BRAZ TAVARES DA HORA JÚNIOR

Molecular phylogeny and population genetics of *Microcyclus ulei*, causal agent of the South American leaf blight of *Hevea brasiliensis*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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(Orientador)

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SUMÁRIO

RESUMO

HORA JÚNIOR, Braz Tavares, D.Sc., Universidade Federal de Viçosa, Agosto de 2012. Filogenia m**olecular e genética de populações *de Microcyclus ulei*, agente causal do mal das folhas de Hevea b*rasiliensis***. Orientador: Eduardo Seiti Gomide Mizubuti. Co-orientadores: Luiz Antonio Maffia e Robert Weingart Barreto.

O mal das folhas da seringueira, doença causada pelo fungo *Microcyclus ulei*, impediu o desenvolvimento de *Hevea* spp. em áreas de monoculturas na região amazônica e, consequentemente, acarretou perdas na produção de borracha natural em sistemas agrícolas na América Tropical. O uso de clones de seringueira com resistência parcial ao mal das folhas é a melhor opção para o manejo da doença. Embora o mal das folhas seja conhecido desde o início do século 20, aspectos básicos da classificação filogenética e da estrutura genética da população do patógeno são desconhecidos. Quatro regiões genômicas (LSU rRNA, mtSSU, MCM7 e ITS) foram usadas em estudos de reconstrução filogenética que suportam a classificação de *M. ulei* na família Mycosphaerellaceae s. str., ordem Capnodiales, em Ascomycota, proximamente relacionado às espécies de *Mycosphaerella*. Da mesma forma o seu anamorfo *Fusicladium heveae* é melhor acomodado em *Pseudocercospora* s. str. A partir desta perspectiva evolutiva, propomos um modelo de ciclo de vida que inicia com espermogônias em folhas próximas à maturidade levando a ascósporos maduros em pseudotécios dentro dos estromas. A variabilidade genética do patógeno em plantações comerciais de seringueiras foi analisada em grande e pequena escalas, utilizando 17 locos microssatélites. Quinze populações locais da Amazônia e de regiões

produtoras no Brasil foram analisadas em larga escala. Constatou-se alta diversidade gênica e associação aleatória de alelos. Algumas populações geograficamente distantes foram geneticamente relacionadas. Isolamento por distância foi evidente apenas para as populações da região amazônica. Os padrões espaciais da variação genética de *M. ulei* são o resultado de fluxo gênico, provavelmente, afetado por fatores antrópicos e deriva genética por falha de conectividade entre os seringais. O estudo em pequena escala foi feito para caracterizar a variabilidade intraespecífica da população do patógeno a partir de isolados coletados de clones de seringueira suscetíveis e resistentes. A análise bayesiana de agrupamento revelou agrupamento de isolados de acordo com o nível de resistência sugerindo que seleção desempenha um papel importante na evolução do *M. ulei*. Além disso, houve diferenciação genética elevada entre populações simpátricas do patógeno em hospedeiros suscetíveis e parcialmente resistentes. A especificidade a hospedeiros parece atuar como barreira eficiente ao fluxo gênico.

ABSTRACT

HORA JÚNIOR, Braz Tavares, D.Sc., Universidade Federal de Viçosa, August, 2012. **Molecular phylogeny and population genetics of *Microcyclus ulei*, causal agent of the South American leaf blight of *Hevea brasiliensis*** Adviser: Eduardo Seiti Gomide Mizubuti. Co-advisers: Luiz Antonio Maffia and Robert Weingart Barreto.

South American leaf blight (SALB) caused by *Microcyclus ulei* prevented the development of large-scale *Hevea* spp. monoculture areas in the Amazon region and consequently led to failure of natural rubber production in managed landscapes in Tropical America. Planting of clones with partial resistance to SALB is the best option to manage the disease. Although SALB has been known since the beginning of the 20th century, basic aspects of its phylogenetic classification and the genetic structure of the pathogen population are unknown. Four genomic regions (LSU rRNA, mtSSU, MCM7 and ITS) were used for reconstructing the molecular phylogeny. The results of these analyses support the classification of *M. ulei* in the family Mycosphaerellaceae s. str, order Capnodiales, in the Ascomycota, closely related to *Mycosphaerella*-like species. Similarly, the phylogeny of its anamorph, *Fusicladium heveae*, placed this species in a different order and it was better accommodated in *Pseudocercospora* s. str. From this evolutionary perspective, we propose a model of the life cycle of the pathogen starting with spermogonia in the near-mature leaf stage and leading to mature ascospores in pseudothecia within stromata. Genetic variability of the pathogen in large and small scale commercial rubber tree plantations was analyzed using 17 microsatellite markers. Fifteen local populations from the

Amazon and the main producing areas in Brazil were analyzed in large scale. The pathogen has high gene diversity and random association of alleles across loci. Some geographically distant populations were genetically related. Isolation by distance was evident only for populations from Amazon region. The spatial patterns of the genetic variation of *M. ulei* are the result of gene flow probably affected by anthropogenic factors and genetic drift enhanced by the lack of connectivity between rubber plantations from the eastern and western regions of Brazil. A small scale study was conducted to characterize the intraspecific variability of the pathogen population associated with susceptible and resistant rubber tree clones. Bayesian clustering analyses revealed that isolates could be grouped according to the clone resistance level suggesting that directional selection plays a role in shaping the evolution of *M. ulei*. Additionally, it was observed a high genetic differentiation between sympatric populations from susceptible and partially resistant clones. In areas where susceptible and partially resistant clones are cultivated in close proximity, clone specificity probably acts as an efficient barrier to gene flow.

INTRODUÇÃO GERAL

A borracha natural é uma *commodity* de alto valor e é a matéria-prima de produtos essenciais e amplamente utilizados em diversos setores. Fatores físico-químicos como elasticidade, plasticidade, resistência ao uso, isolamento e impermeabilidade a líquidos e gases, fazem da borracha natural um produto insubstituível em muitas aplicações (LIEBEREI, 2007). Consequentemente, existe uma demanda crescente por esta *commodity*. As maiores plantações de seringueiras encontram-se instaladas no sudeste da Ásia e 92% da produção mundial de borracha natural mundial provêm da Tailândia, Indonésia, Malásia, Vietnã, Índia e China. Em 2010, o Brasil produziu aproximadamente 220.000 toneladas, o que corresponde a 2% da produção mundial, e importou 260.000 toneladas para atender sua demanda interna (FAO, 2012; MDIC, 2012).

A previsão é que a demanda mundial em 2020 seja de aproximadamente 16,4 milhões de toneladas contra os 10,4 milhões de 2011 (IRSG, 2012). Comparado a outros países, o Brasil possui áreas extensas, propícias ao plantio de seringueiras, mas o mal das folhas, doença causada pelo fungo *Microcyclus ulei* (Henn.) von Arx (Ascomycota) está presente em todas as regiões onde a seringueira é cultivada (GASPAROTTO et al., 1997).

