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# Genetic variability and chromosome-length polymorphisms of the witches' broom pathogen *Crinipellis pernicios* from various plant hosts in South America

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## ABSTRACT

*Crinipellis pernicios* has been classified into at least four known biotypes associated with members of unrelated plant families. In this study, genetic variability is shown for 27 C (Cacao), 4 S (Solanum), and 7 L biotype (Liana) isolates of *C. pernicios* collected from different regions of Brazil and South America. The objective was to investigate the genetic variability of the pathogen in the cacao-producing region of Bahia, Brazil, and elsewhere, through microsatellite analysis, and attempt to identify possible correlations between host specificity and electrophoretic karyotypes. The PCR-banding patterns were found to vary both within and between the different biotypes, and a correlation was established between the PCR-banding patterns and the chromosomal-banding patterns of each isolate. Microsatellite and chromosomal patterns among all of the L and S biotype isolates were distinctly different from the C biotypes analysed. A higher degree of genetic and chromosomal variability was found among C biotype isolates from the Amazon in comparison with C biotype isolates from Bahia, which seems to be comprised of only two main genotypes. This finding has important implications to the current cacao-breeding programme in Brazil.

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## Introduction

*Crinipellis pernicios* (Agaricales, Tricholomataceae) is the causal agent of witches' broom disease (WBD) of cacao (*Theobroma*

cacao). This fungal pathogen is believed to have originated in the Amazon basin and it infects plant species within the families Malvaceae, Solanaceae, Bignoniaceae, Bixaceae, and Malpighiaceae (Evans 1980; Griffith et al. 2003; Griffith & Hedger 1994b,c;

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Purdy & Schmidt 1996; Resende et al. 2000). Since its first reported occurrence in coastal Ecuador in 1894, the disease has spread to cacao plantations throughout the Americas and Caribbean islands, causing severe economic losses (Griffith et al. 2003; Pereira et al. 1996; Purdy & Schmidt 1996). At this time this disease is limited to the Americas, but is a potential threat to all tropical cacao-growing regions of the world, where the cultivation of cacao is typically done by small landholders with limited technological inputs that make them particularly susceptible to this fungal disease.

An example of the devastating impact of the introduction of this fungus into disease free regions can be found in the cacao-producing region of southeastern Bahia, Brazil, where plantations were abandoned or substituted for other crops (Pereira et al. 1996). This has increased the rate of destruction of the Atlantic rainforest ('Mata Atlântica') and has caused serious socioeconomic problems in the region. Currently the only control mechanism is the selection of resistant plants, which are cloned and distributed to the producers. However, this selection is based on limited information about the genetic variability of both the plant and the fungal pathogen.

The broad host-range of *C. perniciosus* has prompted several authors to propose the following classification system based on host specificity: (1) the C biotype infects species of *Theobroma* and *Herrania* (Malvaceae); (2) the S biotype affects several members of the Solanaceae [4]; (3) the L biotype is found on liana vines (especially the species *Arrabidaea verrucosa* – Bignoniaceae) and associated plant debris (Evans 1978; Griffith & Hedger 1994b,c; Hedger et al. 1987).

The C and S biotypes cause the characteristic symptoms of WBD on their respective hosts, whereas the L biotype generally causes no symptoms (Hedger et al. 1987). Another important distinction among the biotypes is that while the C and S biotypes exhibit primary homothallism (Delgado & Cook 1976; Evans 1980; Griffith et al. 2003; Griffith & Hedger 1994c), the L biotype has an outcrossing breeding strategy (bifactorial heterothallism), which is widespread among the agaric fungi (Griffith & Hedger 1994a,b).

RFLP analyses of the mitochondrial DNA, ITS and IGS regions of the rRNA locus has allowed the separation of the C and S biotypes and has revealed a clonal population structure for these biotypes that correlates with their non-outcrossing breeding strategy (De Arruda et al. 2003; Griffith & Hedger 1994b). In contrast, the L biotypes show a non-clonal population structure due to outcrossing and a high degree of genetic variability (Griffith & Hedger 1994b,c; Griffith 1989). Moreover, although the genetic variability between isolates from the different biotypes of *C. perniciosus* have been examined with RAPD (Andebrhan & Furtek 1994; Andebrhan et al. 1999), Enterobacterial Repetitive Intergenic Consensus (ERIC) repetitive element sequence-based PCR (De Arruda et al. 2003) and AFLP (Ploetz et al. 2005), there has been no attempts to correlate the results. In general, diversity is higher among C biotype isolates from the Amazon region than between isolates from Bahia and some isolates from the Amazon clustered together with those from Bahia. These data indicate that the original introductions into Bahia were probably from Amazonian C biotype strains (Andebrhan et al. 1999; De Arruda et al. 2003).

In the present study we analysed the genetic and chromosomal variation of 38 isolates of *C. perniciosus* comprising three

different biotypes (C, S, and L) collected from different geographical locations in Brazil and from both coastal and Amazonian Ecuador. The objectives were to evaluate the genetic variability of the C biotype in Bahia in comparison with isolates from the Amazonian region and to establish whether the genetic differences in the biotypes could be correlated with other factors such as chromosomal variations or possibly host range.

## Experimental procedures

### Fungal strains

Field isolates of *Crinipellis perniciosus* were collected at different times, geographic locations, and from different hosts. The 38 isolates examined are listed in Table 1; 27 isolates correspond to the C biotype and each of these constitutes a single-spore culture obtained from different basidiomes collected in the field; four correspond to the S biotype and were obtained as described for the C biotype; and seven correspond to the L biotype and were obtained by isolation from stipe tissues of basidiocarps collected in the field [including one unmated (monokaryotic) single spore isolate, L7, lacking clamp connections, the progeny of L1]. C biotypes from other countries were not evaluated due to phytosanitary concerns and regulations. S biotypes were taken only from one region of Brazil as they are only found in this region. All isolates were independently tested for pathogenicity on their respective hosts (data not shown). The collection sites are shown schematically in Fig 1. All isolates used in this study can be obtained from the Laboratório de Genômica e Expressão at UNICAMP, as well as further information concerning the collection data.

### Genotypic analysis of isolates by PCR amplification

Isolates were analysed with the microsatellite primers, TeloA1R (CCCTAA)<sup>3</sup> according to Meinhardt et al. (Meinhardt et al. 2002a,b) and TeloC1 (TTTACGG)<sup>3</sup>, a repeat sequence derived from the *Crinipellis perniciosus* sequencing data. A total of eight microsatellite primers were initially tested but only these two are shown in this study because of their specific results. Reactions were conducted as described by Meinhardt et al. (Meinhardt et al. 2002a,b).

