinucleotide repeat motifs were compared to each other using BLAST (Altschul *et al.* 1990) with a cut-off E-value of 1E-100. Those sequences with reciprocal best BLAST hits between the two species were aligned and those with the longest SSR repeat lengths were further investigated for primer design.

The third set of primers was designed from only *L. theobromae*. SSR-containing sequences were extracted from the *L. theobromae* genome and used to design primers. The primer pairs predicted to amplify in *L. theobromae* with the 50 longest SSR repeats were investigated and the best were chosen for synthesis.

2.2.2. *Neofusicoccum* primers

Two sets of primers were developed for the genus *Neofusicoccum*. The first set was developed by mining and extracting SSR containing sequences from the genomes of *N. cordaticola*, *N. kwambonambiense*, *N. parvum* and *N. ribis*. The fragments from each genome were compared with the others using BLAST with cut-off E-value of 1E-100. Those fragments with reciprocal best BLAST hits between these genomes were aligned and the 30 alignments with the longest SSR lengths were further investigated for primer design.

The second set of *Neofusicoccum* primers was developed from the genome of *N. parvum* (UCD646So). These primer pairs were analyzed *in silico* on all four available *N. parvum* genomes. Primer pairs predicted to yield single amplicons and with size polymorphisms between the four *N. parvum* genomes were chosen for further analysis. The predicted amplicons of each primer pair were aligned and it was investigated whether the polymorphism was due to SSR length variation. The twenty best primer pairs of this set were chosen for further analysis.

2.3. SSR marker amplification and assessment of transferability

Synthesized primer pairs were tested using polymerase chain reaction (PCR) to establish their utility in the species for which they were designed, as well as to investigate whether they were transferable to other species in the genus. Primers were tested in twelve *Lasiodiplodia* spp. (Table 2) and eight *Neofusicoccum* spp. (Table 3). Reactions were set up using 1x KAPA Taq Buffer (Kapa Biosystems), 0.5 U KAPA Taq DNA Polymerase (Kapa Biosystems), 200 µM of each dNTP, 0.4 µM of each primer, 50-100 ng of template DNA and PCR-grade water to a volume of 25 µl. Thermal conditions of the PCRs were one cycle of denaturation at 95 °C for four minutes, followed by 35 cycles of denaturation (95 °C for 30s), annealing (60 °C for 30s) and elongation (72 °C for 30s), and a final elongation step at 72 °C for four minutes.

Following PCR amplification, 5 µl of each sample were separated using electrophoresis on a 1.5% agarose gel at 80 V for 30 min. Samples were combined with GelRed (Biotium) and loading buffer prior to loading samples on the agarose gel. Samples were electrophoretically analyzed in conjunction with 2 µl GeneRuler 100bp Plus DNA Ladder (Thermo Fisher Scientific) and were visualized on a Gel Doc™ EZ Imager (Bio-Rad).

2.4. Marker polymorphism tests

Fragment length analyses were performed on PCR amplicons of a selection of both the *Lasiodiplodia* and *Neofusicoccum* markers to determine their polymorphism. SSR markers were PCR amplified from twenty isolates of *Lasiodiplodia mahajangana* and twenty isolates of *N. parvum* using the same conditions as described above, except for the final elongation step that was performed at 60 °C for 30 minutes. Twelve *Lasiodiplodia* and eleven *Neofusicoccum* SSR markers displaying agarose gel electrophoresis size polymorphism were selected and their respective primer pairs were fluorescently labelled. Standard primers were synthesized at Inqaba Biotech (South Africa) and fluorescently labelled primers at LTC Tech (South Africa).

Table 2. Primers designed for amplification of microsatellite containing sequence fragments from *Lasiodiplodia* spp.

Marker*	Primer	Primer sequence (5' - 3')	Tm (C)	Repeat Motif	Predicted fragment sizes (bp)	Number of motif repeats		
						L. theobromae	*L. pseudotheobromae*	*L. gonubiensis*
Set 1:								
Las1	Las1f	TGCATCATCCACCTCCACAT	67	CTG	380-399	9	6	7
	Las1r	GCACCTTCAACTCGGTCTTC	64.2					
Las2	Las2f	AGGCCTTGATCAACGGTACA	64.8	CAA	295-304	24	11	23
	Las2r	CTCTTGGCCTTGTTGTTCCC	66.1					
Las3	Las3f	AGAAGCTATGAGTGCTCGCT	61.4	GAT	366-372	6	5	7
	Las3r	TAAGGAAGGTGGTCTGGCAG	64.5					
Las6	Las6f	CAGCTCTACCTTCACCGACT	61.6	CAG	480-489	9	8	8
	Las6r	GTGTTGTGAGAGATGGACGC	63.9					
Las8	Las8f	TCCACGATGCACAGAGATGG	67.9	ACC	492-501	8	10	4
	Las8r	CTTGATGGCGAGGTCATTGG	68.1					
Las9	Las9f	CCAGAGTCAGCACCCTAAGC	64.3	GAGC	411-424	6	5	4
	Las9r	CGGCAGCAATCTACATACCA	64					
Las10	Las10f	AACGAGTGACGACGAGTGTG	64.6	CTG	458-470	11	12	15
	Las10r	ATCGTGCGTCATACAGTGGA	64.7					
Las11	Las11f	ACGGTTACGTGCCATCTGTT	64.7	A	223-314	14	17	16
	Las11r	ACCTCAGTCACCTGGACACC	64.7					
Las12	Las12f	GATGTTCGAGCAAGGACGAT	64.6	TGG	305-309	5	6	5
	Las12r	GTCCAATGGCAACGCTTACT	64.3					
Las13	Las13f	GTGAGTGCAGGTGTAGGCAA	64.4	GA	193-200	6	8	4
	Las13r	GTGCATGCTTTCGTACGCT	64.4					
Las14	Las14f	TGATGGGAGAGTGAGAAGGC	64.8	AGC	424-430	8	9	10
	Las14r	ACACGTATGTAATGCGCAGC	64.1					
Las15	Las15f	ACAAAGGGCATTCGGAGTTA	63.7	T	364-464	10	17	5
	Las15r	CGTGCGTGATGAAGAAGAAA	64.4					
Las19	Las19f	CTGCGCCTTCCTATTAGACG	64	CT	387-405	14	8	7