As epidemias do mal das folhas foram e continuam sendo responsáveis pela baixa produção de borracha natural em áreas úmidas da América do Sul além de reduzirem a longevidade dos seringais implantados com clones suscetíveis. Em condições ambientais favoráveis, a doença

induz a desfolha e até a morte de clones suscetíveis (HOLLIDAY, 1970; CHEE e HOLLIDAY, 1986; LIEBEREI, 2007).

As estratégias de controle disponíveis são limitadas e a utilização de variedades resistentes é a melhor opção para o manejo da doença (GARCIA et al., 2004). Plantios de clones resistentes e produtivos possibilitam o cultivo de seringueiras em zonas de ocorrência endêmica da doença. A resistência ao mal das folhas da seringueira é o objetivo de vários programas de melhoramento na América Tropical, África e Ásia, mas existem poucos clones comerciais disponíveis por causa da instabilidade da resistência e baixa produção dos clones resistentes selecionados. A variabilidade genética da população do patógeno é um fator que explica o comportamento variável de clones resistentes plantados em diversas áreas (PERALTA et al., 1990; GARCIA et al., 2002).

Atualmente, o enfoque dos programas de melhoramento, como por exemplo, o projeto CIRAD-Michelin-Brazil (CMB), é a obtenção de clones produtivos com resistência quantitativa. Os clones resistentes obtidos são tão produtivos quanto os clones asiáticos e têm a vantagem de poder ser usados em áreas afetadas pelo mal das folhas. Além disso, estes clones seriam interessantes para área indenes como as do sudeste asiático e África, como uma estratégia preventiva para reduzir o potencial impacto econômico da introdução do *M. ulei* (GARCIA et al., 2004).

Apesar de programas de melhoramento da seringueira para resistência ao mal das folhas estarem sendo conduzidos, não existem informações sobre a variabilidade genética na população de *M*. *ulei* e a distribuição geográfica desta variabilidade no Brasil. O conhecimento da

estrutura genética da população do patógeno pode ser usado para entender o desenvolvimento da doença, prever a evolução do mal das folhas e desenvolver estratégias efetivas de melhoramento visando resistência à doença, e, principalmente, prolongar a vida útil dos materiais resistentes selecionados (MILGROOM e FRY, 1997). Estudos sobre a epidemiologia do mal das folhas foram recentemente finalizados (GUYOT et al., 2008; HONORATO JÚNIOR, 2010), mas até o momento, não existem estudos relacionados aos aspectos filogenéticos de *M*. *ulei* e sobre a genética molecular da população do patógeno. Assim, os objetivos do presente trabalho foram: i. Investigar a filogenia de *M*. *ulei* e, ii. Determinar a estrutura genética da população do patógeno no Brasil em grande e pequena escala de amostragem usando marcadores microssatélites.

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CAPÍTULO 1

Unraveling critical characteristics of the life cycle of *Microcyclus ulei*, a highly destructive fungal pathogen of the rubber tree (*Hevea brasiliensis*)

ABSTRACT

Four genomic regions (LSU rRNA, mtSSU, MCM7 and ITS) were used for reconstructing the molecular phylogeny of *Microcyclus ulei*, *a* pleomorphic fungus that causes South American leaf blight (SALB) in *Hevea* spp. Classification based on the teleomorphic and anamorphic morphological traits do not reflect proper evolutionary relationships. The molecular phylogeny of the teleomorph supports the classification of *M. ulei* in the family Mycosphaerellaceae s. str., order Capnodiales (Ascomycota), closely related to *Mycosphaerella*-like species. However, the phylogeny of the anamorph, *Fusicladium heveae*, suggests a different order and it is better accommodated in *Pseudocercospora* s. str. Based on these findings, the life cycle of the pathogen was revisited. Pathogen development was monitored in inoculated leaves under field conditions. '*F. heveae*' was observed from the young leaves (B2 stage) and decreased in the mature leaves (D stage). '*Aposphaeria ulei*' structures were seen in the near mature leaves (C/D stage) preceding *M. ulei* which was fully formed in the mature leaves. 'Pycnospores' did not germinate and were not infective. We propose a model of sexual cycle beginning with '*A. ulei*' as spermogonia in the C/D leaf stage leading to mature ascospores in pseudothecia within pronounced, erumpent ascostromata of *Mycosphaerella*-type.

Key words: South American leaf blight, *Fusicladium*, *Aposphaeria*, spermogonia, molecular phylogeny.

Taxonomic novelties: *Pseudocercospora ulei* (Kuyper), comb. nov.

INTRODUCTION

Molecular phylogenetic approaches have become key tools for the classification of plant pathogens and new features of biological and epidemiological relevance have emerged from studies that used these techniques. DNA polymorphism data provide additional characters for the classification of microorganisms, allowing for the application of the phylogenetic species concept and contributing to improving the understanding of the evolutionary relationships among species from the application of high-resolution analyses (AVISE and WOLLENBERG, 1997; TAYLOR et al. 2000; HIBBETT et al., 2007). Traditionally, the phylogenetic relationships among fungal species have been investigated mainly using morphological characters, however ultrastructural, biochemical and genomic traits can also be used and often contribute to increasing the power of the analyses (MCLAUGHLIN et al. 2009).

Recent studies provide strong evidence that molecular phylogenetic trees accurately reflect the evolutionary history of the Fungi and a consensus classification for many groups is possible (HIBBETT et al., 2007). This is particularly useful for members of an important group of ascomycete fungi, the Dothideomycetes (SCHOCH et al. 2009a; CROUS et al., 2009a; ZHANG et al., 2009a). The molecular phylogenies of several plant and human pathogenic, endophytic, saprophytic and epiphytic species of the Dothideomycetes have been studied. However, the classification and evolutionary issues related to the tropical plant pathogenic Dothideomycetes of economic and quarantine importance have not yet been investigated.

South American leaf blight (SALB) of rubber tree caused by the plant pathogenic Dothideomycete *Microcyclus ulei* (Henn.) von Arx is recognized as the most serious threat to the natural rubber industry worldwide (van BEILEN and POIRIER, 2007). Epidemics of SALB led to the failure of rubber cultivation in managed landscapes in tropical America in the early 20th century (GRANDIN, 2009) and, because of the potential serious economic consequences, there are quarantine measures for preventing SALB in the rubber in the Palaeotropics, especially in southeast Asia, a SALB-free zone (GASPAROTTO et al., 1997; LIEBEREI, 2007). The fungus infects young leaves, stems and fruit tissues of *Hevea brasiliensis* (Willd. ex A. L. Juss.) Muell.-Arg., *H. benthamiana* Muell.-Arg., *H. spruceana* (Benth.) Muell.- Arg., *H. guianensis* Aublet and *H. camporum* Ducke (CHEE and HOLLIDAY, 1986). Immature leaves are the only susceptible stage and lesions are formed 5 to 7 days after infection and the asexual spores (conidia) of the fungus are observed on immature leaves, mainly on the lower side of the leaf, shortly after. The pycnidial form and sexual structures (stromata) appear on the margins of the necrotic lesions once the leaves reach maturity (HOLLIDAY, 1970).