### Chromosomal analysis

Chromosomal DNA preparation from the saprotrophic mycelia of different isolates of *Crinipellis perniciosus*, protoplasting and sample plug preparations were done as described previously (Rincones et al. 2003). Chromosomal separations were carried out in a Contour-Clamped Homogeneous Electric Field (CHEF) system (DR-II, Bio-Rad, Herts) under the conditions described previously (Rincones et al. 2003), with minor modifications. Gels were run at 10–12 °C in 0.5 × Tris-borate-ethylenediamine tetraacetate (TBE) buffer at a constant current of 1.4 V cm<sup>-1</sup> for a total 354 h divided into three blocks. Block 1 consisted of pulse intervals progressively increasing from 2700 s to 5000 s for 172 h; block 2 progressively increasing from 2500 s to 3000 s for 80 h; and block 3 had a constant pulse time of 2200 s for 102 h. Approximate band sizes were calculated from the relative migration of the chromosomes of the *Schizosaccharomyces pombe* size standard

**Table 1 – Isolates used in this study**

Biotype	Isolate designation	Collection no.	Host	Location <sup>a</sup>
C (Bahia)	C01*	CP02	<i>Theobroma cacao</i>	Itajuípe, BA, Br.
	C02	CP09	<i>T. cacao</i>	Ilhéus, BA, Br.
	C03	Belmonte	<i>T. cacao</i>	Belmonte, BA, Br.
	C04	Ilhéus	<i>T. cacao</i>	Ilhéus, BA, Br.
	C05	SA	<i>T. cacao</i>	Santo Amaro, BA, Br.
	C06	FA42	<i>T. cacao</i>	Itabuna, BA, Br.
	C07	FA276	<i>T. cacao</i>	Itabuna, BA, Br.
	C08	FA277	<i>T. cacao</i>	Itabuna, BA, Br.
	C09	FA278	<i>T. cacao</i>	Itabuna, BA, Br.
	C10	FA281	<i>T. cacao</i>	Aiquara, BA, Br.
	C11	FA287	<i>T. cacao</i>	Inema, BA, Br.
	C12	FA300	<i>T. cacao</i>	Ibirataia, BA, Br.
	C14	FA311	<i>T. cacao</i>	Itagiba, BA, Br.
	C15	FA317	<i>T. cacao</i>	Ilhéus, BA, Br.
	C16	FA562	<i>T. cacao</i>	Itabuna, BA, Br.
	C17	FA563	<i>T. cacao</i>	Itabuna, BA, Br.
	C18	BP10	<i>T. cacao</i>	Itapebi, BA, Br.
C (Amazon)	C19	FA551	<i>T. cacao</i>	Tabatinga, AM, Br.
	C20	ESJOH1	<i>T. cacao</i>	Marituba, PA, Br.
	C21	ESJOH2	<i>T. cacao</i>	Ouro Preto, RO, Br.
	C22	ESJOH3	<i>T. cacao</i>	Belem, PA, Br.
	C23	ESJOH4	<i>T. cacao</i>	Altamira, PA, Br.
	C24	ESJOH5	<i>T. cacao</i>	Medicilândia, PA, Br.
	C25	ESJOH6	<i>T. cacao</i>	Ariquemes, RO, Br.
	C26	ESJOH8	<i>T. cacao</i>	Ji-Parana, RO, Br.
	C27	ESJOH9	<i>T. cacao</i>	Alta Floresta, MT, Br.
S	S1	FA104	<i>Solanum cernum</i>	Rio Pomba, MG, Br.
	S2	FA607	<i>S. lycocarpum</i>	Coimbra, MG, Br.
	S3	FA608	<i>S. cernum</i>	Rio Pomba, MG, Br.
	S4	FA609	<i>Solanum</i> sp.	Poços de Caldas, MG, Br.
L	L1	SCFT	<i>Arrabidaea verrucosa</i>	San Carlos, NA, Ec.
	L2	LEP1	<i>A. verrucosa</i>	Pichilingue, RI, Ec.
	L3	SCL4	<i>A. verrucosa</i>	San Carlos, NA, Ec.
	L4	LA10	<i>A. verrucosa</i>	Pichilingue, RI, Ec.
	L5	LA17	<i>A. verrucosa</i>	Pichilingue, RI, Ec.
	L6	LC3	<i>A. verrucosa</i>	Pichilingue, RI, Ec.
	L7	SCFT48	<i>A. verrucosa</i>	San Carlos, NA, Ec.

\*Reference isolate used for the genome project ([www.lge.ibi.unicamp.br/vassoura](http://www.lge.ibi.unicamp.br/vassoura))

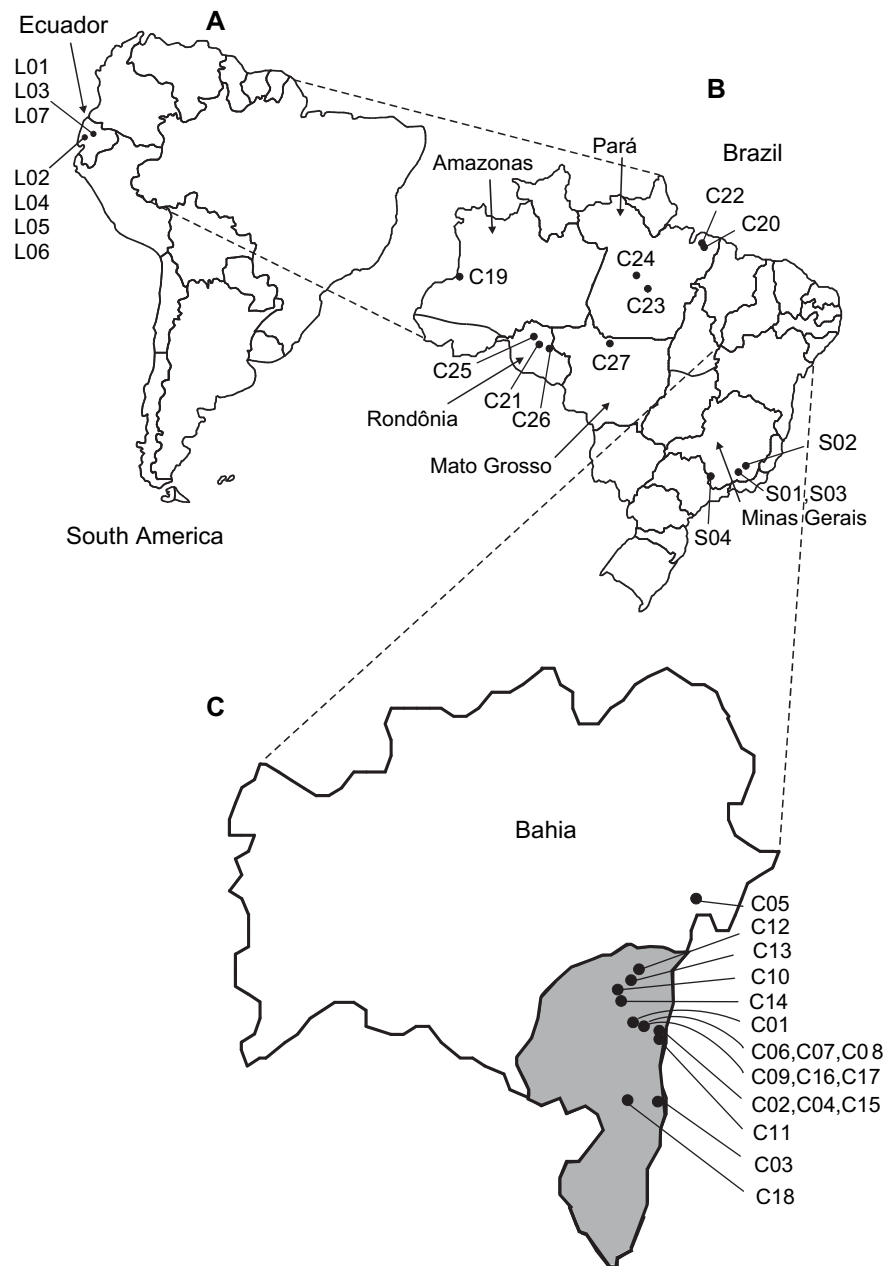
a Origin of isolates: Br., Brazil; BA, Bahia State, except for C05, all C (Bahia) isolates were collected within a region of approximately 90 km<sup>2</sup> of Southeastern Bahia; the States of Amazonas (AM), Mato Grosso (MT), Rondônia (RO), and Pará (PA) correspond to the Brazilian Amazon; the State of Minas Gerais (MG) is located in Southeastern Brazil; Ec., Ecuador; NA, Napo Province in Amazonian Ecuador; RI, Los Rios Province in Coastal Ecuador (Fig 1).