	Las19r	AGTGTGTCGTCCTTAGCGGT	64.1					
Las21	Las21f	CTCGTCTCCAACACCAACG	64.9	GCA	408-438	2	6	6
	Las21r	AGCCTCGACATCCTTCAAGA	64.3					
Las22	Las22f	CATGAGCAGACCAATCCTCC	65.1	CCG	326-409	11	11	12
	Las22r	CGGTCATGTCATACTGGCAC	64.6					
Las23	Las23f	GGTTGATGTCGCAAGTGATG	64.7	GA	440-490	5	9	16
	Las23r	CACTTCCAAGTCCACCCACT	64.5					
Las24	Las24f	GTTGGAGACGTAGAGCTGGC	64.4	CCG	273-284	6	7	7
	Las24r	ACCCTTTCGAACCAGAACCT	64.1					
Las25	Las25f	CTGCGGTAGAGGTTCGACTC	64.3	A	251-354	6	16	6
	Las25r	CTTCACCTTCTTCGAGGGC	64.3					
Las26	Las26f	TGCATGCAATAGCTCCTGTC	64.4	GT	307-326	13	16	9
	Las26r	TCTTACCGGCACTACGGAAC	64.3					
Las27	Las27f	AGAAGAGACGCCTAGGACCC	64	A	366-465	11	15	6
	Las27r	GAAGAGAATGAGCGTCAGGG	64.3					
Las28	Las28f	GCACCCAAGGAGATTCGTTA	64.3	A	394-481	17	17	11
	Las28r	CGATCAGATTCGCCTTCTTC	64.2					
Set 2:								
TPs1	TPs1f	CTTTCACATGCCTGCCCTAT	64.3	ATC	279-347	32	11	
	TPs1r	TTGCGTCAGTAGGAGCCTTT	64.2					
TPs2	TPs2f	CGGTAACGATTACCCTCACG	64.5	AGG	349-379	12	6	
	TPs2r	AATGAGTCGGCTCTTCTCCA	64.3					
TPs3	TPs3f	ATCGCGGAGTCAGCTGATAG	64.8	AGG	380-397	12	6	
	TPs3r	GTCGTCGTCGTCGATGGT	65.6					
TPs5	TPs5f	GCCTGCTGGAAACGATAAAC	63.8	ATC	106-122	11	6	
	TPs5r	GGCATTAGCATTAGCGTCAG	62.7					
TPs6	TPs6f	CATCATCGCAGTCCTCTGAA	64.5	AGC	391-394	15	10	
	TPs6r	ATCCTCGAGCTTGTTGCTGT	64.4					
TPs7	TPs7f	TCCGGAGAAATGAAGCAGTC	64.7	AGC	365-368	10	5	

	TPs7r	CTCGTCGGTCTCCTGGTCT	65				
TPs8	TPs8f	TACGCCGAGTATTCAGTTGC	63.2	ACC	118-130	10	6
	TPs8r	ATCAAACGTATCCAGCGACA	63.5				
TPs9	TPs9f	GTGGACTCGGAAATTGAGGA	64.4	AGC	384-384	10	13
	TPs9r	CAAGGTCAAGCAGGACATGA	64.4				
TPs10	TPs10f	CTTGACCCGGTGCTCGAT	66.8	AGC	346-361	10	6
	TPs10r	CATTGCTTCACCCTCACACA	65.3				
TPs11	TPs11f	AACCCGGATTCCTTTGAGAT	63.8	AGC	361-382	9	9
	TPs11r	CAACGTGGTCGTGGATAGG	64.5				
TPs12	TPs12f	ACGAACGAGTCCGTCCAC	64.5	AGC	530-548	10	5
	TPs12r	CCCCAATTCGACCTAGTCC	63.7				
TPs13	TPs13f	AGGTCCACCGTCTCCTTCTT	64.4	ACG	353-383	10	5
	TPs13r	GGGAAGCTGGTGGAGAGG	65.4				
TPs14	TPs14f	CATCGCTCCAGACATCCAC	65	CCG	292-298	10	8
	TPs14r	ATCAACCCGCTCTTCCTG	63.4				
TPs15	TPs15r	TGCAGTCTGATTCAGCGTCT	64.3	AAG	302-336	14	not present
	TPs15f	GTCCCGGTCAATTGTGATCT	64.2				
TPs16	TPs16f	TATCTCGACGAACCGGAAAC	64.3	AAG	215-251	9	21
	TPs16r	CCAACACGAAACTCCAAGGT	64.3				
TPs17	TPs17f	CAAGGTGGGGGTATGAGTTG	64.5	AAC	212-224	9	5
	TPs17r	CTCGTGGGTCCCATTTCTAA	64.1				
TPs19	TPs19f	CGACTTACCAAACGGACACG	65.7	CCG	357-366	5	15
	TPs19r	CATGCATGATGGCGCACT	67.7				
TPs21	TPs21f	GGATTCTTTGAGACGCTGGT	63.6	AGC	329-335	9	7
	TPs21r	GCAGGGTTGAAGAGACGAAG	64.3				
TPs22	TPs22f	TTGGGTAACTCCACGCAAAT	64.5	AGC	365-383	9	18
	TPs22r	AGTGCATCCTAGAGGTCAACG	63.6				
TPs23	TPs23f	AATCCCGACGTTCCCAAG	65.3	AAG	271-394	5	30
	TPs23r	GGCTCGATGGTGTAGGAGAG	64.2				
TPs24	TPs24f	CTGCCTACGCTCCTCCTG	64.3	AAG	515-587	7	24

