Agrotrópica 17: 61 - 64. 2005.
Centro de Pesquisas do Cacau, Ilhéus, Bahia, Brasil

DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE GENETIC ANALYSIS OF *Crinipellis perniciosa*

Karina Peres Gramacho[1], A. M. Risterucci[2], C. Lanaud[2], Cássia Bahia[1], Uilson V. Lopes[1], Abelmon da Silva Gesteira[3], Marcos Renato Araújo[4]

[1]CEPLAC/CEPEC, Caixa Postal 07, 45600-970, Itabuna, Bahia, Brasil; [2]Cocoa Research Center, CIRAD - France; [3]UESC; [4]UNICAMP/Laboratório de Genômica e Expressão

Witches' broom disease of cacao (*Theobroma cacao* L.), caused by *Crinipellis perniciosa* (Stahel) Singer, is the most important disease of cacao in the cacao growing areas of South America and Caribbean Islands. Very little is known about the genetic biology of the pathogen population, and this information is very important to the host improvement programs and deployment of resistant planting material. A collaborative America Countries international program was initiated to identify and describe the genetic diversity of *Crinipellis perniciosa* in South America. Microsatellites (SSR) constitute highly informative genetic markers for population genetic studies due to their co-dominant and multiallelic nature and distribution in the genome. SSR primers were searched in the *C. perniciosa* genome data base and designed as potential candidates to define an efficient, standardized, molecular fingerprinting protocol for this pathogen. These primers have been evaluated for reliability, widespread distribution across the *C. perniciosa* genome and their ability to discriminate isolates of this fungus . The final objective here is to study the diversity structure of the *C. perniciosa* population from the main cacao growing area in South America countries. Preliminary results are reported.

Key words: Witches' broom, *Theobroma cacao,* genetic diversity.

Desenvolvimento de marcadores microsatélite para análise genética de *Crinipellis perniciosa*. A doença vassoura-de-bruxa do cacaueiro (*Theobroma cacao* L.), causada pelo fungo *Crinipellis perniciosa* (Stahel) Singer, é a principal enfermidade desta cultura na América do Sul e Ilhas do Caribe. Pouco se sabe sobre a biologia e o comportamento genético deste fitopatógeno, informação importante para o desenvolvimento de variedades resistentes à vassoura-de-bruxa. Um consórcio internacional foi iniciado para descrever a diversidade genética do *Crinipellis perniciosa* na América do Sul. Microsatélites (SSR) constituem marcadores genéticos altamente informativos para estudos genéticos de população, devido a sua natureza co-dominante e distribuição no genoma. SSR foram procurados na base de dados do C. *perniciosa* e selecionados como candidatos potenciais para o estabelecimento de um protocolo de *fingerprinting* para este fitopatógeno. Estas regiões de SSR foram avaliadas quanto a sua distribuição no genoma do *C. perniciosa* e poder descriminativo entre os isolados da espécie. O objetivo final deste trabalho foi estudar a estrutura genética em populações de *C. perniciosa* provenientes de regiões produtoras de cacau de países da América do Sul. Os resultados preliminares deste trabalho são descritos.

Palavras-chave: Vassoura-de-bruxa, *Theobroma cacao*, diversidade genética.

Recebido para publicação em 01 de outubro de 2003. Aceito em 14 de dezembro de 2004.

Introduction

In 1989, witches'broom disease (*Crinipellis perniciosa)* was introduced in Bahia (Pereira et al., 1989), causing a tremendous drop in yield. The severity of the disease was higher than in other regions due to a combination of several factors favorable to the disease, such as a continuous large area (600 thousand hectares) cultivated as a monoculture, with highly susceptible varieties and with a climate favorable to the development of the epidemics.