Although SALB has been known since the beginning of the 20th century, some critical aspects of the life cycle of the fungus remain unknown, especially the function of the pycnidial stage and its role in the development of sexual structures. Koch's postulates were performed by LANGFORD (1945) and confirmed recently by GUYOT and DOARÉ (2010). In both studies, the authors inoculated conidia and ascospores to reproduce the symptoms of SALB, but the role of pycnospores in the pathogen life cycle

remains unclear.

The fungus was first observed in 1900 in the Amazon rainforest in Peru and Brazil and was later described by HENNINGS (1904). Initially, two spore stages were described: the teleomorph as *Dothidella ulei* and a pycnidial form (Coelomycetes) as *Aposphaeria ulei*. The conidial stage (Hyphomycetes) was described by J. Kuyper in Surinam in 1912 as *Fusicladium macrosporum*. In 1917, G. Stahel observed the connection of hyphae from different fungal structures within the leaf tissue and linked the teleomorph and anamorph stages of the fungus and renamed the teleomorph as *Melanopsammopsis ulei* (HOLLIDAY, 1970). Later, MÜLLER and von ARX (1962) transferred the teleomorph to the genus *Microcyclus* and suggested a close relationship with the genus *Mycosphaerella* based on the morphology of the *Passalora*-type conidia. In *Microcyclus*, the development of erumpent stromata on living leaves (or other plant parts) from a substrate is due to the proliferation of a foot-like hypostroma and some of the *Mycosphaerella* spp. associated with pine trees have similar prominent ascostromata (EVANS, 1984).

The taxonomy of the causal agent of SALB is confusing and the lack of DNA sequence data for all three stages of the life cycle has prevented better elucidation of its classification. The genus *Microcyclus* used to be classified as belonging to the Mycosphaerellaceae (order Capnodiales) as a stromatic counterpart of the family (von ARX and MÜLLER 1975; ERICSSON and HAWKSWORTH 1993), but in 1996 it was re-classified in the Planistromellaceae (order Dothideales) (BARR 1996; LUMBSCH and HUHNDORF, 2007). After a morphological taxonomic review of the conidial

stage, the anamorphic name was changed to *Fusicladium heveae*, a species that belongs to the Venturiaceae family (order Venturiales) (ZHANG et al. 2011a). The anamorphic genus *Aposphaeria* is recognized as a member of the family Lophiostomataceae (order Pleosporales) as a well-supported group (MUGAMBI and HUHNDORF, 2009; ZHANG et al., 2011b).

Thus, there are questionable issues regarding the classification of the teleomorph and anarmorph stages at the genus, family and order levels of the causal agent of SALB. Additionally, knowledge about the evolutionary history of the pathogen and of related species is scarce. Molecular studies could help to resolve the true affinity of this fungus (EVANS, 2002; SCHUBERT et al., 2003; KIRK et al., 2008) and the objective of this study was to investigate the phylogenetic relationships of *M. ulei* using molecular approaches to shed light on its classification and on the various stages of its life cycle, particularly on the function of the intermediate pycnidial stage.

MATERIAL AND METHODS

Sampling, Isolation and DNA extraction

Leaves with typical lesions of South American leaf blight were sampled in commercial fields of rubber in Brazil. Sampling was aimed at areas with records of high incidence of SALB in the states of Acre, Rondônia, Mato Grosso, Minas Gerais, Espírito Santo and Bahia between 2008 and 2010 (Figure 01 and Table 01). Single conidia were transferred from fungal structures formed on lesions to culture media, using a sterilized fine-needle under a dissecting microscope. Monosporic cultures of *F. heveae* were grown on potato sucrose agar (PSA) containing sucrose (30mM), potassium phosphate monobasic (14.7mM), in double distilled water to 1000 ml (pH 5.0 ± 0.2) supplemented with cysteine (10000 ppm), tryptophan (2500 ppm), threonine (2500 ppm) and chloramphenicol (50µg/mL), for 20 days at 24 ±1ºC in the dark (CARLOS MATTOS, personal communication). Isolates were cultivated on M4 culture medium (JUNQUEIRA et al., 1984) in the dark for 2 months at 24 ± 1 ºC. Pycnidial stromata of *A. ulei* and ascostromata of *M. ulei* were excised from one lesion of an infected leaf with a sterilized razor blade. Each structure was examined under the microscope to check for possible contamination by other fungi and these stromata were transferred to a microtube (1.5 mL). The procedure was repeated from another lesion on the same leaf. This method was chosen in order to avoid contamination of the sample with other fungal species and plant material. In order to break up the melanised cell walls, the microtubes containing fungal material (mycelium, pycnidia or ascostromata) were placed in liquid nitrogen and

macerated using a micropestle. DNA extraction was carried out following standard cetyltrimethyl ammonium bromide extraction procedures (DOYLE and DOYLE, 1990).

DNA phylogeny

All phylogenetic analyses were performed using molecular loci of nuclear ribosomal as the first 600 bp at the 5' end of the 28S rRNA gene (LSU) using primers LR1 and LR4 (O'DONNELL 1992) and the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, and the second ITS region (ITS2) using primers ITS1 and ITS4 (WHITE et al., 1990).; mitochondrial ribosomal gene as the 650 bp internal region of the mtSSU-rDNA using primers NMS1 and NMS2 (LI et al.,1994); and the single-copy protein-coding MCM7 genes using the primers Mcm7-709for and Mcm7-1384rev (AGUILETA et al., 2008; SCHMITT et al., 2009; RAJA et al., 2011).

The polymerase chain reaction (PCR) reaction was done with a mixture containing 20 g of DNA, 0.2 µM of each primer and 1X of DreamTaq™ DNA Polymerase Master mix as described by the manufacturer (Thermo Fisher Scientific). PCR cycles were carried out in a PTC100 thermal cycler (MJResearch, Incline Village, NV) and consisted of a 5min denaturation step at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC for LSU, mtSSU and ITS primers or 57 ºC for MCM7 primers and 1min at 72 ºC with a final extension of 10min at 72 ºC. PCR products were visualized by ultra- violet fluorescence following 1% agarose gel electrophoresis in 1X TBE buffer and GelRed™ (Biotium) staining. Single-band products were purified using the E.Z.N.A cycle-pure kit (OMEGA Bio-tek). DNA

concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The same primers used for PCR amplification were used for the sequencing reactions using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare) according to the manufacturer's recommendations. The purified PCR products were sequenced using a MegaBACE^{TM} 1000 DNA Sequencing System (GE Healthcare). Sequences were manually edited with The Staden Package, ver. 1.6.0 (STADEN, 1996) to generate a consensus sequence. Additional sequences used in the analyses were obtained from GenBank and Fungal Genomics Portal of Joint Genome Institute (GRIGORIEV et al., 2011) (Table S1). Sequences were aligned with the Muscle® v. 3.6 software (EDGAR, 2004) implemented in the Mega 5.0 program (TAMURA et al. 2007). The final data matrices comprised of the LSU dataset with 62 taxa with 551 unambiguously aligned characters, mtSSU dataset with 43 taxa and 621 aligned characters, MCM7 dataset with 37 taxa and 468 aligned characters and the ITS dataset with 45 taxa and 462 aligned characters.