	TPs24r	CCTGCCCTTGCCCTTCTT	66.9				
TPs25	TPs25r	GCGACGAGGACAACTCGTA	64.6	AGC	388-388	5	15
	TPs25f	AGACCTCCACCCAACCTCTC	64.9				
TPs26	TPs26f	GGCGAAGGTCCAGTACGATA	64.3	AGC	299-320	8	14
	TPs26r	CATACTGCGGGTATTGCTGTT	64.1				
TPs27	TPs27f	AATCAAGCTGGTCGAGCAAT	64.1	AGC	282-300	5	11
	TPs27r	GCCAAGTCGTCCAGTTTCTT	63.6				
TPs28	TPs28f	CACAGCTGGTTGGATGATTG	64.7	ACC	269-284	5	11
	TPs28r	TGTATCACCGCTCCTTCTCC	64.6				
TPs29	TPs29f	GACGCCAGTCGAGTTGATCT	64.9	ACC	115-140	7	11
	TPs29r	TCTCAATCCACCAACCAACA	64.5				
Set 3:							
LT1	LT1f	GCAACTCCGATCAGATGTCA	64.4	CAG	342	19	
	LT1r	TGACAGCTTCTTCAGACGGA	64.3				
LT2	LT2f	ACGCACCTCTCACTTCGACT	64.5	AAC	433	30	
	LT2r	GTGCTGATTCCGTTTGCTTT	64.4				
LT5	LT5f	CAGTATTTCCAGTGCGGGAT	64.2	TCT	267	26	
	LT5r	GTCTTCGGTGGAGGCATCT	64.7				
LT6	LT6f	TCTCATCTCAGGGCAGAGGT	64.5	GAA	340	25	
	LT6r	CCGTCTGAGCAAGGAATAGC	64.2				
LT7	LT7f	CAAGCTCATCTGCTCCACAA	64.7	CTT	458	22	
	LT7r	GACGAGGATGTCGGCTTTC	65.2				
LT8	LT8f	ACCACCAAACATTTCCCAAA	64.3	CTT	354	25	
	LT8r	ACGAAGAGCTGGAAGAGCTG	64.2				
LT9	LT9f	GCCGAAGATGACACTTCCAT	64.5	GGCAAT	415	8	
	LT9r	GGTTGACCTGGTGGTACGTC	64.7				
LT10	LT10f	TTGACTGCTGGACACGCTAC	64.6	TTG	463	15	
	LT10r	AGAGCTGCGGTCATGATTCT	64.4				
LT11	LT11f	TGTGAATGAGAGTATCGCGG	64.3	CCA	351	14	

	LT11r	AGCATCATGAAGAGGTTGGG	64.4			
LT12	LT12f	GCGTTGCTGTTAGTCAGACG	64.1	CCA	171	13
	LT12r	GGTTGTGGAGGAGCAGAAGA	64.8			
LT13	LT13f	TGGTGTGTCGTGGTGGTTAG	65.1	GTGA	223	12
	LT13r	TGAACATCAAGCACCTGAGC	64.6			
LT14	LT14f	GGGTCCACTGAGGAGCATAC	64	GCT	220	13
	LT14r	TACACATGGCCTCAGAGCAG	64.6			
LT15	LT15f	GGAGGATTGTCAGCGTTTGT	64.5	GGA	240	15
	LT15r	GGACAATTTCCTTTCTGCGA	64.4			
LT16	LT16f	GAGAGTGATCGATTTGCCATC	64	GAG	263	13
	LT16r	CAACCGCCCTTTAACGAATA	63.9			
LT17	LT17f	TAGTTGTCGCTGGGATAGGG	64.3	GAG	230	14
	LT17r	ACGAAGAAACTCGCCACTGA	65.3			
LT18	LT18f	CCGTCTTCTCCTGAATCTGC	64.3	CTCCTG	442	6
	LT18r	GGGATTGTGAGTGAGAGGGA	64.6			
LT19	LT19f	CACACACGGTTCTATGCCAG	64.7	CTG	432	16
	LT19r	CGTGATAGCCATTCTGCTCA	64.4			
LT20	LT20f	GAATGCGTGTTTGGATGATG	64.4	GTG	342	13
	LT20r	CAACTCCGAGATCAAGAGCC	64.3			
LT21	LT21f	TCCCGTTTGTCCTTTACCAG	64.1	CCA	239	13
	LT21r	TGAGGAAGGAGGTGAAGGTG	64.7			
LT22	LT22f	ACCAAGAAACAACAGGCTGC	64.6	ATC	364	14
	LT22r	CGATCCGATAGTTGAAGGAC	61.5			
LT23	LT23f	CGGGATCACTTCTTTCCAGA	64.5	GTT	309	13
	LT23r	AGGTAGTTAGCTACGCCGCA	64.1			
LT24	LT24f	GACCGGAGATACGCAACAAT	64.2	TATT	381	11.5
	LT24r	CCGTCTCTAATAGCTAGCGGC	64.3			
LT25	LT25f	TGGATCTCGACAAATCCTCC	64.4	CTG	183	14
	LT25r	CTTCGACTGGGATCGAGAAC	64.2			
LT26	LT26f	TAACGGCACGACGTATACCA	64.2	TCC	380	13