and the reference isolate C01, whose chromosome sizes had been reported previously (Rincones *et al.* 2003). Densitometric analysis of the ethidium bromide-stained pattern of the chromosomal bands was performed using the software Image Master<sup>®</sup> VDS (Amersham Bioscience, Little Chalfont). CHEF gels were transferred to membranes (Hybond N+, Amersham Biosciences) and probed with PCR labelled-amplified fragments of: (1) ITS regions (White *et al.* 1990) of the ribosomal DNA (rDNA) of *C. pernicioso*; and (2) the insert of a genomic clone from isolate C01 showing significant sequence similarity (BLASTx, E value of 9E-13) to a reverse transcriptase–RNase H integrase of *Tricholoma matsutake* (GenBank accession no. AY661428) (Rincones *et al.* 2003). Prehybridization, hybridization, and washing of the membranes were conducted according to the manufacturer's instructions.

## Results

### Microsatellite-based PCR analysis

The microsatellite PCR primer TeloC1 revealed genotypic differences between the various isolates of the L and S biotypes, but failed to show any difference for C biotype isolates used in this study (Fig 2). This was particularly interesting as it gave the same specific banding pattern for all of the C biotype isolates, regardless of their origin, allowing its differentiation from the other biotypes, therefore suggesting that this primer could be used as a C biotype indicator (Fig 2C). The primer TeloA1R (CCCTAA)<sup>3</sup> (Meinhardt *et al.* 2002a,b) was able to



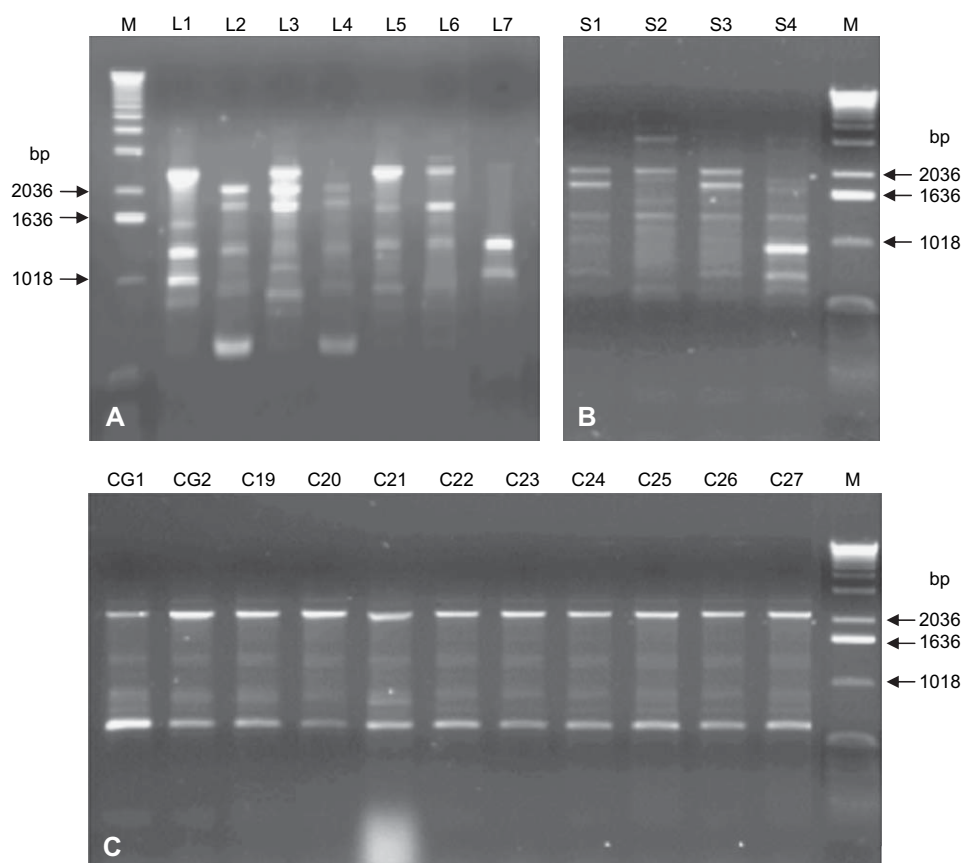
**Fig 1 – Maps detailing the sites for the collection of the isolates of *Crinipellis perniciososa* listed in Table 1. A. Map of South America detailing the sites for the collection of isolates of *C. perniciososa* in Ecuador. B. Map of Brazil shows the sites of isolate collection in the Brazilian Amazon and Southeastern Brazil. C. Map of Bahia shows sites of isolate collection in the southeastern cacao-growing region of this state. The shaded area represents the cacao-growing region in the state of Bahia.**

separate most of the biotype C, L and S isolates into specific groups (Fig 3B–C). The C-biotype isolates from Bahia were separated into two groups with the TeloA1R primer, CG1 (comprising isolates C01, C02, C03, C04, C08, C09, C11, C14, C15 and C17) and CG2 (comprising isolates C05, C06, C07, C10, C12, C13, C16, and C18). The nine Amazon isolates were separated into seven groups with the three isolates from Para C20, C22 and C23 representing a single group (Fig 3A). All of the L and S biotype isolates showed genetic variations with the TeloA1R primer (Fig 3B–C). In the case of the L biotype isolates, this primer revealed a somewhat similar banding

patterns for L1 and its progeny L7 indicating that alterations in a given population could possibly be monitored with this primer. All PCR amplicons for the different primers ranged from 3000 bp to 500 bp in size.