Crinipellis perniciosa is a very unique basidiomycete, as it belongs to a restricted group of basidiomycetes, that is hemibiotrofic and has a homomictic reproductive strategy (Griffith and Hedger, 1994), features found in only 1% of all basidiomycetes. While much research has been conducted on population structure of fungal pathogens (Mc Donald 1997), knowledge on the population structure of *C. perniciosa* is sparse and most work has been focused primarily on understanding the origin of the fungus in Bahia (Anderbrhan et al. 1999; Gomes *et al.* 2000; Arruda et al. 2003).

Simple sequence repeat (SSR) markers are potentially valuable tools for studying genetic variation within genotypes. Microsatellites are highly polymorphic due to their variable number of repeat and highly reproducible and easy to score. The primers also provide specificity for a particular species; therefore, the markers can be applied to samples therefore avoiding the potential problems associated with nonspecific markers on contaminated samples. They are ideal molecular markers for studying genetic variation within populations (Jarne and Lagoda 1996).

At present, library development and screening is the common way to isolate relatively large quantities of SSRs, but the investments in time and money can make this process expensive. Database screening provides the most readily available source of SSRs to anyone with access to a sequencing data base. As part of our research in population dynamics *Crinipellis perniciosa*, we initiated this study to develop locus-specific microsatellite markers, and applied these to a collection of 600 isolates of *C. perniciosa.* The genome database was searched for the presence of SSR. The objective of this search was to assess the frequency of different motifs and to check the general applicability of these primers in amplifying DNA from other biotypes, and other closely related species (*Moniliophthora roreri).*

Materials and Methods

Fungal Isolates and DNA extraction

One-hundred isolates of *C. perniciosa* were used in this study. The isolates are listed in Table 1.

Table 1. Isolates of *Crinipellis perniciosa* and *Moniliophtora roreri* screened for SSR amplification

C- biotype	Bahia (91)* Amazonas (2) Peru (1) Ecuador (1)
S- biotype	*Solanum lycocarpum (1)* *Solanum rugoso* (1) *Ormosea arborea* (1)
Moniliophthora roreri	Peru(1) Ecuador(1)
	Total: 100 isolates

* Numbers of isolates within parentheses.

Infected tissues, taken from vegetative brooms and diseased pods, were harvested, surface-sterilized and incubated on *Crinipellis* nutrient selective medium for 3-8 days. The morphology of the derived cultures was carefully observed in order to confirm their identity. All fungal cultures were grown in Malt 50% for 12 days. Mycelia were filtered through Whatman No. 1 filter paper, washed twice with TE buffer (10mM Tris-HCl; 1mM EDTA, pH 8.0), lyophilised and total DNA was extracted as described by Zolan and Pukila (1986). The amount of DNA was quantified by electrophoresis and spectrophotometry. The DNA samples were diluted in 100 µl sterilized water adjusted to 1 ng μl^{-1} and stored at –21°C for further use. Samples that showed degradation of DNA in electrophoresis gels were discarded and DNA extraction was repeated. RNA was eliminated by adding 3 µl of RNAse.

Isolation of microsatellites

Crinipellis perniciosa database (http://www.lge.ibi.unicamp.br/vassoura/) was screened for the presence of SSR. Primer pairs complementary to the flanking regions of the microsatellite sequences were designed using either the computer program PRIMER3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or oligo 4.0. Primers were designed in a way of yielding PCR products of three different sizes. Sixty six microsatellites with di-tri and tetra motifs were screened in *Crinipellis* sequences (Table 2). Sixty-three primers were designed and tested in 3% agarose gel.

PCR Analysis and Amplification

PCR reactions were carried out in a volume of 20 µL containing 5 ng of DNA template, 0.4 µM of each primer,

1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1X reaction buffer and 1 unit of *Taq* DNA polymerase (Amersham Pharmacia Biotech or PeqLeb). PCR products were first checked using 3% agarose gel stained with ethidium bromide. Primers pairs with good amplification were screened for polymorfism in 5% polyacrylamide gels.