	LT26r	GTGCTGCTGGTTGAGGAAGT	64.9			
LT27	LT27f	CTCTTCTTCGACAGCCTTGC	64.6	CAGCCT	497	7
	LT27r	CTGATCCTGAACCCATCGTT	64.3			
LT28	LT28f	GGTCATCGTCGGATCCTCTA	64.4	ACTC	166	11
	LT28r	TAAGGGTTGGTAGAGGCGAA	63.8			
LT29	LT29f	GAATCGGGATCCTCATCAGA	64.4	CTC	249	14
	LT29r	GCCGAAGGAGATCGACTACA	64.7			
LT30	LT30f	TACCACACAACCCAGCATTG	64.9	CTT	205	14
	LT30r	AGGTGTCGGTCAGCCAGTAG	64.7			
LT31	LT31f	CGCCATTGATGAGGAAAGAT	64.3	TTG	355	16
	LT31r	ATACTCTGGGTTTGGCATCG	64.2			
LT32	LT32f	GCTGTTCGTCATCGTCTTCA	64.6	AGA	468	16
	LT32r	AGCACGGACTTGTTCAACCT	64.1			

* Entries in boldface indicate markers that were selected for polymorphism assessment

Table 3. Primers designed for amplification of microsatellite containing sequence fragments from *Neofusicoccum* spp.

Marker*	Primer	Primer sequence (5' - 3')	Tm (C)	Repeat Motif	Predicted fragment sizes (bp)	Number of motif repeats			
						N. cordaticola	*N. kwambonambiense*	*N. parvum*	*N. ribis*
Set 1:									
Neo1	Neo1f	ACGATGCAAGTCTGGACCTC	64.7	CTG	307-334	19	10	13, 17	13
	Neo1r	GGAGAAACCGTGACAAGCAT	64.5						
Neo4	Neo4f	CGCATATACTCAGCCTGCAA	64.2	CTG	350-380	17	12	8, 12, 18	12
	Neo4r	GCTCAACAGCCACCTCTACC	64.2						
Neo5	Neo5f	TGCAGTTTCGATGTTTGAGC	64.4	GTG	405-429	16	11	10, 11, 12	9
	Neo5r	CTCGCCGTATTTGGAGGTT	64.3						
Neo7	Neo7f	GTACCAAGACGCCCAGAAG	62.8	TTCC	327-335	7	5	6, 7	6
	Neo7r	CAGACTTGAGGCCAGTCAGG	65.5						
Neo8	Neo8f	CGGCGCTCTTGGTAGGAG	66.4	TGCTGG	268-280	4	4	6, 7	5
	Neo8r	CCCTCGACACCAGCAGAG	65.4						
Neo9	Neo9f	GCCATCGAGAAGGTCGAGAT	66	GGT	389-428	5	7	4, 6, 7	5
	Neo9r	GACCTTTAAGACGGCGTGTC	64						
Neo10	Neo10f	TGTTCGCGCACAATGTAGTC	65.3	GGC	162-168	10	9	8, 9	8
	Neo10r	GCAGCCCGTTCCTGTCTT	65.9						
Neo11	Neo11f	CCGCTTTAGCCTCAATCTCA	64.6	GA	171-187	14	-	8, 9, 16	21
	Neo11r	GAGGGGTGTTGCTGTCTTTG	65.1						
Neo12	Neo12f	TATTGGCTCGTCTTCGGACT	64.1	GCG	216-223	9	8	4, 5	6
	Neo12r	AGAATGGCGACTGGTTTGAC	64.5						
Neo14	Neo14f	CAGCAGAGGAGCAGCAGAT	64.1	TGCTCG	247-256	4	2	2	2
	Neo14r	TCCGACCGATGTACCTTGAC	65.3						
Neo15	Neo15f	CGTCAATGGAGCATCGTG	64.7	CGCCAA	315-327	4	4	3, 4, 5	3
	Neo15r	AGTTAGGCACGGGTGCAG	64.4						
Neo16	Neo16f	CGAGGGCAGCTTGAGGTT	66	TGGT	370-398	10	7	6, 8, 14	7
	Neo16r	CCTCCCTGTTCCACTAGCAT	63.5						