#### Electrophoretic karyotype

Fig 4A shows the four different karyotypes obtained for the 18 C biotype isolates (C01–C18) collected in various regions of Southeastern Bahia: C01–C18 (Table 1). Most of the isolates from this region could be grouped into two different



**Fig 2 – Microsatellite primer based analysis with the primer TeloC1. A. The banding profile for the seven L biotype isolates amplified with the TeloC1 primer. B. PCR banding pattern of the four S biotype isolates amplified with primer TeloC1. C. Comparison of nine out of the 11 C biotype groups, CG1 and CG2 correspond to the two chromosomal groups from Bahia and the other isolate designations are as listed in Table 1; isolates C08 and C15 showed the same amplicon band pattern seen for CG1. The isolate designations are as stated in Table 1. M indicates the 1 kb DNA standard from Invitrogen.**

karyotypes that were designated as CG1 (comprising isolates C01, C02, C03, C04, C09, C11, C14, and C17) and CG2 (comprising isolates C05, C06, C07, C10, C12, C13, C16, and C18). The other two karyotypes were exhibited by isolates C08 and C15 and they are both very similar to karyotype CG1: in the case of isolate C08, its karyotype showed an extra band of approximately 4.5 Mb in size (arrow on Fig 4A) while the karyotype of isolate C15 presented a slightly smaller fifth band (arrow on Fig 4A). In contrast, all nine isolates collected in the Brazilian Amazon (C19–C27, Table 1) exhibited different karyotypes, varying in band number and sizes, as shown in Fig 4D.

Fig 5A shows the karyotypes obtained for all isolates of the S and L biotypes. The CG1 karyotype is shown for comparison purposes. All isolates examined for these two biotypes exhibited different karyotypes, varying in band number and sizes.

A densitometric analysis of the ethidium bromide-stained banding pattern was performed for all karyotypes. Two of these analyses are shown as examples (Fig 4B–C) to illustrate that the relative intensity of the ultraviolet fluorescence was higher for some bands. This result suggests that each of these bands represent at least two chromosomes, which could be homologous or heterologous chromosomes of similar sizes.

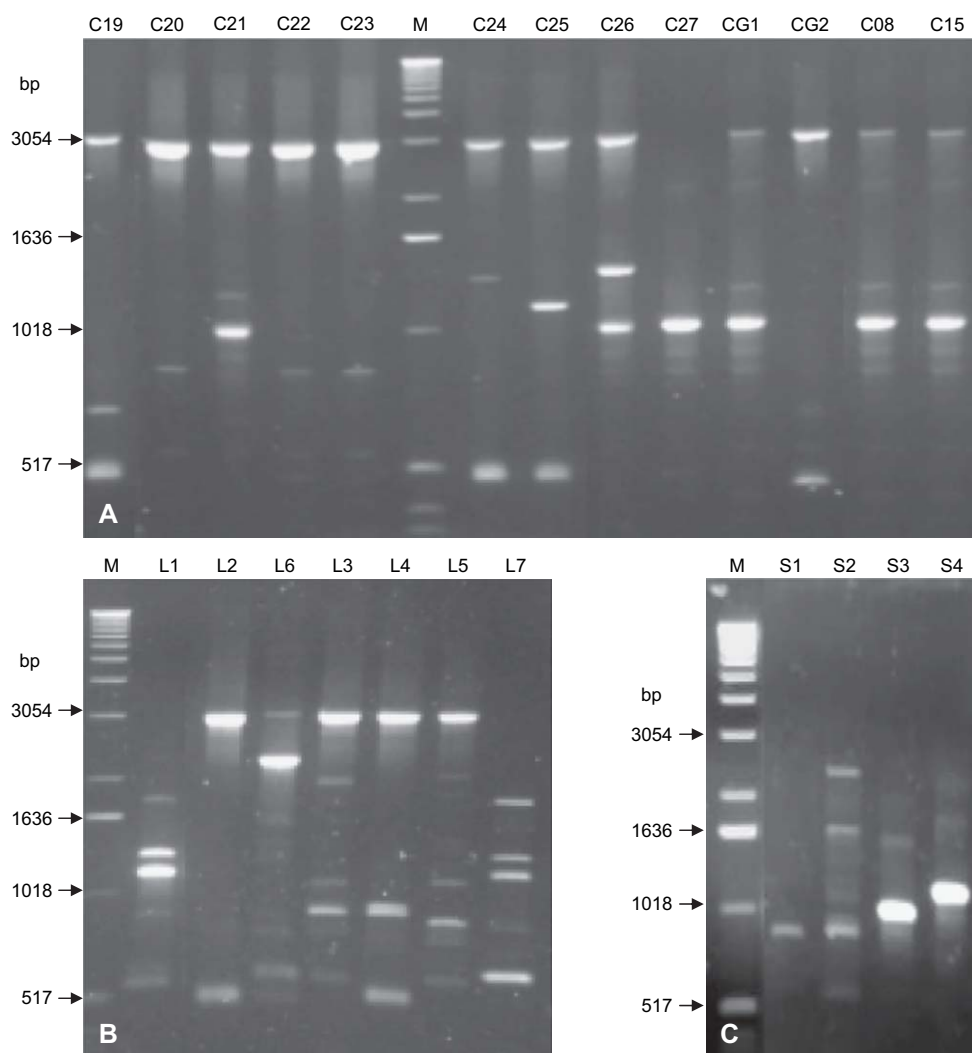
Results from the densitometric analysis were taken into consideration when calculating total genome sizes for each isolate (Table 2). Table 2 lists the approximate sizes of the bands and summarizes the electrophoretic karyotypes for each isolate, grouped by biotypes and geographic origin.

### Southern hybridization

Fig 5B illustrates the results of some of the Southern hybridizations; however, all hybridization results are summarized in Table 2. Southern hybridization of blots from the pulsed-field gels with the ITS probe shows that the rDNA repetitions were usually clustered to a single chromosome, independent of the biotype (Fig 5B, Table 2). Isolate L6 was the only exception with the ITS probe hybridizing to two chromosomal bands (Fig 5B, Table 2). The rDNA repetitions were usually found on one of the two largest chromosomes, except in isolate C25, where the ITS probe hybridized to the third chromosomal band (Fig 5B, Table 2).

The probe containing a sequence similar to a reverse transcriptase–RNase H integrase hybridized to most bands of every isolate examined with the exception of the smaller bands in some isolates (Fig 5B, Table 2). This result suggests





**Fig 3 – Microsatellite primer-based analysis with the primer TeloA1R. A.** Comparison of the banding patterns for C biotype isolates from the Amazon C19 to C27 and the representative chromosomal groups from Bahia CG1 and CG2 and the two CG1 subgroups, C08 and C15 amplified with the microsatellite primer TeloA1R. **B.** PCR banding profiles for the seven L biotype isolates. **C.** Banding profile for the four S biotype isolates from Minas Gerais. The isolate designations are as listed in Table 1. M indicates the 1 kb DNA standard from Invitrogen.

