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PERMANENT GENETIC RESOURCES

Microsatellite markers for the *Eucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*

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Abstract

Ten microsatellite markers were developed for the fungus *Kirramyces gauchensis*, which causes an important stem canker disease of *Eucalyptus* trees in plantations. Primers for 21 microsatellite regions were designed from cloned fragments. Fourteen of the primer pairs provided single amplicons and 10 of these were polymorphic for *K. gauchensis*. Allelic diversity ranged from 0.21 to 0.76 with a total of 30 alleles. None of the markers was able to amplify in the phylogenetically distinct but morphologically similar species *Kirramyces zuluensis*. The 10 characterized polymorphic microsatellite regions will be studied to determine the population structure of *K. gauchensis* in plantations of different countries.

Keywords: ascomycete, *Eucalyptus* stem canker, *Kirramyces gauchensis*, *Kirramyces zuluensis*, M-FIASCO, microsatellites

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Species of *Kirramyces* include important pathogens of *Eucalyptus* leaves, shoots and stems (Andjic *et al*. 2007). *Kirramyces* (= *Colletogloeopsis*) *gauchensis* is the causal agent of a serious stem canker disease on *Eucalyptus* trees (Cortinas *et al*. 2006c). This fungus is very similar to, but phylogenetically distinct from, *Kirramyces* (= *Colletogloeopsis*) *zuluensis*, which is also an important *Eucalyptus* stem canker pathogen. *K. gauchensis* has a wide geographical distribution and has been recorded on *E. grandis*, *E. tereticornis*, *E. camaldulensis* and different *Eucalyptus* hybrids in plantations of South American and African countries as well as in Hawaii. The fungus has never been found in the native range of *Eucalyptus* or infecting trees of other genera. At present, the origin of this fungus is unknown. *K. gauchensis* is a haploid ascomycete. The genus name refers to the asexual reproductive structures (pycnidia) found in nature. As is the case in *K. zuluensis*, sexual reproductive structures have never been reported for the fungus. However, other closely related species of *Kirramyces* have *Mycosphaerella* sexual states, and phylogenetic inference suggests that the same will be true for *K. gauchensis* and *K. zuluensis*.

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Microsatellite markers have been useful in understanding the population biology of many fungal pathogens (e.g. McDonald 1997; Zhan & McDonald 2004; Feau *et al*. 2005). Initial studies on *K. gauchensis* were frustrated by the fact that microsatellite primers developed for *K. zuluensis* did not amplify any amplicons (Cortinas *et al*. 2006a, c). However, this fact and multilocus phylogenetic analyses led to the discovery that isolates initially treated as a single species actually represented distinct taxa (Cortinas *et al*. 2006c). The objective of this study was therefore to isolate and characterize microsatellite loci that can be used to study the population structure of *K. gauchensis* collected from diseased trees in different countries.

The microsatellite-containing regions were isolated using a modified form (Cortinas *et al*. 2006a) of the (fast isolation by AFLP of sequences containing repeats) FIASCO technique of Zane *et al*. (2002). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. To screen for repetitive sequences, 1 μg of genomic DNA was pooled from the isolates CMW 7474, CMW 7300, CMW 7279 of *K. gauchensis*. Genomic DNA was extracted from cultures using phenol-chloroform following the method described by Cortinas *et al*. (2006b, c). The biotinylated probes $(TC)_{15}$ $(CA)_{15}$ and $(GATA)_6$ were used to enrich the genomic DNA. All

Table 1 Locus and primer names, GenBank Accession numbers, primer sequences, repeat motif, annealing temperature (T_a), $MgCl_2$ concentration, number and size range of alleles and observed (H) allelic diversity (Nei 1973) of the ten polymorphic loci analysed in this study using 42 isolates of *Kirramyces gauchensis*

Locus name	Primer names	GenBank Accession no.	Primer sequences (5′–3′)	Repeat motif	T_a	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean H	No. of isolates tested
K. gauchensis 1	Kgauche 1F Kgauche 1R	DQ975190	NED-CTCCATTGCATCGGGTCTCATG GGTGGCAAGTTCGAGCTTCA	$(AG)_{24}$	59 °C	3.5	6	290–327	0.76	42
K. gauchensis 2	Kgauche 2F Kgauche 2R	DQ975191	PET-CAAATCCTCGGCTGCGTCATGG CACTGCGCTTTCGTCTCTACCGA	$(GA)_4TA(GA)_4$	54 °C	3.5	3	148–183	0.50	42
K. gauchensis 3	Kgauche 3F Kgauche 3F	DQ975192	NED-AGATGGCTGTACGAAGAATGTCC AAGCCAATCCACGCGTCAAGG	$(CT)_7AC(TC)_{17}$ $TTTCT(GT)_{12}$	60 °C	3.5	3	211–266	0.42	42
K. gauchensis 4	Kgauche 4F Kgauche 4R	DQ975193	VIC-CCGCGAGAGAAACAACATCC GATAGGAGGCACATAACCCAAG	$(GA)_{10}$	59 °C	3.5	2	251–260	0.24	42
K. gauchensis 5	Kgauche 5F Kgauche 5R	DQ975194	FAM-TTGGCCAGCAGGAACATGAGC CACTCATTCACTTGACCGCCTC	$(GTGGT)GGT$ $(GTGGT_3(GGT)_2$ $(GTGGT)_2$	62 °C	2.0	2	288–294	0.43	42
K. gauchensis 6	Kgauche 6F Kgauche 6F	DQ975195	FAM-CGCCTTATGCCTTTGATGGTTGC GATTCCTAAATCGACCATCCGC	$(GT)_{15}$	56 °C	3.5	4	165–203	0.43	42
K. gauchensis 7	Kgauche 7F Kgauche 7R	DQ975196	VIC-ACCAGGGATGCCGTATGTGCAG CATCACACACCGTCCTCCCAC	$(TG)_9$	60 °C	3.5	2	107–109	0.46	42
K. gauchensis 8	Kgauche 8F Kgauche 8R	DQ975197	PET-ATCATCTGCCCTTGGACGGACG CCATCACCACACGAAACATCAAG	$(TG)_9$	59 °C	2.0	3	134–150	0.21	42
K. gauchensis 9	Kgauche 9F Kgauche 9R	DQ975198	FAM-GATCACGCAATGAGAGTGTCTCC GGTTTCCGACTGATTGGTTCATC	$(ACAG)_5$	54 °C	3.5	2	89–98	0.52	42
K. gauchensis 10	Kgauche 10F Kgauche 10R	DQ975199	PET-ATAGTAAGAAGATAAATAAGGCG GCGAAGTAGACTATATAAGTATC	$(AAG)_{53}$	52 °C	3.5	3	134–143	0.40	42