Neo17	Neo17f	CGTTACAGTGGCGCGTAGAT	65.4	CTGGTG	291-309	4	5	4, 5, 6	4
	Neo17r	ACGAAACACCTCATGGATGG	65.2						
Neo18	Neo18f	GTGACGTCGCAAGGTTCC	65	CAG	179-203	9	7	5, 7, 13	7
	Neo18r	ATAATAGGCAGGCCCGAAGA	64.8						
Neo19	Neo19f	TATGACACACGCCTGAAAGG	64.1	GCA	312-321	9	10	7, 8	8
	Neo19r	CGACTGCTGGAGAGGTTAGG	64.3						
Neo20	Neo20f	CCAGTAGTAGCCTGCGAAGC	64.3	GTGGCC	433-445	5	4	5, 6	4
	Neo20r	CTCGACCTCCTCCCAATCC	66.3						
Neo21	Neo21f	CAGCCTCTTCGTCCTCTCC	64.6	GCA	427-451	9	8	7, 8, 12	9
	Neo21r	GTCTGGGATGGATGGTAGCC	65.9						
Neo22	Neo22f	TGATCTATATTCTGGGCGGG	64	TG	433-450	9	10	3, 4	6
	Neo22r	TCAGTCGTTGAGGTGACGAG	64.7						
Neo23	Neo23f	GGAAGCTTCTGGCTGAACAC	64.3	GCT	293-308	7	6	5, 9	7
	Neo23r	CTCCCTTCTCACGACTCAGG	64.4						
Set 2:									
Neo24	Neo24f	CAGTGGGAAATACGGCTTGT	64.2	ATG	224-257			7, 16, 18	
	Neo24r	ATACCACGGAGTGTCAAGGG	64.2						
Neo26	Neo26f	ACAGAGGACGGAAGAAAGCA	64.3	AGA	244-265			5, 6, 8, 12	
	Neo26r	ACATCTGGATCGGAGATTCG	64.4						
Neo27	Neo27f	TGACGTGGAACGAGTTTCTG	64.4	CTT	147-170			10, 11, 12, 18	
	Neo27r	TACGCAACACTGAACTTGGC	64.3						
Neo28	Neo28f	ATCATGTAGGGAATGCAGCC	64.2	CTG	477-492			8, 10, 11, 13	
	Neo28r	AATTGCCCTACCAACAGTCG	64.2						
Neo29	Neo29f	CTCGTCCTTCTTCTCCAACG	64.3	AGC	360-372			7, 9, 11	
	Neo29r	GGGATGAACACCACCTTCAG	64.8						
Neo30	Neo30f	GCGTGTCCCTCTGTCAAACT	64.7	TCT	354-369			6, 8, 11	
	Neo30r	GACGAACAAGACGCAAACAA	64.3						
Neo31	Neo31f	ACCCGTAGTACAGCAGCCAC	64.5	CCG	380-395			4, 6, 8, 9	

	Neo31r	TCACAGCACATCAAGCATCA	64.8			
Neo32	Neo32f	TGAGACTGTTCGCGTGTAGG	64.5	CTG	327-339	8, 9, 12
	Neo32r	CCTCAATCCTGCTCCTTCTG	64.3			
Neo34	Neo34f	CCTGAAGACCGTTGGTCAAT	64.3	TTG	477-495	6, 9, 12
	Neo34r	TTACTCGTTACGCCTCCCAC	64.3			
Neo37	Neo37f	TTCTGAACCACAATGACCCA	64.5	AGC	296-317	6, 10, 13
	Neo37r	CTCGGATTCGATATGTGCCT	64.3			
Neo38	Neo38f	AGTCGAGGTTGGACATCAGG	64.6	CGG	301-313	5, 8, 9
	Neo38r	CCGCTCAGCTATAGGCAATC	64.1			
Neo39	Neo39f	AGCTAGACGCAGACGAGGAC	64.1	GCA	408-423	7, 8, 12
	Neo39r	GGTCGAGAACGTCAGAAACC	64.1			
Neo41	Neo41f	TCGAGATTGACGTTGTGCTC	64.6	TGC	295-310	5, 6, 10
	Neo41r	CCTCGTGGAGTTGGAGACAT	64.6			

* Entries in boldface indicate markers that were selected for polymorphism assessment

Fluorescently labelled primer pairs were used in PCR amplification from the twenty *L. mahajangana* and *N. parvum* isolates using the same conditions as described above. Fragment analysis of the labelled amplicons was performed with GeneScan on an ABI 3500xl Autosequencer (Thermo Fisher Scientific). All samples were analyzed in conjunction with a GeneScan 500 LIZ (Applied Biosystems) internal size standard. Alleles were scored using GeneMapper v. 4.1 (Applied Biosystems). Marker diversities were assessed by calculating the gene diversity (Nei 1973) using GenAlEx v 6.5 (Peakall *et al.* 2006) through the formula $H=1- \Sigma x_k^2$, where x_k is the frequency of the k^{th} allele.

3. Results

3.1. Primer development

SSR containing sequences were successfully extracted from the genomes of three *Lasiodiplodia* spp. and four *Neofusicoccum* spp. (Figure 1). These genomes contained between 2220 and 3113 microsatellite repeats. On average the density of SSRs within a genome was 63.9 repeats/Mb and the average repeat length was 20.58 bp. The composition of SSR repeat motifs were similar among genomes of the same genus, with the exception of *L. theobromae* that had relatively fewer mono- and more trinucleotide repeats than the other two *Lasiodiplodia* species. If the SSR motif compositions differ greatly between taxa, the development of transferable markers could be complicated due to a low amount of shared SSR loci. Trinucleotide repeats were the most frequent motif length in all the above genomes.

