PUBLISHED VERSION

Cardoso, Jose Emilson; Wilkinson, Michael James
Development and characterisation of microsatellite markers for the fungus Lasiodiploidia theobromae, *Summa Phytopathologica*, 2008; 34(1):55-57.

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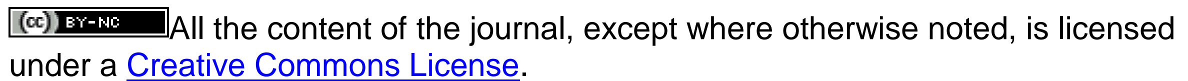
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14th August 2013

http://hdl.handle.net/2440/69171

NOTAS CIENTÍFICAS

Development and characterisation of microsatellite markers for the fungus *Lasiodiplodia theobromae*

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Data de chegada:09/11/2006. Aceito para publicação em: 08/10/2007

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RESUMO

Cardoso, J.E.; Wilkinson, M.J. Desenvolvimento e caracterização de marcadores microsatélites para o fungo *Lasiodiplodia theobromae*. ***Summa Phytopathologica,*** v.34, n.1, p.55-57, 2008

Lasiodiplodia theobromae é um importante patógeno de vegetais superiores nas regiões tropicais e sub-tropicais, sendo capaz de infectar diferentes hospedeiros sob as mais variadas condições ambientais, o que sugere uma grande variabilidade genética. O objetivo deste estudo foi o de desenvolver marcadores moleculares microsatélites polimórficos de um isolado brasileiro que possa ser usado na caracterização da população de *L. theobromae* e fungos correlatos. Nove microsatélites foram identificados no genoma deste isolado, o que permitiu o desenvolvimento de seis marcadores polimórficos em uma população de nove isolados patogênicos de plantas tropicais do Brasil. Testes preliminares revelaram uma acentuada variabilidade genética entre os isolados testados, confirmando a grande variabilidade da população brasileira deste fungo. Estes marcadores poderão ser usados em estudos evolutivos em epidemiológicos desta espécie.

Palavras chave: seqüência simples repetida (SSR), marcador molecular.

ABSTRACT

Cardoso, J.E.; Wilkinson, M.J. Development and characterisation of microsatellite markers for the fungus *Lasiodiplodia theobromae*. ***Summa Phytopathologica,*** v.34, n.1, p.55-57, 2008

Lasiodiplodia theobromae is an important fungal pathogen of higher plants from tropical and sub-tropical regions. The fungus infects divergent hosts in a wide range of environmental conditions, suggesting that it is highly variable. The aim of this study was to develop new polymorphic microsatellite markers from a Brazilian isolate of *L. theobromae* that can be used in population studies of this and related fungi. The nine microsatellite markers developed included six that revealed allelic polymorphisms among nine isolates of the disease collected from infected plants in Brazil. Preliminary evaluation of the markers suggested substantial genetic variability among Brazilian *L. theobromae* populations. These markers have potential utility for evolutionary and epidemiologic studies of this fungus.

Additional keywords: simple sequence repeat, molecular marker.

Lasiodiplodia theobromae (Sin. *Botryodiplodia theobromae,* teleomorphic form *Botryosphaeria Rhosdina (Berk. & M.A. Curtis)Arx AMX* is one of the most important fungal plant pathogens in northern Brazil. This fungus is both ubiquitous and plurivorous and is able to infect over 500 plant species; causing symptoms ranging from seed rot to the discolouration of timber (7, 8). Infection is generally limited to wounded or stressed-weakened plants (1, 4, 5, 6). The species reproduces mainly by asexual mitospores, with the sexual stage rarely being observed under field conditions (7).

The current taxonomic treatment of *L. theobromae* is based entirely on culture characteristics and asexual reproductive structures (*e. g.* pycnidium, conidiophore, and conidiospores) (7). The extensive list of synonyms that apply to the species illustrates the confusion that exists over its taxonomic and phylogenetic status. Therefore, it is important to determine whether different genetic variants have evolved to specialise towards the infection of particular hosts or if the entire aggregate is characterised by genotypes that all possess the capacity for a broad host range. In order to distinguish between these alternative scenarios, it is first important to develop the capability to discriminate between closely related pathotypes.

Simple Sequence Repeat (SSR) or microsatellite analysis is widely acknowledged as the method of choice for molecular studies of population genetic structure, kinship, genotype diagnosis and genetic evolution. Whilst some SSR markers have recently been developed for species closely related to *L. theobromae* from South Africa (2, 3, 9), the aim of this study was to develop polymorphic microsatellite markers from Brazilian isolates that can be used in population studies of *L. theobromae* and related fungi.