The software Modeltest v.3.7 (POSADA and CRANDALL, 1998) was used to determine the best-fit model of evolution for Maximum Likelihood (ML) analyses and model selection was based on the Akaike Information Criterion. The ML analyses were carried out for each of the four datasets using RAxML (STAMATAKIS, 2006) employing GTR+I+G model of evolution and bootstrap support was obtained by running 1000 pseudo replicates (STAMATAKIS et al., 2008). Bayesian analysis was conducted with MrBayes v.3.1.2 (RONQUIST and HUELSENBECK, 2003) on the same aligned datasets after MrModeltest v.2.2 (NYLANDER, 2004) was used to determine

the GTR+I+G as the best nucleotide substitution model. The Markov Chain Monte Carlo (MCMC) analysis used four chains that started with a heating parameter of 0.2 from a random tree topology and lasted 50 million generations. Trees were saved each 100 generations, resulting in 500,000 saved trees. Burn-in was set at 12,500,000 generations after which the likelihood values were stationary, leaving 375,000 trees from which the 50 % majority rule consensus trees and posterior probabilities were calculated. Quality of mixing and convergence to the stationary distribution were assessed from three independent runs using Tracer v1.5 (RAMBAUT and DRUMMOND, 2007).

Assessments of the pleomorphic development of *Microcyclus ulei* under natural conditions.

At the Michelin Plantation of Bahia (Brazil), 93 leaves at the B2 developmental stage (HALLÉ and MARTIN, 1968) of 8 rubber trees of the RO38 clone were tagged with a label and observations were made until maturity (stage D), from December 15, 2011 to February 24, 2012. A total of 1,353 assessments were made during the experiment. Scoring of sporulation in lesions naturally infected was performed at every four days using a 1–6 scale for sporulation intensity of the anamorph (*Fusicladium*) adapted from JUNQUEIRA et al. (1988), where 1 = necrotic non-sporulating lesions, 2 = chlorotic non-sporulating lesions, 3 = slight sporulation on lower side of the leaflets, 4 = moderate sporulation on lower side of the leaflets, 5 = high sporulation on lower side of the leaflets, and 6 = high sporulation on both sides of the leaflets. Pycnidial and ascostromata density was assessed at the

same time interval using a 0–4 scale where 0 = no stroma, 1 = 1–5 stromata per leaflet, 2 = 6–15 stromata per leaflet, 3 = 16–50 stromata per leaflet, and 4 = more than 50 stromata per leaflet.

Test of infectivity and germination of pycnosporos of *Microcyclus ulei* under controlled conditions

Suspension of pycnospores was obtained from pycnidia formed in C/D leaves of the RO38 rubber clone. There were no conidia or ascospores. The lower surface of three young leaves from the Fx 3864 rubber tree clone were spray-inoculated with an inoculum suspension of pycnospores or conidia separately using a HS Airbrush Complete set (Paasche Airbrush company) in an inoculation chamber with a temperature maintained at 24°C, relative humidity superior to 85%, artificial daylight of 2.000 lux and 12 h photoperiod. The inoculum suspension was made of $2x10^5$ spore/ml of water plus Tween 80 at 0.05%. Water with 0.05% Tween 80 was used as a negative control. Sporulation was scored after 12 days on all inoculated leaves. The suspensions of pycnospores and conidia were incubated in the dark at 25 ºC on water agar PSA supplemented as described above. Germination assessments were conducted at 6, 12, 24 and 120 h of incubation at 24 ±1ºC. The experiment was conducted twice.

RESULTS

Phylogeny

Analyses using maximum likelihood and bayesian methods with Dothideomycetes members resulted in phylogenies with similar topologies. Strongly supported clades confirmed that the holomorph *M. ulei* belongs to the family Mycosphaerellaceae s. str., order Capnodiales (Figure 02).

The RAxML search of the partial LSU alignment yielded a most likely tree (Figure 02) with a log likelihood value of -4021.9. The alignment of the 62 OTUs had 551 sites including alignment gaps, of which 203 sites were parsimony-informative, 32 were variable and parsimony-uninformative, and 311 were constant. Members of the Lophiostomataceae (Pleosporales), among them *Aposphaeria populina*, and species of the genus *Fusicladium* (Venturiaceae, Venturiales) formed well-supported monophyletic groups, while the anamorphs *A. ulei* and *F. heveae* were grouped together in the *Pseudocercospora* s. str. clade of the Mycosphaerellaceae with *Mycosphaerella pyri* as the nearest relative.

The dataset for the mtSSU sequences had 43 taxa and 621 characters (202 sites parsimony-informative and 35 singletons) and the log likelihood value of the most likely tree was -3620.4 (Figure 03). In this analysis, the holomorph *M. ulei* had as nearest relatives members of the genus *Pseudocercospora*. The phylogeny reconstructed with the partial sequence of the MCM7 region (Figure 04) (log likelihood of -6817.7) was based on a dataset with 468 characters (244 parsimony-informative sites and 256 variables sites) and corroborated that the holomorph *M*. *ulei* is closely related to *Mycosphaerella musicola* and *M. fijiensis*, within the *Pseudocercospora*

clade. The Bayesian analysis of LSU, mtSSU, MCM7 and ITS are shown in Supplementary Figures (S1 to S4).

Function of intermediate pycnidial stage in the life cycle of *Microcyclus ulei*.

F. heveae was found in 7.0% of the leaves in the B2 stage, 100% in B2/C, C and C/D; and 38.8% in D leaves (Figure 05; Table S2). Most commonly, the occurrence of *F. heveae* was recorded after January 19th. *A. ulei* first emerged from the upper side of infected leaves in the C/D stage in December 26th and it was found in 41.8% of the leaves in this stage and in 97.4% in D stage leaves. Ascostromata arose on January 12th and were found only in the upper side of D leaves in 62.6% of the observations. Only during the period of January 12th to January 19th, the three types of pathogen structures were concomitantly observed.

The main weather descriptors during the course of the experiment (72 days) were: average maximum temperature 29 ºC and average minimum of 22 ºC, the average relative humidity during the day and at night were 72% and 94%, respectively. Total (cumulative) rainfall was 267 mm.

The suspension of pycnospores did not cause lesions (Figure S4A), but the pathogen sign was visible with infection from conidia after 12 days of inoculation (Figure S4B). The pycnospores did not germinate in *in vitro* assays, while conidia germination started at 6 h of incubation (data not shown).