For the first set of primers developed for *Lasiodiplodia*, 2740 primer pairs were designed from SSR containing sequences of *L. pseudotheobromae*. *In silico* primer analysis predicted that 219 of these primer pairs could amplify a fragment in *L. gonubiensis, L. pseudotheobromae* and *L. theobromae*. Twenty-nine of these primer pairs were selected for synthesis. The second *Lasiodiplodia* primer set compared 1597 and 1918 trinucleotide SSR containing fragments from *L. pseudotheobromae* and *L. theobromae*, respectively. Analysis

of BLAST results identified 700 fragments that were common between these two species and 30 primer pairs were designed from the fragments with the largest repeat sizes. The third set of *Lasiodiplodia* primers were selected from an initial 2225 primer pairs designed from SSR containing sequences of *L. theobromae.* From the analysis of the predicted amplification fragments, 33 primer pairs were selected for synthesis.

BLAST analysis of SSR containing sequences from the four *Neofusicoccum* genomes indicated that 259 fragments were best reciprocal hits. Twenty-three primer pairs were developed from these fragments. The second *Neofusicoccum* primer set was selected from an initial 1677 primer pairs designed from SSR containing fragments of *N. parvum* UCD646So. Of these primers, 335 were predicted to produce amplicons with size differences in the other *N. parvum* genomes. Twenty primer pairs were designed on predicted amplicons where the length variation was within the SSR sequences.

3.2. Primer validation and cross-species amplification

The 92 synthesized *Lasiodiplodia* primer pairs were validated against twelve *Lasiodiplodia* spp. Of these, 77 primer pairs successfully amplified a fragment of the expected size in at least one of the twelve species (Supplementary Table 2). The 43 synthesized *Neofusicoccum* primer pairs were tested against eight *Neofusicoccum* spp. and 32 of these primer pairs amplified fragments of the expected size (Supplementary Table 3). Sequences of the primer pairs that allowed successful amplification are reported for the *Lasiodiplodia* primer sets (Table 2) and the *Neofusicoccum* primer sets (Table 3).

A large number of developed primer pairs successfully amplified fragments from multiple species (Figure 1). Of the primers tested on the twelve *Lasiodiplodia* spp. between 26 (*L. rubropurpurea*) and 70 (*L. theobromae*) primer pairs successfully amplified per species with more than half of the primer pairs able to amplify in nine or more of the twelve species. Three primer pairs amplified only a single *Lasiodiplodia* spp., i.e. not transferable. Between