Ten primers were radiolabelled and checked on acrylamide gel. This step was done at CIRAD. Radioactive PCR were as follow: 5 µL forward primer (100 µM) was end-labelled using 10 µL [ã-^{32}P] –ATP, 2 µL of T_4 kinase buffer, and 3 µL of T_4 DNA kinase were brought to a final volume of 30 µL with sterile ddH_20. The contents were mixed and incubated at 37°C for 1 h. The reaction was stopped by incubation at 70°C by 10 min. For the radioactive PCR reaction, 3 µL of radioactive primer and 5 µL of nonradioactive primer (together, 4 µM) were used following the PCR protocol. Eletrophoresis of radioactive products was carried out in 5% denaturing TBE acrylamida gel. The gels were dried at 80°C for 1.5 h.

All the amplifications were performed with a Touch Down PCR, encompassing 10 cycles of denaturation at 94°C for 4 min, primer annealing at 60-48°C using 1.0°C decrements, and extension at 72°C for 1 min, followed by 20 cycles at 94°C for 4 min, 48°C for 1 min, and 72°C for 1 min, and a final 4-min extension at 72°C.

Results and Discussion

Sixty-three primer pairs, each flanking a distinct microsatellite locus, were first tested on a set of 16 isolates including isolates of the two biotypes . Most of the primer pairs produced locus-specific amplicons in PCR. Of the primer pairs designed, 28 were tested on polyacrilamide gel. Six loci were polymorphic and another 13 remain to be screened for polymorphism (Figure 1). Almost all alleles found differed in size by three base pairs or multiples of two base pairs. There is one case where three successive alleles differed by only one base pair (Table 2).

Table 2. Characterization of microsatellite products in isolates of *Crinipellis perniciosa.*

	PRIMER 15	PRIMER 16	PRIMER 23	PRIMER 19	PRIMER 14	PRIMER 22
Nb. alleles	7	3	3	3	2	2
1º	176	209	202	197	235	198
2º	177	212	206	200	239	201
3º	180	221	214	206		
4º	186					
5º	194					
6º	197					
7º	200					

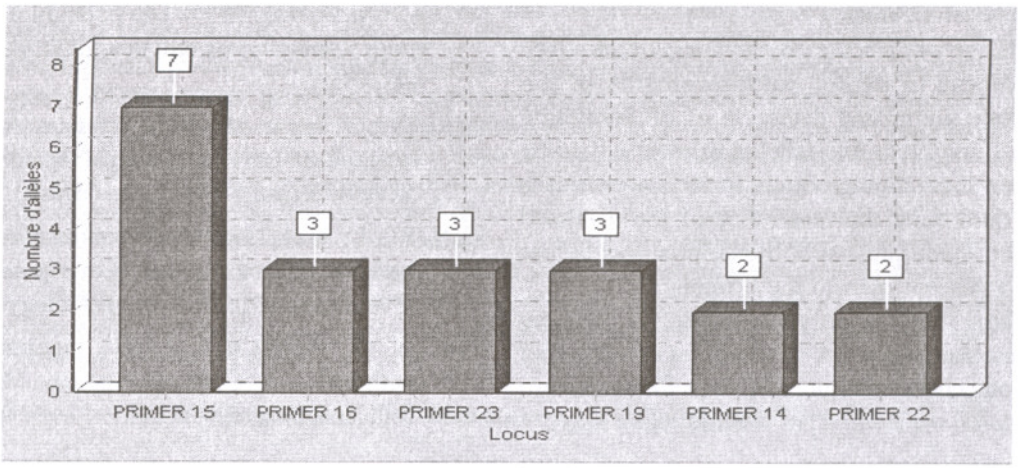

Figure 1. Histogram depicting the number of alleles that occurred at each locus.

Overall tetranucleotide repeat microsatellites showed higher levels of polymorphism than three dinucleotide repeat regions. Average heterozygosities ranged from 0.02-0.5. Preliminary results indicated that Bahia population do indeed have reduced genetic diversity when compared with the others populations. Primer sets were also tested to check the general applicability of these primers in amplifying DNA from closely related biotypes and species. Cross species amplification was successful in all loci. We have identified three loci very useful to distinguish isolates from different biotypes, and a fourth one (primer 23) that identified the most pathogenic isolate. Four primers differentiated isolates from other countries.