A mycelial mat (200mg) was cultured from an isolate (Ao1) from a gummosis-infected cashew plant from northeastern Brazil (Piauí state) and grown in liquid still culture of potato dextrose broth (PDB) for 15 days. The culture was then macerated and its DNA extracted using the DNeasy kit (Qiagen[R]) according to the manufacturer's protocol. A genomic library was constructed by first double-digesting

Table 1. Primers designed for the amplification of microsatellites of *Lasiodiplodia theobromae*.

Locus	SSR primer pair		Sequence 5' - 3'	Annealing temperature °C (*)	Fragment size(bp)	Core sequence	Number of alleles (**)
LAS 01	LT1	F: R:	GAGGGTTTTGTGCTCCATGT GGAAAACGGTGGTCAAAGAA	52-62	202	$(CA)_6$	3
LAS 02	LT2	F: R:	ATTGGAAAGGAGGGAAAGGA GCGCGCTTCTTCCAGAAA	55-60	102	$(CA)_8$	3
LAS03	LT3	F: R:	GTAGATGTGGTCGCGGAGTT TCCCCCATGTATACCAGGTC	55-62	234	$(GGT)_8$	4
LAS 04	LT4	F: R:	GTTGCTCCTCATCTGCACCT GTAGATGTGGTCGCGGAGTT	54-62	255	$(CCA)_7$	3
LAS 05	LT5	F: R:	GGCTACCCGTAACATGTGGA AAGAAGGCACGACGGATGA	52-62	204	$(CA)_{14}$	1
LAS 06	LT6	F: R:	TCCATCGTCTCTCTCAGCAG AAGAAGGCACGACCGATG	53	346	$(CA)_{15}$	1
LAS 08	LT8	F: R:	CTCGTTAGGAAGGAAAGCAT GAACTATCCCCGCATCTACT	54	188	$(GGT)_7$	2
LAS 09	LT9	F: R:	GGGAAAATAAAATGGTCTGG GAAACCCTTGTTCCATGC	55-57	143	$(GA)_9$	2
LAS 10	LT10	F: R:	CAAGGATACGATGTGGACTG AAGATGGAAAGGAGAACGAG	52-61	156	$(GTC)_8$	2

(*)Annealing temperature intervals. (**) Number of alleles among nine isolates of the fungus.

fungal genomic DNA using restriction enzymes *Sau*3AI and *Hind* III to generate fragments typically 600-800bp and then ligation of the products directly into the pGEM vector using the manufacturer's instructions (Promega, Madison, Wis, USA) before transforming the plasmids into *Escherichia coli* (Stratagene), by heat shock. Clones with inserts containing SSRs were selected by colony blotting with a variety of P^{32} radioactively labelled oligonucleotide probes (*e g* CA_{15}, AGG_{15}, GA_{15}), and exposing to X-ray film overnight at -80°C.

Inserts of 'positive' colonies were amplified by PCR using the plasmid-specific M13 universal forward and reverse primers. The reaction mix for the PCR comprised of 1.0ìl 10x reaction Buffer, 0.3ìl $MgCl_2$, 0.2ìl deoxynucleotide triphosphate (dNTP), 0.1ìl Taq DNA polymerase, 0.3ìl each primer pairs as described, 1 ìl bacterial suspension, and 6.80ìl deionised water. PCRs were performed on a Gene Amp[â] PCR System 2700 (Applied Biosystems) thermal cycler using an initial 94°C denaturing step for 1 min followed by 35 cycles of 1 minute to the 94°C, 1 minute at 55°C (annealing) and 4 minutes at 72°C(extension), and finally 10 minutes at 72°C. PCR amplicons from specific clones were size-estimated after electrophoresis through a 1.5% (w/v) agarose (midi) gel at 120V for around 2h 30minutes.

Strong amplicons in the expected size range were purified using QIAprep(-Qiagen) protocol and subjected to cycle sequencing after PCR using the M13 primers and BigDye terminator (Perkin-Elmer Applied Biosystems) cycle sequencing kit (4.0ìl BigDye 3.1, 1.6ìl 10ìM M13, SSR primers, and 4.4ìl of template DNA). The reaction was programmed for 30s denaturation at 95°C, followed by 30 cycles of 30 s (annealing) at 50°C, and 4 min at 60°C (extension). Sequencing was performed on a ABI PrismTM (Perkin-Elmer Applied Biosystems) and base sequence was analysed using ChromasPro 2.3 software (http://www.technelysium.com.au/chromas.html). A total of 41 clones were sequenced, 11 sequences containing microsatellites were identified and flanking primers were designed to amplify these regions by using the Primer3 software (Whitehead Institute Biomedical Research, available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.).