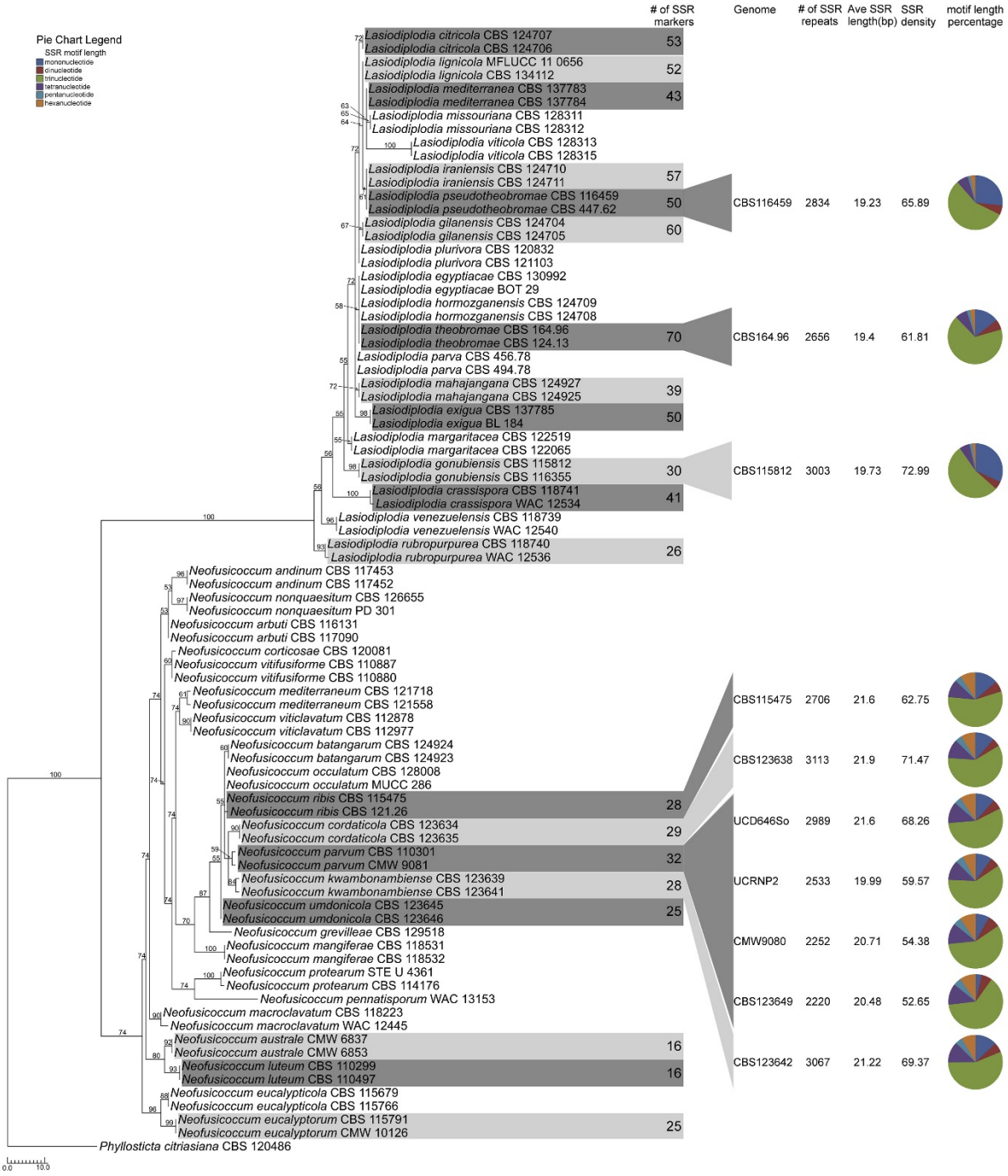

Fig. 1. Maximum parsimony phylogenetic tree of *Lasiodiplodia* spp. and *Neofusicoccum* spp. based on the internal transcribed spacer (ITS) region, including summary information on the number of developed microsatellite (SSR) markers compatible with each species and repeat information for species with genome sequences. Bootstrap support values are indicated as branch labels. Species used to test the transferability of markers are indicated using grey blocks with the number of transferable primers indicated. Species with genome sequences used in this study are indicated with grey wedges and information regarding the total number of perfect repeats, the average repeat length, the average repeat density in the genome as well as a pie chart indicating the SSR motif length composition is given.

sixteen (*N. australe* and *N. luteum*) and 32 (*N. parvum*) primer pairs amplified in the eight *Neofusicoccum* species and of these more than half of the primer pairs amplified seven or more of the eight species. A single marker (Neo31) was not transferable to other species of *Neofusicoccum*.

3.3. Marker polymorphism

The sizes for twelve *Lasiodiplodia* and eleven *Neofusicoccum* SSR markers were determined for 20 isolates of *L. mahajangana* and *N. parvum,* respectively. All the markers were polymorphic with an average number of alleles of 4.5 for the *Lasiodiplodia* markers (Table 4) and 3.2 for the *Neofusicoccum* (Table 5) markers. Gene diversity (H) is a measure of the amount of allelic variation within a population; i.e. the probability that two alleles of a specific locus, chosen at random from the population will be different (Nei 1973). The *Lasiodiplodia* markers had higher average gene diversities than the *Neofusicoccum* markers. In total the *Lasiodiplodia* markers identified 54 alleles that distinguished sixteen multilocus genotypes. The *Neofusicoccum* markers identified 35 alleles and fifteen multilocus genotypes.

4. Discussion

This study is the first to use genomic sequences to produce transferable SSR markers for species of *Lasiodiplodia* and *Neofusicoccum*. From genome derived SSR containing sequences, 77 *Lasiodiplodia* and 32 *Neofusicoccum* SSR markers were developed. These markers were transferable to numerous other species in each of the two genera. A selection of these markers were also shown to be polymorphic and thus of use as population genetic markers in *L. mahajangana* and *N. parvum*.

The markers developed in this study significantly increases the number of available population genetic markers in the genera *Lasiodiplodia* and *Neofusicoccum.* These genera contain many important plant pathogenic species (Slippers & Wingfield 2007). However, the

Table 4. *Lasiodiplodia* microsatellite marker polymorphism assessment using 20 isolates of *L. mahajangana*

Marker	Fluorescent label	Number of alleles	H*
Las2	FAM	7	0.81
LT16	FAM	2	0.42
LT28	VIC	3	0.56
LT31	PET	9	0.80
TPs1	VIC	4	0.69
TPs5	NED	3	0.46
TPs9	FAM	4	0.65
TPs11	NED	7	0.83
TPs22	VIC	5	0.75
TPs26	NED	2	0.18
TPs27	PET	2	0.46
TPs28	NED	6	0.77
Number of isolates			20
Number of alleles			54
Number of multilocus genotypes			16

*Nei's (1973) gene diversity

Table 5. *Neofusicoccum* microsatellite marker polymorphism assessment using 20 isolates of *N. parvum*

Marker	Fluorescent label	Number of alleles	H*
Neo5	NED	5	0.49
Neo7	VIC	3	0.41
Neo8	FAM	4	0.48
Neo10	PET	3	0.41
Neo11	VIC	4	0.27
Neo15	NED	3	0.52
Neo16	FAM	2	0.48
Neo17	VIC	3	0.57
Neo18	FAM	3	0.34
Neo19	PET	3	0.51
Neo21	VIC	2	0.26
Number of isolates			20
Number of alleles			35
Number of multilocus genotypes			15

*Nei's (1973) gene diversity

only SSR markers available prior to this study were developed for *L. theobromae* (Burgess *et al.* 2003; Cardoso & Wilkinson 2008) and for *N. parvum* (Slippers *et al.* 2004). Some of these markers were demonstrated to be transferable to other species in each genus (Slippers *et al.* 2004; Sakalidis 2011; Begoude Boyogueno *et al*. 2012; Cruywagen 2016). The small number of available SSR markers in the *Lasiodiplodia* and *Neofusicoccum* make population studies in these two genera very difficult. The markers designed in this study will significantly reduce the amount of work necessary to perform population analyses on a range of species in these genera, by negating the need for marker development.