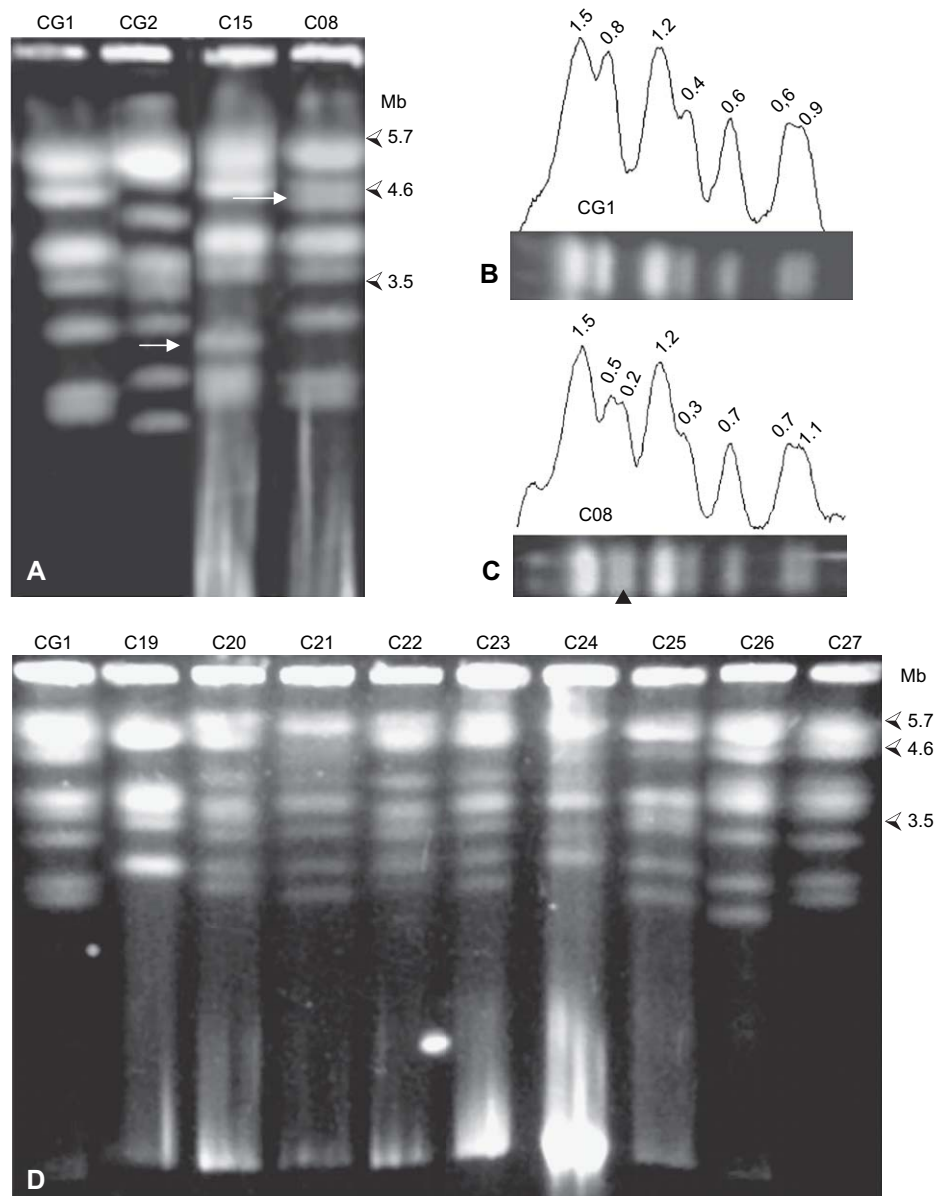
that retrotransposons are widespread throughout the genome of all isolates examined.

## Discussion

In this study, we report genetic and chromosomal-level variability of isolates within and among three different biotypes of the *Crinipellis perniciosa* species complex, collected from different geographic locations. This constitutes one of the most comprehensive studies reported to date for this species with regards to the number of isolates from different basidiomes, geographic distance between collection points for the various isolates, and number of different biotypes examined. We also present for the first time the electrophoretic karyotypes of S and L biotypes of *C. perniciosa*. This study is the first in a series of reports that will attempt to form a cohesive understanding

of genetic variability found in the various biotypes of this fungal pathogen, which will be used to formulate how the variability affects the pathogen–host interaction as well as disease development.

RAPD fingerprinting by Andebrhan et al. (1999) of isolates collected at the beginning of the WBD outbreak in Bahia, suggested that there had been two discrete introductions of the pathogen (i.e. two independent genotypes), a hypothesis that correlates with the presence of only two different karyotypes among more recently collected samples (Rincones et al. 2003) and only two populations detected in Bahia through AFLP analysis (Ploetz et al. 2005). This evidence clearly shows the stability of the C genotypes in Bahia, which have remained almost unchanged from the time of their introduction. Due to this natural genetic stability of the clonal populations of the C and S biotypes and their non-outcrossing reproductive strategy, it is possible to supplement the genetic variability