polymerase chain reactions (PCRs) were carried out using an iCycler (Bio-Rad) using the thermal profiles described in Cortinas *et al*. (2006a).

Of 384 sequenced clones, 21 contained repetitive regions. Primers for these 21 loci were designed visually. OLIGO Analyser 3 (Integrated DNA Technologies) was used to check the melting temperature (Tm), formation of hairpins, self-dimers and hetero-dimers. When the designed primer pairs were tested, 14 primer pairs resulted in single amplicons of the expected size range. One primer from each of the 14 primer pairs was labelled with fluorescent dyes using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems), to allow detection on an ABI 3100 sequencer. PCR amplifications were performed in 25 μL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 μM of each primer, 0.2 μL (1 U) *Taq* Polymerase (Roche), 1× buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$ or 3.5 mM $MgCl_2$, pH (8.3) (Roche) (Table 1) and 18.0 μL of distilled water. The thermocycling conditions were as follows: initial denaturation at 96 °C for 4 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72 °C for 1 min, followed by 30 cycle repetitions of denaturation at 94 °C for 30 s, annealing temperature according to Table 1 for 30 s and extension at 72 °C for 1 min (with 5 s increments every two repetitions). A final extension was carried out at 72 °C for 45 min. Fragment size analysis was carried out after electrophoresis using the software GENEMAPPER, version 3.0 (Applied Biosystems) and LIZ 500 (–250) size standard (Applied Biosystems).

To assess the level of polymorphism, 21 isolates from Argentina (CMW4915, CMW14336, CMW14337, CMW14338, CMW14339, CMW14343, CMW14345, CMW14347, CMW14348, CMW14349, CMW14351, CMW7345, CMW14510, CMW14510, CMW14511, CMW14512, CMW14515, CMW14516, CMW7342, CMW1458, CMW15835), and an equal number of isolates from Uruguay (CMW17561, CMW1495, CMW1501, CMW1502, CMW7270, CMW7272, CMW7275, CMW7276, CMW7277, CMW7278, CMW7281, CMW7282, CMW7287, CMW7290, CMW7292, CMW7293, CMW7298, CMW7299, CMW7305, CMW7306, CMW7309) were genotyped. Of the 14 designed primers pairs, 10 loci were polymorphic, two were monomorphic and two yielded complex stutter patterns that were difficult to interpret.

The allelic diversity (Nei 1973) of the 10 polymorphic loci ranged from 0.21 to 0.76 with a minimum of two, and a maximum of six alleles per locus (Table 1). Thirty alleles were found across the 10 loci. The expected heterozygosity at the loci studied ranged from 0.21 to 0.76. Linkage disequilibrium (LD) was calculated using MULTILOCUS 1.2 (Agapow & Burt 2001). Significant LD ($P < 0.05$) was detected for some loci pair comparisons (data not shown), suggesting little evidence for recombination. This indicates that clonal reproduction could be playing an important role

in the reproductive structure of *K. gauchensis*. Confirmation of this result will, however, need to be tested in more comprehensive population genetic studies. Cross-species amplification between *K. gauchensis* and the closely related *K. zuluensis* (25 isolates) produced negative, incorrect size bands or smeared amplifications, suggesting that the two species no longer share these loci. These primers also failed to amplify amplicons when tested as diagnostic markers on two other related species, *M. nubilosa* and *M. molleriana.* The primers are thus not only useful as population markers but also have the potential to be used as species-specific markers to identify *K. gauchensis* in the development of a DNA-based identification technique.

In this study, 10 microsatellite loci have been characterized and shown to be specific for *K. gauchensis.* These markers can now be applied to populations of the pathogen from different parts of the world, as part of an effort to understand its global diversity and population biology. Such studies will hopefully also enhance efforts to reduce the impact of the pathogen on *Eucalyptus* forestry.

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