The isolate from *Moniliophthora* gave repeatable and scored patterns in all loci. Our results may therefore indicate a recent common ancestor for the *Moniliophthora roreri* studied. These preliminary results suggest the usefulness of the primers in population studies may go beyond the present study of the *C. perniciosa* and a have applications in population genetics of *M. roreri*.

Microsatellites clearly exist in large numbers in the genome of *C. perniciosa*, but their variability is lower than reported for other fungi. This low level of variability is manifested in two ways. First, the number of polymorphic loci was low. Among the 28 loci screened, only seven were found to be polymorphic. Second, the number of alleles at the polymorphic loci was low. The number of alleles in each locus ranged from 2 to7 among isolates of *C. perniciosa* from a wide geographic area. In contrast, in *Venturia inaequalis,* seven polymorphic loci were found in 23 sequenced clones and the number of alleles in each locus ranged from 2 to 48 (Tenzer et al 1999). Also in *Aspergillus fumigatus,* 10-23 different alleles were detected in four microsatellite loci among 102 isolates (Bart-Delabesse *et al.*1998).

Although, We will test the effectiveness of the silver staining process to detect polymorphisms in the remaining loci, automated detection of microsatellite alleles with fluorescent dye-labeled primers would be most efficient for studying populations. However, it is prudent to determine the feasibility of the designed primers and polymorphisms of the defined loci before attaching fluorescent dyes to the primers.

Given their level of polymorphism, the primers presented here should prove to be useful for investigating the population genetic structure of *Crinipellis perniciosa* and *Moniliophthora rorei.* This work is under way.

Acknowledgements

Special thanks to the technicians José dos Santos Reis and Joselito de Moraes, for helping us on collecting the isolates and to SEAGRI-BA and CFC/ICCO/BIOMOL for supporting this project and the Project Genoma Consortium and CIRAD.

Literature Cited

ANDERBRHAN, T. et al. 1999. Molecular fingerprinting suggests two primary outbreaks of witches' broom disease (*Crinipellis perniciosa*) of *Theobroma cacao* in Bahia, Brazil. *European Journal of Plant Pathology* 105: 167-175.

ARRUDA, M. C. C. et al. 2003. Nuclear and mitocondrial DNA variability in *Crinipellis perniciosa* from different geographic origins and hosts. Mycological Research 107:25-37.

BART-DELABESSE, E. et al. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates.*Journal of Clinical Microbiology* 36: 2413-2418.

GOMES, L.M. et al. 2000. Genetic diversity of *Crinipellis* from the south region of Bahia-Brazil using RAPD molecular markers. Fitopatologia Brasileira 25:377.

GRIFFITH,G.W.; HEDGER, J. N. 1994. The breeding biology of biotypes of witches´ broom disease of cocoa (*Crinipellis perniciosa*). Transactions of the Bristish Mycological Society 88.

JARNE, P.; LAGODA, P.J.L.1996. Microsatellites, from molecules to population and back. Trends in Ecology and Evolution 11: 424-429.

MCDONALD, B.A. 1997. The population genetics of fungi: tools and techniques. Phytopathology 87: 448-453.

PEREIRA, J. L.; et al. 1989. Primeira ocorrência de vassoura-de-bruxa na principal região produtora de cacau do Brasil. Agrotrópica (Brasil) 1: 79-81.

TENZER, I.; et al. 1999. Identification of microsatellite markers and their application to population genetics of Venturia inaequalis. Phytopathology 89: 748-753.

ZOLAN,.M.; PUKILA, A. P. 1986. Inheritance of DNA methylation in Coprinus cinereus. Molecular and Cellular Biology 6: 195-200.