DISCUSSION

DNA sequences of the three stages of the life cycle of *M. ulei* collected in a wide geographic area in Brazil confirmed the anamorph-teleomorph connection of this fungal species, but the current classification of the pathogen in the Planistromellaceae (BARR 1996; LUMBSCH and HUHNDORF, 2007) was not supported by any of the phylogenies. The analysis conducted in the present study based on nuclear and mitochondrial ribosomal rDNA as well as protein-coding gene support the classification of *M. ulei* in the family Mycosphaerellaceae s. str., in the order Capnodiales of the phylum Ascomycota. Mycosphaerellaceae is a well-supported family within the Capnodiales with *Mycosphaerella punctiformis* as the type species (VERKLEY et al. 2004; CROUS et al., 2009a). DNA sequence data of *M. punctiformis* were included in all our analyses, corroborating the classification of the pathogen at the family level and revealing a close relationship of *M. ulei* with *Mycosphaerella*. Currently, robust multi-gene phylogenetic analysis support *Mycosphaerella* as a polyphyletic group (CROUS et al., 2007a, 2009b, SCHOCH et al., 2009a, b), suggesting that *Mycosphaerella* s. l. should be subdivided to reflect natural groups (genera) as defined by their anamorphs since *Mycosphaerella* s. str. is today restricted to species with *Ramularia* anamorphs (CROUS et al. 2009b).

The classification of *Microcyclus* as a *Mycosphaerella*-like organism was discussed previously (CANNON, 1995). The *Microcyclus* genus has ellipsoidal, hyaline, 1-septate ascospore in clavate, bitunicate asci typical of the genus *Mycosphaerella* Johanson (EVANS, 2002). The development of

stromatic tissue in *Microcyclus* appears to be the only character that contributes to its separation from the genus *Mycosphaerella* (CANNON, 1995). However, some *Mycosphaerella*-like species have similar erumpent ascostromata (EVANS, 1984, 2002). As already demonstrated for *Mycosphaerella*, the genus *Microcyclus*, as currently circumscribed, may also be polyphyletic, given the variety of anamorphs associated with the assigned species (CANNON, 1995).

The conidial stage of *M. ulei, F. heveae,* is morphologically indistinguishable from the anamorphic stage of *Venturia* (SCHUBERT et al. 2003). Other species of *Fusicladium* s. l. (including *Spilocaea* and *Pollaccia*) and the teleomorphic genus *Venturia* form a monophyletic group in Venturiaceae (BECK et al., 2005; ZHANG, et al. 2011a), but some *Fusicladium*-like species belong to Sympoventuriaceae (ZHANG, et al. 2011a). Although *F. heveae* has already been treated as a species of the *Passalora*-type and, therefore, a cercosporoid fungus (CROUS and BRAUN, 2003), our molecular data demonstrate that this species is better accommodated in *Pseudocercospora* s. str., within the Clade 16 of CROUS et al. (2009b) and Clade 14 in CROUS et al. (2012) in which the type species, *Pseudocercospora vitis*, resides (Figure 02 and S1). Whilst in the Mycosphaerellaceae, many anamorph forms evolved in more than one clade and represent different genera (CROUS, et al., 2007a; CROUS et al., 2009b) the anamorph convergence of '*F. heveae*' is at the order level. This observation is also evident for '*A. ulei*' (Figure 02).

Pseudocercospora s. str. is a well-defined genus in Mycosphaerellaceae based on both DNA sequence and morphology data

(CROUS et al., 2009b; CROUS et al., 2012). *Pseudocercospora* is now recognized as a holomorphic genus, and several species have *Mycosphaerella*-like teleomorphs. As observed for '*F. heveae*' in the present study, when the phylogenetic species concept is applied to other species of the genera *Paracercospora*, *Cercostigmina*, *Phaeoisariopsis* and *Stigmina,* they are reduced to synonymy with the genus *Pseudocercospora* (STEWART et al. 1999, CROUS et al. 2001a, BRAUN and HILL 2002, CROUS et al. 2006a).

Recognizing that the anamorphic form of causal agent of SALB belongs to *Pseudocercospora* s. st. has important implications, at least the fact that it is a close relative of well-known highly destructive plant pathogens that affect important crops worldwide (CROUS et al., 2012). This fact allows for the adoption of a comparative epidemiology and genomics approaches, using better studied pathogenic species such as *P. fijiensis*, the causal agent of the black leaf streak disease of banana (CHURCHILL, 2011).

From an evolutionary perspective, and based on the molecular phylogenetic evidence, the life cycle of *M. ulei* was re-examined with special attention to its intermediate pycnidial stage. After the proof of the anamorph-teleomorph connection provided by molecular phylogenetic analyses, the development of the pathogen in the rubber leaf was monitored under environmental conditions favorable to the development of SALB. Physiological data indicate that leaves at the B and C stages act as sinks with high respiration rates and are almost lignin-free (LIEBEREI, 2007). The conidial lesions are the first signal of the disease and fertile pycnidia occur three to five weeks later on mature or near-mature diseased leaves

(LANGFORD, 1945; CHEE and HOLLIDAY, 1986). Ascocarps become mature at about four to six weeks and the formation of ascospores is correlated with effete pycnidia (HOLLIDAY, 1970). In the present study, mature pycnidia were seen after three weeks on the upper surface of leaves in the C/D and D stages in the area occupied by the conidial (*Pseudocercospora*) lesions. After five to six weeks, the stromata become more visible and increase in number and size.

In contrast to a previous finding (HOLLIDAY, 1970), but in accordance to another study (LANGFORD, 1945), our results confirm that the pycnospores do not germinate *in vitro* and fail to infect rubber leaves. These observations corroborate the hypothesis that the supposedly erumpent pycnidia structures are in fact spermogonia and they are involved in the initial stages of the sexual cycle (LANGFORD, 1945; CHEE and HOLLIDAY, 1986; EVANS, 2002). Generally, fungi in the Mycosphaerellaceae produce spermogonia and the spermatia are thought to act as male sexual elements because of their small size, inability to germinate and infect the host plant, and pseudothecial development beginning from protoascomata concurrently (or a few days later) with spermogonia and the two structures are similar in size and shape (HIGGINS, 1920; SNYDER, 1946; DRING, 1961; INMAN et al., 1991). The production of spermatia is reported to occur in *P. fijiensis* (LIBERATO et al., 2009), and they are considered as male gametes, formed in spermogonia, which usually develop from the substomatal chambers before the formation of pseudothecia, although the cytological details of spermatization and ascospore development have not yet been fully described (CHURCHILL, 2011). Similar fertilization stage could take place in the *M*. *ulei*.

Based on a highly likely phylogenetic position of the pathogen, a revised version of the life cycle of this pleomorphic fungus that causes SALB of the rubber tree is presented (Figure 06). Only one anamorphic stage, which belongs to *Pseudocercospora* s. str., is present and it infects young leaves being responsible for the secondary cycles of the disease in the field. The sexual cycle begins with spermogonial developing in the leaf (from stage C/D) and finishes with mature ascospores in pseudothecia within pronounced, erumpent ascostromata of the *Mycosphaerella*-type.

TAXONOMY

Pseudocercospora ulei (Kuyper) Authors to be defined later **comb. nov.**

≡ *Fusicladium heveae* K. Schub. & U. Braun, in Crous & Braun, *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodiversity Series 1: 481 (2003)

≡ *Fusicladium macrosporum* Kuyper, Recueil Trav. Bot. Néerl. 8: 374 (1911).