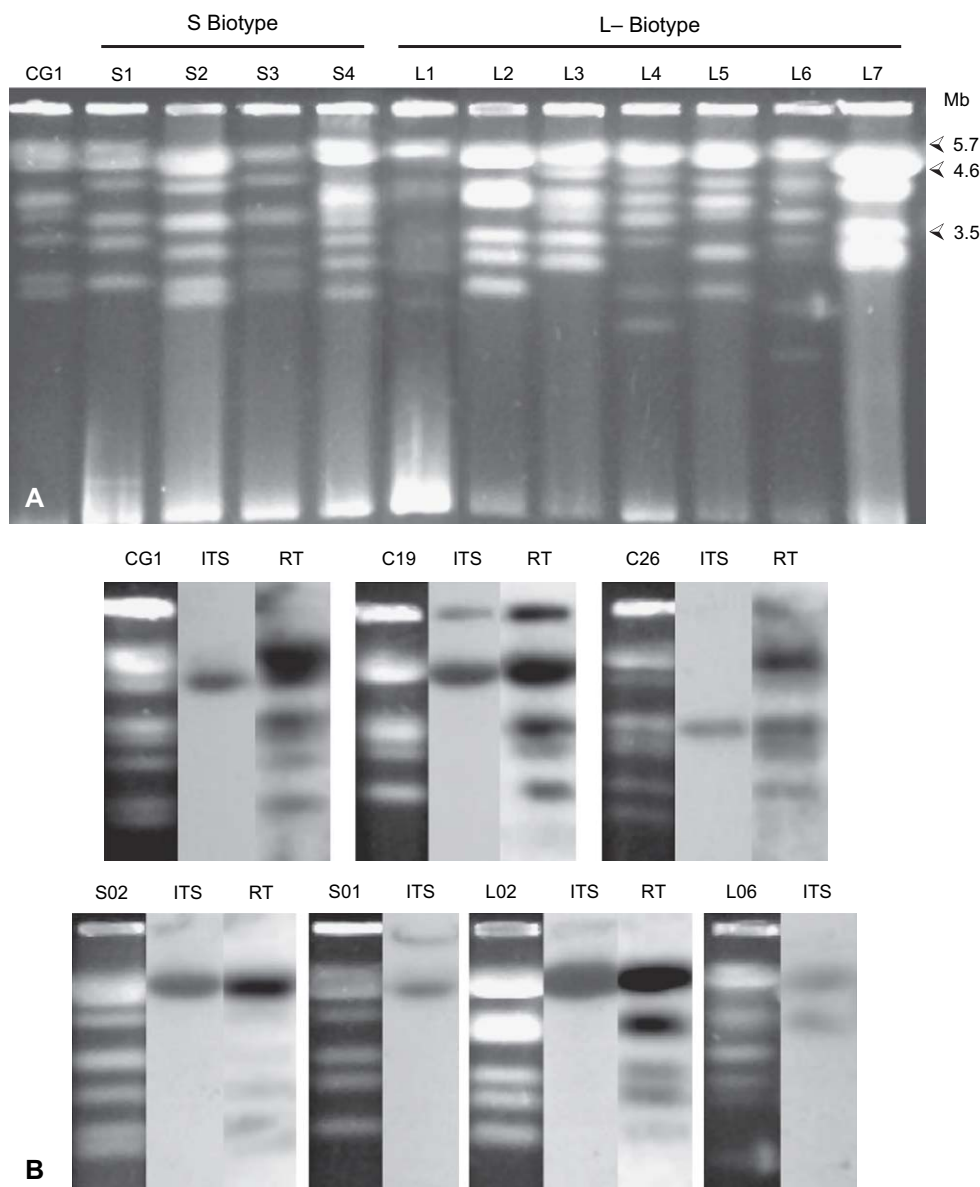


**Fig 4 – CLPs between isolates of the C biotype of *Crinipellis pernicioso* from Bahia-Brazil. A.** Four different karyotypes obtained for the 18 C biotype isolates collected in Southeastern Bahia (C01–C18, [Table 1](#)). **B.** Densitometric analysis of the ethidium bromide-stained pattern of karyotype CG1, numbers represent the integrated optical density (IOD) for the respective peaks. **C.** Densitometric analysis of the ethidium bromide-stained pattern of the karyotype of isolate C08, numbers represent the IOD of the respective peaks. CG1: karyotype of the C biotype group 1 consisting of isolates C01, C02, C03, C04, C09, C11, C14, and C17. CG2: karyotype of the C biotype group 2 consisting of isolates C05, C06, C07, C10, C12, C13, C16, and C18. Sizes correspond to the relative migration of the three large chromosomes of *Schizosaccharomyces pombe* (strain 972h, Bio-Rad).

analysis with chromosomal analysis via electrophoretic karyotyping.

With a single exception, all C biotype isolates collected in Southeastern Bahia (C01 to C18) possessed eight chromosomes and a total genome size of approximately 30 Mb; only one isolate (C08) possessed nine chromosomes with an estimated total genome size of 34.8 Mb. In fact, C biotype isolates collected in this region were very homogeneous in their karyotypes, with eight isolates presenting karyotype CG1 and another eight isolates presenting karyotype CG2 ([Table 2](#)). These two karyotypes are identical to those previously

reported in Bahia, comprising only four isolates that were included in this work ([Rincones et al. 2003](#)) and these two chromosomal groups (CG1 and CG2) are supported by the microsatellite primer, TeloA1R, which separated all of the C-biotype isolates from Bahia into two groups that were identical to the chromosomal groups. The two additional karyotypes for this region represented by isolates C08 and C15 are remarkably similar to karyotype CG1. C15 differs from CG1 in the size of the fifth band, which appears slightly smaller, whereas isolate C08 differs from CG1 with an additional band of approximately 4.5 Mb. Furthermore,



**Fig 5** – Analysis of the CLPs between isolates of the S and L biotypes of *Crinipellis perniciosus* from South America. **A.** CLPs between isolates of the S and L biotypes of the *Crinipellis perniciosus* species complex together with reference karyotype CG1. Sizes correspond to the relative migration of the three large chromosomes of *Schizosaccharomyces pombe* (strain 972h, Bio-Rad). **B.** Examples of the Southern hybridization of the gels shown in Figs 4A, 4D and 5A with the two probes tested. ITS: probe consisting of the ITS-amplified region of the rRNA locus of reference isolates C01. RT: probe consisting of a PCR-amplified insert of a genomic clone from isolate C01 showing high sequence similarity (BLASTx, E value 9E-13) to a reverse transcriptase–RNase H integrase of *Tricholoma matsutake* (GenBank accession no. AY661428). Isolate designations are as in Table 1. Results of all hybridization experiments are summarized in Table 2.

polymorphisms associated with the hybridization of the rDNA between these four karyotypes from Bahia lends support to the CG1 origin of C08 and C15. The data showed that this marker hybridized to the second largest chromosome (4.6 Mb) in karyotypes CG1, C08 and C15 and the largest chromosomal band (5.3 Mb) in the CG2 karyotype (Fig 5B, Table 2). These apparent subgroups of CG1 could not be detected by any of the microsatellite markers tested, which is a limitation of PCR-based analysis, especially in the

detection of chromosomal rearrangements that can only be revealed by pulsed-field gel electrophoresis.

These subgroups of CG1 are possibly the result of very recent differentiation events and the chromosomal differences between isolates C08 and C15 in comparison to CG1 are subtle and could have arisen from the CG1 karyotype in a single event: either a deletion or a translocation of a portion of the fifth chromosome in the case of isolate C15; and a duplication in the case of isolate C08. It is also probable that band 2



**Table 2 – Estimated sizes and number of chromosomes of the 38 isolates examined of the *Crinipellis pernicioso* species complex. The chromosomal bands were numbered arbitrarily according to size in decreasing order**