Ten SSRs were found to contain 6 to 15 uninterrupted repeats and so specific primer pairs were designed to target the flanking regions of these loci by reference to the insert sequence.

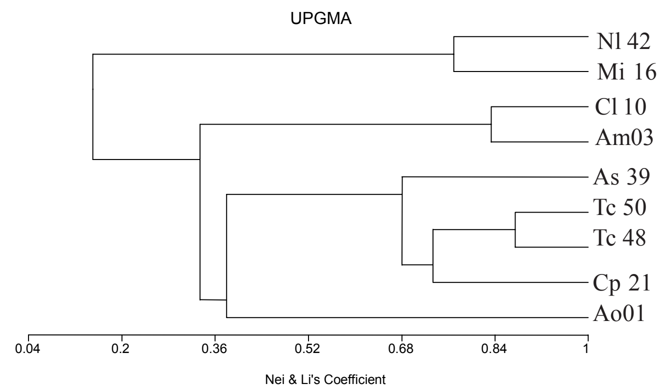

Figure 1. Unweighted pair-grouped tree generated from microsatellite polymorphic data among isolates of *Lasiodiplodia theobromae* collected from infected plants, namely Ao = *Anacardium occidentale* (cashew), Am = *Annona muricata* (soursop), As = *Annona squamosa* (sweetsop), C*l* = *Citrus limon* (lemon), Cp = *Copernicia prunifera* (wax palm), Mi = *Mangifera indica* (mango), N*l* = *Nephelium lappacearum* (rambotam), Tc = *Theobromae cacao* (cocoa).

A gradient thermocycler (Whatman-Biometra) was then used to determine the optimal annealing temperature for each SSR primer pair; genomic DNA of isolate Ao1 acted as template for all PCRs. Nine of the ten primer pairs generated amplicons, with annealing temperatures ranging from 52 to 62º C (Table 1). Primers targeting locus 7 failed to generate any PCR products.

These SSR markers were then evaluated for their ability to reveal allelic polymorphisms between genotypes of the fungus using the following nine Brazilian isolates of *L. theobromae* collected from divergent plant hosts: Ao (*Anacardium occidentale,* cashew), Am (*Annona muricata.* soursop), Cp (*Copernicius prunifera,* wax palm), Mi (*Mangifera indica,* mango), C*l* (*Citrus limon,* lemon), As (*Annona squamosa,* sweetsop), N*l* (*Nephelium lappacearum,* rambotam), and two isolates of Tc (*Theobromae cacao,* cocoa). For this, one of the primer pair was fluorescently labelled with HEX or FAM (CMWG), submitted to PCR and fractionated by capillary electrophoresis on a ABI Prism™, automated sequencer. The size of all amplicons detected was determined using the Genotyper® package software.

In spite of the limited number of samples included in this test, the PCR products yielded by seven primer pairs included polymorphisms in allele length among genotypes (Table 1). Data from Genotyper were then compiled into Excel files (Microsoft),with a data matrix of characters being compiled for each isolate by scoring the presence or absence of each allele at each locus. These data were then used to generate an Unweighted pair-group method with arithmetic mean (UPGMA) genetic distance tree based on Nei and Li's unbiased genetic distance and using the statistical package MVSP (Multi-Variate Statistical Package v.3.1) (Kovach Computing Services, Anglesey, Wales).

The resultant dendrogram revealed variability among all nine isolates. Interestingly the two isolates collected from *Theobroma cacao* showed markedly lower levels of genetic divergence than that between isolates from distantly related hosts (Fig. 1).

Thus, the primers developed in this study can be used to determine the genetic diversity of *L. theobromae* isolates and will also have value to provide diagnostic tests to distinguish ecotypes of this fungus.

ACNOWLEDGEMENTS

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, MCT-Brazil) and the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) - Brazil for financial assistance . We also thank F.C.O. Freire, F.M.P. Viana, E.D.N.L. Martins and A.A. dos Santos for providing fungus isolates and Adam Croixford, Sara Hughes, Moy Robson, J.J.V.Cavalcanti, and Joel Allainguillame for technical assistance.

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