= ?*Passalora heveae* Massee (nom. nud.) sensu Stahel, Bull. Dept. Landb. Suriname 34: 34 (1917).

Teleomorph: *Microcyclus ulei* (Henn.) Arx, in Müller & Arx, Beitr. Kryptogamenfl. Schweiz 11: 373 (1962).

≡ *Dothidella ulei* Henn., Hedwigia 43(4): 254 (1904).

≡ *Melanopsammopsis ulei* (Henn.) Stahel, Bull. Dep. Landb. Suriname 34: 1-111 (1917)

Note:

This species is better accommodated in *Pseudocercospora* (Mycosphaerellaceae, Capnodiales) than in *Fusicladium* (Venturiaceae, Venturiales) (SCHUBERT et al. 2003), based on phylogenetic analysis.

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FIGURE LEGENDS

Figure 01. Sampled areas (dots) located in the rubber producing regions in Brazil. States from which samples were collected are shaded.

Figure 02. Maximum likelihood phylogeny of *Microcyclus ulei* based on the first 551 bp at the 5' end of the 28S rRNA gene (LSU) of 62 taxa using RAxML (log likelihood = -4021.879653). Thickened branches indicate significant Bayesian posterior probabilities ≥ 95%; numbers refer to RAxML bootstrap support values ≥ 70% based on 1000 replicates. The scale bar shows 0.08 expected changes per site. The tree was rooted to Pleosporales members.

Figure 03. Maximum likelihood phylogeny of *Microcyclus ulei* based on the mtSSU-rDNA (621pb) of 43 taxa using RAxML (log likelihood = -3620.369300). Thickened branches indicate significant Bayesian posterior probabilities ≥ 95%; numbers refer to RAxML bootstrap support values ≥ 70% based on 1000 replicates. The scale bar shows 0.2 expected changes per site. The tree was rooted to Venturiales

Figure 04. Maximum likelihood phylogeny of *Microcyclus ulei* based on the gene MCM7 (468 bp) of 37 taxa using RAxML (log likelihood = -6817.743065). Thickened branches indicate significant Bayesian posterior probabilities ≥ 95%; numbers refer to RAxML bootstrap support values ≥ 70% based on 1000 replicates. The scale bar shows 0.3 expected changes per site. The tree was rooted to *Aspergillus nidulans* and *Aspergillus carbonarius*.

Figure 05. Pleomorphic development of the life cycle of *Microcyclus ulei*. Weighted average based on score of sporulation and pycnidial and stromata

density during the leaf development of RO38 clone rubber in field conditions in the period of December 15, 2011 to February 24, 2012.

Figure 06. A proposed model of the life cycle of *Microcyclus ulei*. Arrows indicate of leaf development; Dotted arrows indicate the enlargement of fungal structure; and dashed arrows the young leaves as prevalent to start of infection.