All of the markers that were investigated in the fragment length analyses were polymorphic (*Lasiodiplodia* markers, 2-9 alleles; *Neofusicoccum* markers, 2-5 alleles) and most markers had moderate to high levels of gene diversity (*Lasiodiplodia* markers, $0.18<H<0.83$; *Neofusicoccum* markers, $0.26<H<0.57$). This is similar to SSR markers previously developed for some species of *Botryosphaeriaceae*, e.g. *N. parvum* (2-15 alleles, $0.05<H<0.84$), *M. phaseolina* (2-13 alleles, $0.41<H<0.85$) and *B. dothidea* (13-57 alleles, $0.77<H<0.97$) (Slippers *et al.* 2004; Baird *et al.* 2010; Manawasinghe *et al.* 2018). In contrast, another study that developed SSR markers for *Diplodia sapinea* displayed lower number of alleles (2-4) and levels of gene diversity ($0.05<H<0.4$) (Bihon *et al.* 2011). Gene diversity estimates should be carefully interpreted when they are used to assess SSR markers because they are dependent on the population being investigated. For example, markers will exhibit low gene diversity if the population is clonal or inbred, however the same markers would have larger gene diversities where they are applied in a more genetically diverse population. Care should also be taken with cross-species transferable markers, as high marker polymorphism in one species does not necessarily predict the same level of polymorphism in other species.

A large number of SSR markers were developed for *Lasiodiplodia* and *Neofusicoccum* in this study. These were substantially more than what have previously been developed for the *Botryosphaeriaceae*. Most of the previous studies used inter-SSR (ISSR) PCR and gave rise

to between five and 21 markers (Burgess *et al.* 2001; Burgess *et al.* 2003; Slippers *et al.* 2004; Bihon *et al.* 2011). In a similar study, twelve markers were developed for *Macrophomina phaseolina* through screening SSR enriched genomic libraries (Baird *et al.* 2009). Manawasinghe *et al.* (2018) used an approach similar to that in the present study to develop 29 SSR markers for *Botryosphaeria dothidea.* Clearly, as genome sequences for more genera of *Botryosphaeriaceae* become available, SSR markers for species of fungi in this family will become increasingly available. The markers produced in this paper might, however, make it unnecessary to develop more markers for species in *Lasiodiplodia* and *Neofusicoccum*. For both the common species and the less common species (which genomes might not be available for some time) this would be a big advantage.

Most markers developed in this study were transferable within the genus for which they were developed and they represent the largest collection of transferable SSR markers thus far developed for the *Botryosphaeriaceae* and one the largest of such developed on fungi. Only two studies that developed SSR markers for the *Botryosphaeriaceae* have determined the transferability of their markers. Slippers *et al.* (2004) developed eight SSR markers for *N. parvum* and seven of these were transferable to at least eight other species within the family and one marker was transferable to two other species. Of the twelve SSR markers developed by Baird *et al.* (2009), two markers were transferable to five other species and one marker was transferable to four other species of *Botryosphaeriaceae* (Baird *et al.* 2010). Cross-species transferable SSR markers have also been developed in other fungi, however these studies have also reported a low number of transferable markers (between four and eleven) (Wadud *et al.* 2006; Cristancho & Escobar 2008; Benichou *et al.* 2009; Leyva-Madrigal *et al.* 2014). The largest collection of cross-species transferable SSR markers in a fungal genus, other than the present study is that of Bhat *et al.* (2018), which developed thousands of SSR markers that were predicted to be capable of amplification across eight *Colletotrichum* species. The markers developed in the present study were not tested on species outside the genus for which they were developed. Given the fact that they were

designed to amplify across species, targeting conserved primer binding sites, it is possible that they could be useful in other genera too.

Genome mining for SSRs results in significantly higher numbers of developed primers than conventional methods. The availability of thousands of SSR containing sequences retrieved from the *Lasiodiplodia* and *Neofusicoccum* genomes allowed us to develop primers for thousands of these regions. From these primers we could then select primers for specific needs; i.e. cross-species transferability. Other studies have also used SSR-containing sequences, extracted from fungal genome sequences, to develop SSR markers (Jia *et al.* 2015; Mercière *et al.* 2015; Fortuna *et al.* 2016; Wang & Chilvers 2016; Yu *et al.* 2016; Vaghefi *et al.* 2017; Bhat *et al.* 2018; Mlonyeni *et al.* 2018). These studies generally develop much larger numbers of SSR markers than studies using other approaches.

The markers developed in this study have limitations that should be taken note of. The transferability of markers were tested on single isolates of species. Researchers adopting these markers will thus still need to confirm the amplification and polymorphism of these markers in the target population. Even though many studies have demonstrated SSR marker transferability among various fungal taxa (Wadud *et al.* 2006; Cristancho & Escobar 2008; Benichou *et al.* 2009; Leyva-Madrigal *et al.* 2014; Bhat *et al.* 2018), only a few have demonstrated the polymorphism of these markers in the non-target species (Cristancho & Escobar 2008; Bhat *et al.* 2018). Despite the additional steps needed to select appropriate markers from collections presented here, the transferable markers still represent a useful starting point for marker development that is expected to significantly reduce time and cost.

Simple sequence repeat markers continue to be a popular and feasible option for performing population studies, despite the increased prevalence of genome-wide approaches (Davey *et al*. 2011). SSR markers are popular because they are highly polymorphic, cost-effective and do not require extensive bioinformatics knowledge and infrastructure to analyze (Guichoux *et*

al. 2011; Hodel *et al*. 2016). Genome-wide sequencing approaches are still too costly for most species of *Lasiodiplodia* and *Neofusicoccum* to apply widely. Mining genome sequences for SSRs represents a significant reduction in marker development cost compared to sequencing of SSR enriched libraries. Making use of pre-existing markers such as those presented in this study saves the time and funds associated with marker development, leaving the cost of fluorescent marker labelling as the largest expense (Hodel *et al*. 2016).

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