Biotype	Isolate	Chromosomal bands: Mb (n)									Total number of chromosomes	Minimal size	Total size
		1	2	3	4	5	6	7	8	9			
C Bahia	CG1 <sup>a</sup>	5.2 (1) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	3.9 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.8 (1)	-	-	8	26.4	30.3
	CG2 <sup>b</sup>	<u>5.3 (1)<sup>c</sup></u>	4.7 (1) <sup>c</sup>	4.2 (1) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	2.7 (1)	-	8	30.4	30.4
	C08	5.2 (1) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	4.5 (1) <sup>c</sup>	3.9 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.8 (1)	-	9	30.9	34.8
	C15	5.2 (1) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	3.9 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	<b>3.2 (1)<sup>c</sup></b>	3.0 (1) <sup>c</sup>	2.8 (1)	-	-	8	26.3	30.2
C Amazon	C19	<u>5.0 (2)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.8 (2) <sup>c</sup>	3.4 (1) <sup>c</sup>	3.0 (2) <sup>c</sup>	-	-	-	-	8	19.3	31.1
	C20	5.5 (1) <sup>c</sup>	<u>4.9 (1)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.6 (2) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.8 (1)	-	-	8	27.2	30.8
	C21	5.4 (2) <sup>c</sup>	<u>4.5 (1)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.7 (2) <sup>c</sup>	3.4 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	2.9 (1)	-	-	9	27.1	36.2
	C22	5.5 (1) <sup>c</sup>	<u>4.9 (2)<sup>c</sup></u>	4.0 (1) <sup>c</sup>	3.6 (2) <sup>c</sup>	3.4 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	2.9 (1)	-	-	9	27.4	35.9
	C23	5.5 (1) <sup>c</sup>	5.0 (2) <sup>c</sup>	4.1 (1) <sup>c</sup>	3.8 (2) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	2.9 (1)	-	-	9	27.9	36.7
	C24	5.3 (2) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.8 (2) <sup>c</sup>	3.4 (1) <sup>c</sup>	3.1 (2) <sup>c</sup>	-	-	-	9	24.3	36.5
	C25	5.2 (2) <sup>c</sup>	4.6 (1) <sup>c</sup>	<u>3.8 (2)<sup>c</sup></u>	3.5 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.9 (1) <sup>c</sup>	2.7 (1)	-	-	9	25.7	34.7
	C26	5.2 (2) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	3.9 (1) <sup>c</sup>	3.7 (2) <sup>c</sup>	3.3 (1) <sup>c</sup>	2.8 (1) <sup>c</sup>	2.6 (1) <sup>c</sup>	-	-	9	26.1	35.0
	C27	5.2 (2) <sup>c</sup>	<u>4.6 (1)<sup>c</sup></u>	3.9 (1) <sup>c</sup>	3.8 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	2.9 (1) <sup>c</sup>	2.7 (1)	-	10	30.0	39.0
S	S01	5.3 (1) <sup>c</sup>	<u>4.8 (2)<sup>c</sup></u>	4.2 (1) <sup>c</sup>	3.6 (2) <sup>c</sup>	3.2 (2) <sup>c</sup>	2.8 (2) <sup>c</sup>	-	-	-	10	23.9	38.8
	S02	<u>4.9 (2)<sup>c</sup></u>	4.6 (1) <sup>c</sup>	4.1 (2) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.2 (1) <sup>c</sup>	2.8 (1) <sup>c</sup>	2.7 (1) <sup>c</sup>	-	-	9	25.8	34.8
	S03	<u>5.0 (2)<sup>c</sup></u>	4.3 (1) <sup>c</sup>	3.6 (2) <sup>c</sup>	3.2 (1) <sup>c</sup>	2.9 (1) <sup>c</sup>	2.8 (1) <sup>c</sup>	-	-	-	8	21.8	30.4
	S04	5.3 (1) <sup>c</sup>	<u>4.9 (2)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.9 (2) <sup>c</sup>	3.7 (1) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.8 (2) <sup>c</sup>	12	34.5	46.1
L	L01	<u>5.1 (1)<sup>c</sup></u>	4.8 (2) <sup>c</sup>	4.1 (1) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	2.6 (1) <sup>c</sup>	-	9	29.5	34.3
	L02	<u>4.9 (2)<sup>c</sup></u>	4.1 (1) <sup>c</sup>	3.5 (2) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.1 (1) <sup>c</sup>	2.8 (1) <sup>c</sup>	-	-	-	8	21.7	30.1
	L03	<u>4.9 (2)<sup>c</sup></u>	4.5 (1) <sup>c</sup>	4.0 (2) <sup>c</sup>	3.7 (1) <sup>c</sup>	3.3 (2) <sup>c</sup>	3.0 (2) <sup>c</sup>	-	-	-	10	23.4	38.6
	L04	<u>5.2 (1)<sup>c</sup></u>	4.8 (2) <sup>c</sup>	4.4 (1) <sup>c</sup>	4.0 (1) <sup>c</sup>	3.8 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	-	10	32.1	40.7
	L05	5.2 (1) <sup>c</sup>	<u>4.8 (2)<sup>c</sup></u>	4.4 (1) <sup>c</sup>	3.9 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.3 (1) <sup>c</sup>	2.7 (1) <sup>c</sup>	2.4 (1) <sup>c</sup>	-	10	30.1	39.0
	L06	<u>5.3 (1)<sup>c</sup></u>	4.8 (2) <sup>c</sup>	<u>4.3 (1)<sup>c</sup></u>	3.9 (2) <sup>c</sup>	3.6 (1) <sup>c</sup>	3.1 (1)	2.7 (1)	-	-	9	27.7	36.4
	L07	5.2 (1) <sup>c</sup>	<u>4.8 (2)<sup>c</sup></u>	4.1 (2) <sup>c</sup>	3.5 (1) <sup>c</sup>	3.2 (1) <sup>c</sup>	3.0 (1) <sup>c</sup>	-	-	-	8	23.8	32.7

a CG1 refers to all C biotype isolates from Bahia that exhibit the karyotype CG1: C01, C02, C03, C04, C09, C11, C14, and C17.

b CG2 refers to all C-biotype isolates from Bahia that exhibit the karyotype CG2: C05, C06, C07, C10, C12, C13, C16, and C18. Mb, approximate size of the band expressed in millions of base pairs; (n), relative number of chromosomes per band as detected by the densitometric analysis. Minimal Size, minimal genome size expressed in Mb. Total Size, estimated total genome size expressed in Mb. The rDNA genes are located on italic chromosomal bands.

c Chromosomes marked by the RT probe. Chromosomes in bold indicate differences between the C08 and C15 karyotypes in relation to the CG1 karyotype.

of the CG1 karyotype represents two chromosomes, and a small portion of one of these chromosomes was translocated, thus generating two different sized chromosomes in isolate C08. The densitometric analysis of isolate C08, which shows bands 2 and 3 are less intense than bands 1 and 4 (Fig 4C), supports this latter hypothesis and suggests that the CG1 karyotype could have doublets at chromosomes 1, 2, and 3. This would increase the total genome size of the CG1 isolates to 40.1 and the total number of chromosomes to 10. Although this is a large increase in relation to the size reported previously, the total genome size determined by the Feulgen staining technique would support such an increase ( $32.98 \pm 7.94$  Mb) (Rincones et al. 2003). Furthermore, bioinformatic analyses of the shotgun assemblies have generated a 37 Mb estimation of the total genome size (unpublished data), which also supports the hypothesis of a larger genome.

The microsatellite analysis of the nine C biotype isolates collected in the Brazilian Amazon region (C19 to C27, Table 2) with the PCR primer TeloA1R separated all of them into discrete groups, which corresponds to chromosomal variability except for isolates C20, C22 and C23 (Figs 3A, 4D).

Karyotypes for the C biotype Amazon isolates differed among each other with respect to the number of

chromosomes, sizes, and total genome size. These isolates had eight to ten chromosomes and their total genome sizes varied from 30.8–39 Mb (Table 2). The ITS probe hybridized in most cases to band 2, with the exceptions of isolate C19, in which the ITS probe hybridized to the largest chromosome, and isolate C25, in which the rDNA chromosome was located on band 3 (Fig 5B, Table 2). The size of the rDNA chromosome for the Amazonian C biotype isolates varied considerably more, from 3.8 and 5.0 Mb, in comparison with the Bahia isolates in which the size of the rDNA chromosome was either 4.6 or 5.3 Mb. This variation in the size of the rDNA chromosome may be involved with unequal crossing over events that would accumulate over time and would result in alterations that persist in new clonal populations. The isolates C20, C22 and C23, which were grouped together by the TeloA1R primer, are from the same state and two of the isolates are from locations in close proximity, suggesting that the similarity observed could have been derived from a common ancestor. This agrees with the findings of Ploetz et al. (2005), who identified genetic homogeneity in the isolates of specific geographic regions. We detected only slight chromosome variations (Fig 4D) among these isolates, which supports this possibility, and this could be a situation analogous to the subgroups of CG1 found in

Bahia. If so, this could suggest that chromosomal rearrangements are the first step in population differentiation. Reproduction of the *C. perniciosus* species complex is strictly sexual via meiosis within basidia (Delgado & Cook 1976; Griffith & Hedger 1994c), and in agreement with the meiotic maintenance hypothesis proposed by Kistler and Miao (1992), in Bahia, where the fungus has been established for at least 15 y, only a very low chromosomal variation (chromosome length polymorphisms – CPLs) could be detected. Although the meiotic maintenance hypothesis is supported by observations in several fungal species (Kistler & Miao 1992; Pöggeler et al. 2000; Zolan 1995) there are reports of sexually reproducing fungi, such as *Coprinus cinereus* (Zolan et al. 1994), *Septoria tritici* (McDonald & Martinez 1991), *Leptosphaeria maculans* (Plummer & Howlett 1993), *Ophiostoma ulmi* (Dewar & Bernier 1995), and *Pythium sylvaticum* (Martin 1995), with extensive CLPs that are generated and maintained through meiotic processes without apparent reduction in fertility. The C and S biotypes exhibit primary homothallism (non-outcrossing) and any polymorphisms that arise could in principle be perpetuated by autofertilization, which could explain the origin of C08 and C15. If so, further analysis of isolates from Bahia may reveal additional subgroups that have originated from this process.

In addition to the TeloA1R microsatellite primer that correlates with chromosomal variation, a C-biotype specific PCR primer (TeloC1) was found (Fig 2). Data derived from this primer show that the C S and L biotypes appear to be distinct groups within the species complex, thus reinforcing the differentiation into the various biotypes.

All four of the S biotype isolates and the seven L biotype isolates examined revealed distinctly different microsatellite groups and karyotypes. (Figs 3B–C, 5A, Table 2). The S biotype showed the most variation, with karyotypes that exhibited between eight and 12 chromosomes and their total genome sizes varied from 30.4–46.1 Mb. In the case of the L biotype, the karyotypes presented between eight and ten chromosomes and the genome sizes ranged from 30.1 and 40.7 Mb. Except for the presence of bands smaller than 2.7 Mb in some isolates of the L biotype, no significant differences were observed between the karyotypes of the three biotypes that would allow a general correlation of karyotype with host specificity. However, the limited chromosomal-level genetic variability observed in the C biotype isolates from Bahia in comparison with C biotype isolates from the Amazon and to the S biotype isolates found in the neighbouring state of Minas Gerais, Brazil, support the hypothesis that C biotypes were introduced into Bahia from the Amazon region (Andebrhan et al. 1999).

Southern hybridization of the CHEF gels blots for the S biotype revealed that the ITS probe hybridized to the first or second largest band, but the size of this band varied only from 4.8–5.0 Mb among the four isolates (Fig 5B, Table 2). The situation was very similar in the L biotype, with the rDNA chromosomes varying only from 4.8–5.2 Mb in size, with a single remarkable exception, isolate L6, in which the ITS probe hybridized to bands 1 and 3 (5.3 and 4.3 Mb, respectively). Isolate L6 was the only one of the 38 isolates examined that exhibited two rDNA chromosomes (Fig 5B, Table 2). This situation could have arisen from a duplication of part of the

rDNA chromosome; however, our data are too limited to allow any conclusions on this matter. Polymorphism in the rDNA chromosome has been reported in numerous species of fungi and may be produced by a number of mechanisms (Zolan 1995). CLPs among L biotype isolates were detected even between isolates collected only a few meters apart. These isolates belonged to different somatic compatibility groups but may have been related as evidenced by shared mating type factors, as was the case with isolates L6 and L7 collected from the same vine in Bosque Viejo, Pichilingue Province, Ecuador, which shared two mating type factors (A9 and B10) and thus may have been sib-related (Griffith & Hedger 1994b). A comparison of the karyotypes of the parental isolate L1 and its monokaryotic progeny L7 shows that some of the bands from the parental isolate (L1) are conserved but different-sized bands arise in the progeny (L7: bands 1, 3 and 5; Fig 5A, Table 2).

The Southern hybridization of the CHEF gel blots to a genomic probe showing high sequence similarity to a reverse transcriptase from C01 marked most of the chromosomal bands of all the isolates analysed (Fig 5B, Table 2). In addition, preliminary gene expression analysis of this same clone through DNA microarray technology suggests that the presence of cocoa extracts activate these genes (M. Sabha, pers. comm.), and this could be a mechanism for introducing genetic variation, given the fact that this fungus must undergo meiosis in order to produce basidiospores (Delgado & Cook 1976; Evans 1980). The role of transposable elements in the reorganization of a fungal genome through ectopic recombination or simply by their activation has already been reported in several fungal species, such as *Fusarium oxysporum* (Davière et al. 2001), *Magnaporthe grisea* (Dobinson et al. 1993), *Schizosaccharomyces pombe* (Levin et al. 1990), *Saccharomyces cerevisiae* (Boeke 1989), and *Neurospora crassa* (Kinsey & Helber 1989). A similar mechanism might be at work in *C. perniciosus* and several families of transposable elements have already been detected in the genome of this phytopathogen (Araujo et al. 2004; Pereira et al. 2003).

This study shows that genetic variation between isolates within a given biotype and between biotypes can be determined with microsatellite-based PCR combined with chromosomal analysis. Further studies of additional isolates of this pathogen, along with detailed mapping of locations and hosts from Bahia and from the entire Amazon region would allow for a more complete genetic variability evaluation of the fungus. Future studies are now needed to utilize this information together with pathogenicity data to develop an overall picture of how genetic variability within this species complex affects the pathogen–host interaction.

Furthermore, our results suggest that the chromosomal rearrangements observed in *C. perniciosus* are generated through meiotic processes, which may involve the presence of multiple copies of retrotransposons. Therefore, it is important that isolates comprising as many C biotype genetic variability groups/karyotypes as possible should be taken into consideration when assessing resistance of cacao clones. Our results indicate the fragility of the current breeding programme being conducted in Brazil. All cacao clones selected have been challenged against only the two main genotypes of *C. perniciosus* present in Bahia, which may be a very limited

strategy that would render the culture very susceptible to new introductions from the Amazon. Therefore, it is imperative that a new concept for this programme be considered and that phytosanitary measures are strengthened in order to prevent the entrance of new *C* biotype isolates into Bahia, thus possibly extending the time for resistance breakdown (Bartley 1986) of the newly-planted resistant cacao clones.

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