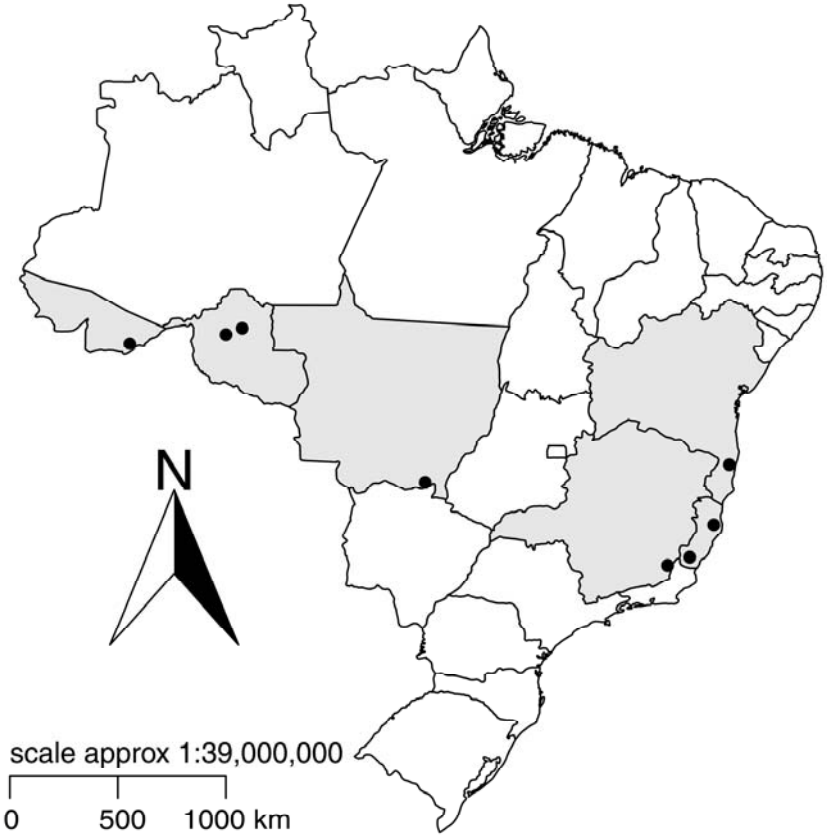

Figure 01. Hora Júnior et al

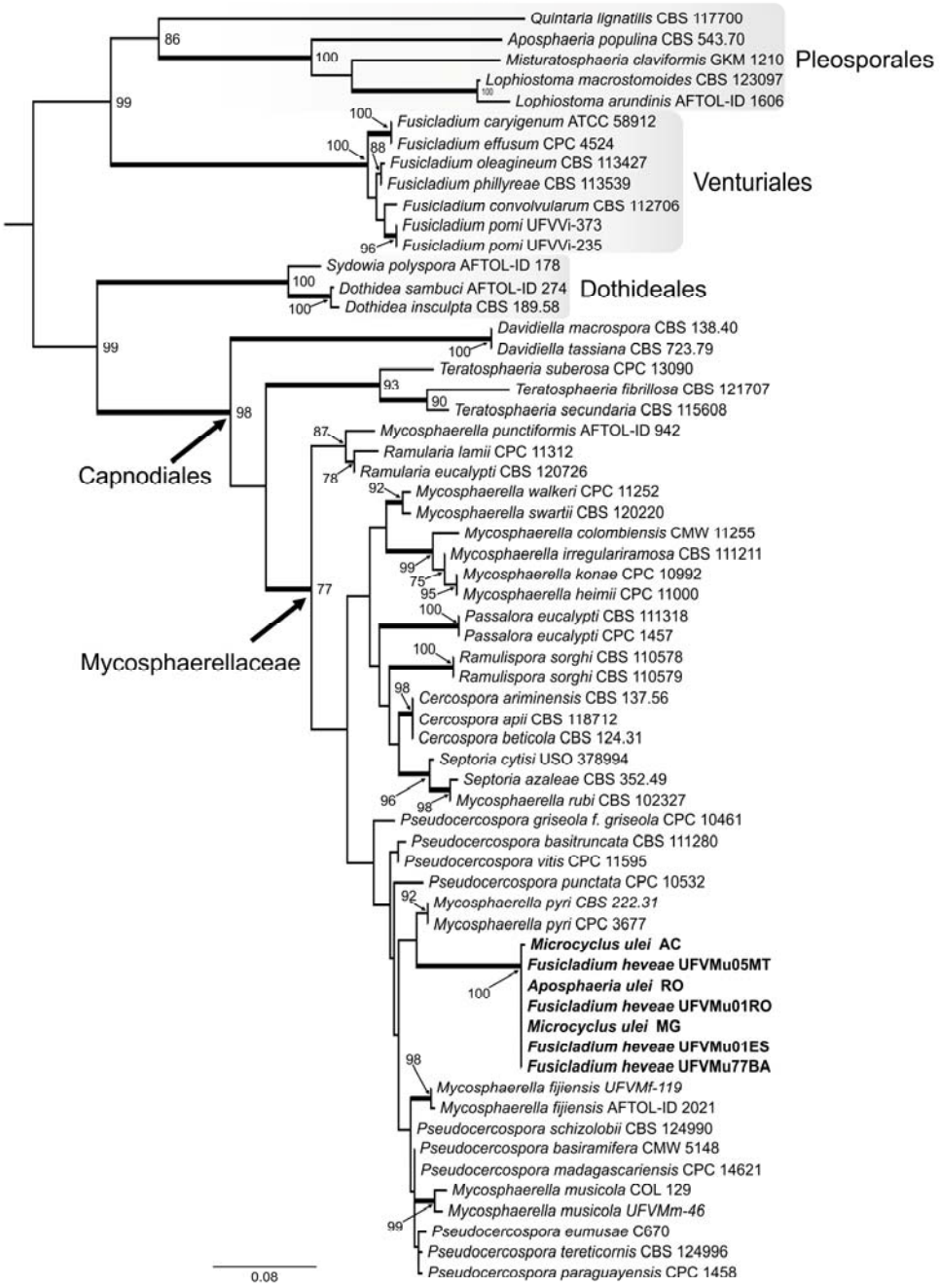

Figure 02. Hora Júnior et al

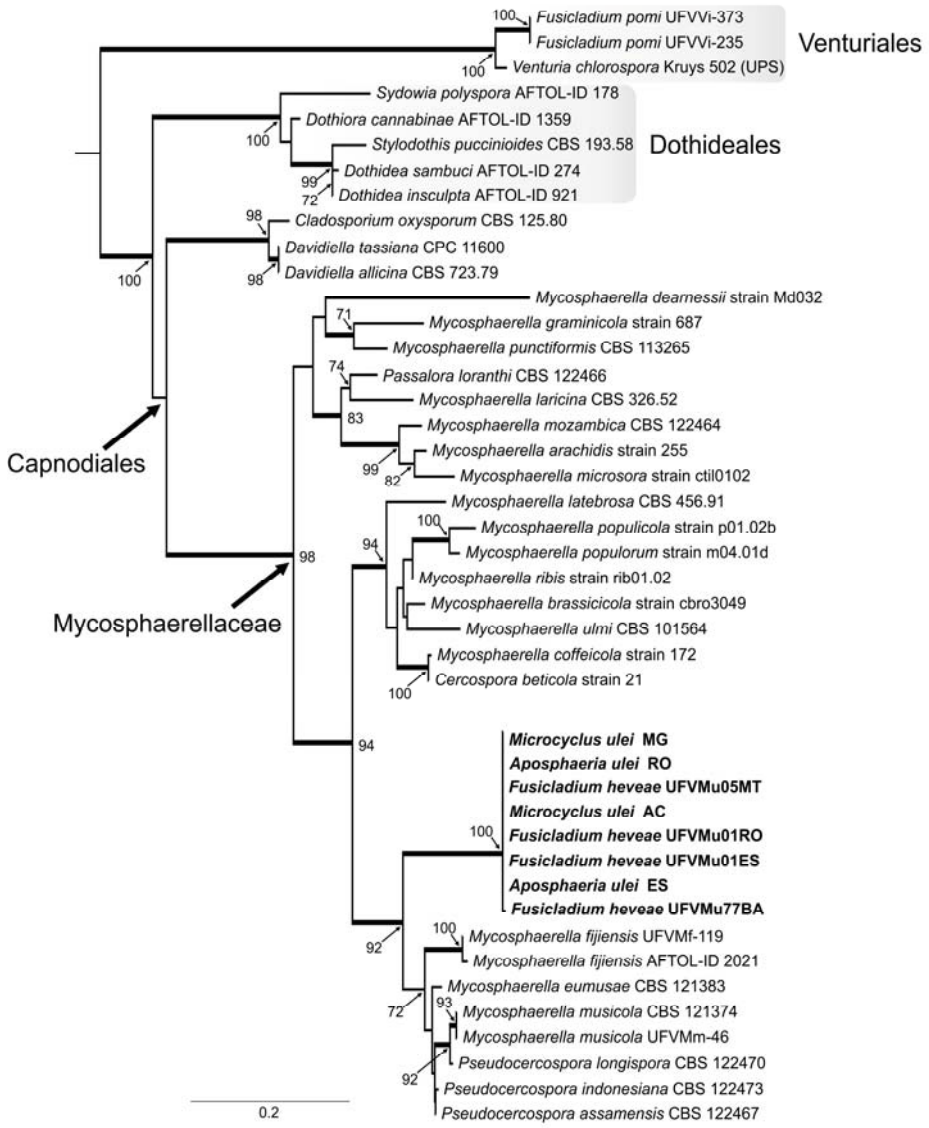

Figure 03. Hora Júnior et al

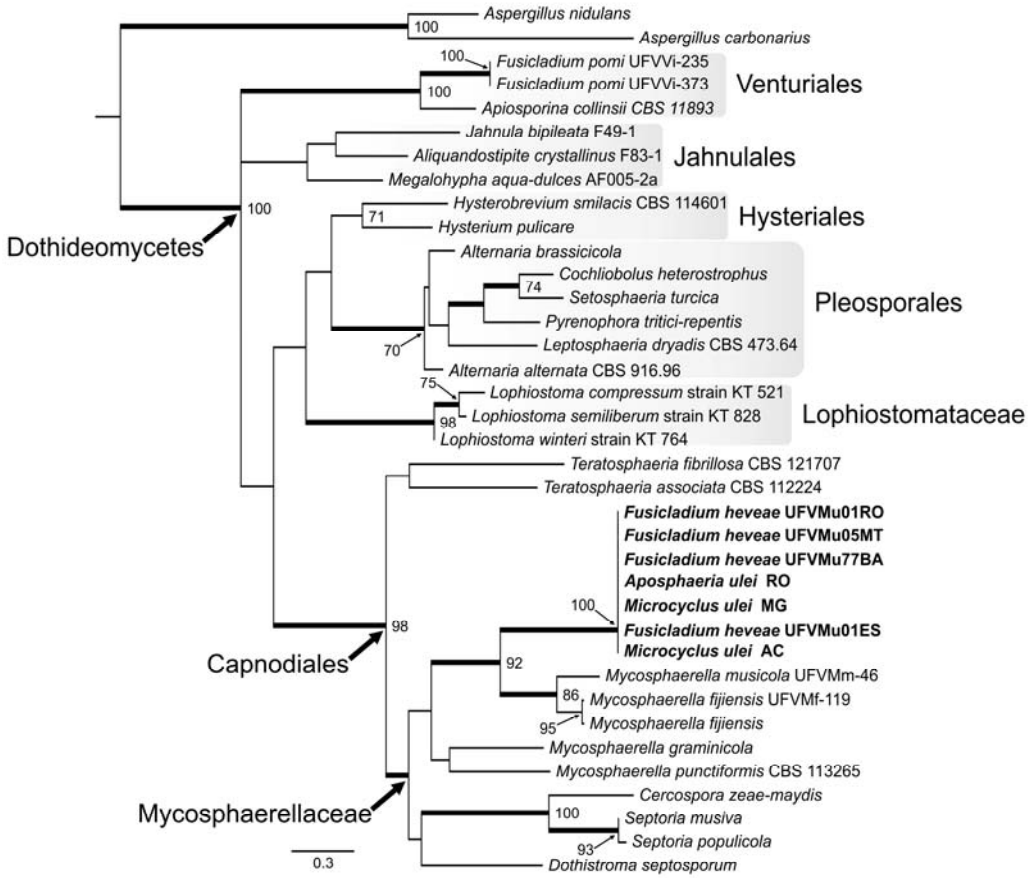

Figure 04. Hora Júnior et al

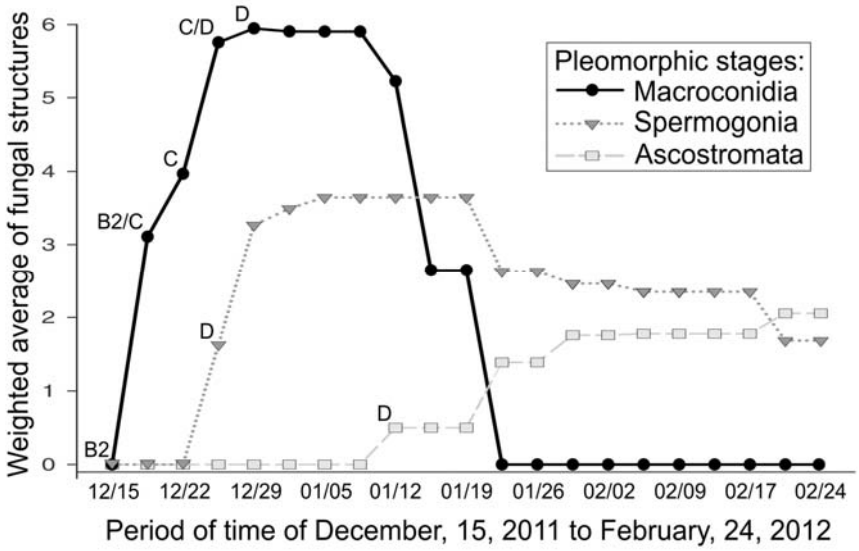

Figure 05. Hora Júnior et al

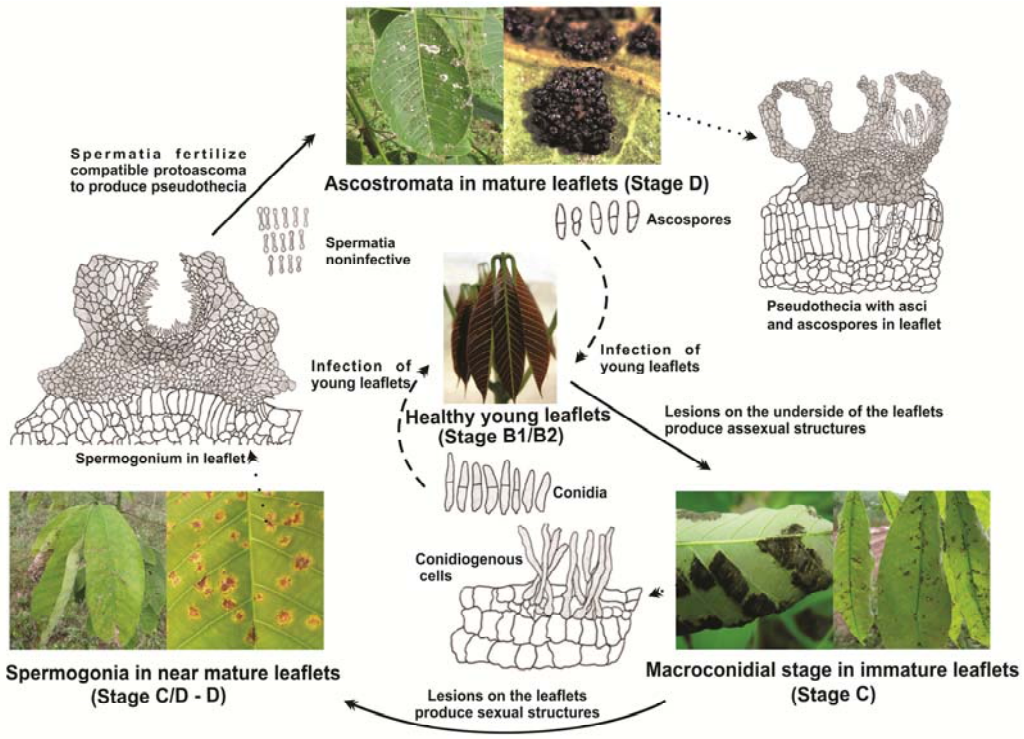

Figure 06. Hora Júnior et al

Table 01. Origin of the *Microcyclus ulei* isolates.

Isolate	Location	Coordinates (Lat/Lon)	Collector	Sampling Date
Aposphaeria ulei ES	Cachoeiro do Itapemirim-ES	-20.752609/-41.290358	B.T. Hora Júnior	2010
Aposphaeria ulei RO	Ariquemes-RO	-9.913333/-63.040833	J. Honorato Júnior	2010
Fusicladium heveae UFVMu77BA	Porto Seguro-BA	-16.378001/-39.366433	B.T. Hora Júnior	2008
Fusicladium heveae UFVMu01ES	Sooretama-ES	-19.220087/-40.121414	B.T. Hora Júnior	2009
Fusicladium heveae UFVMu05MT	Itiquira-MT	-17.208889/-54.150000	B.T. Hora Júnior	2009
Fusicladium heveae UFVMu01RO	Buritis-RO	-10.211944/-63.828889	J. Honorato Júnior	2010
Microcyclus ulei AC	Xapuri-AC	-10.651944/-68.503889	B.T. Hora Júnior	2010
Microcyclus ulei MG	Oratórios-MG	-20.415833/-42.908889	B.T. Hora Júnior	2010

SUPPORTING INFORMATION

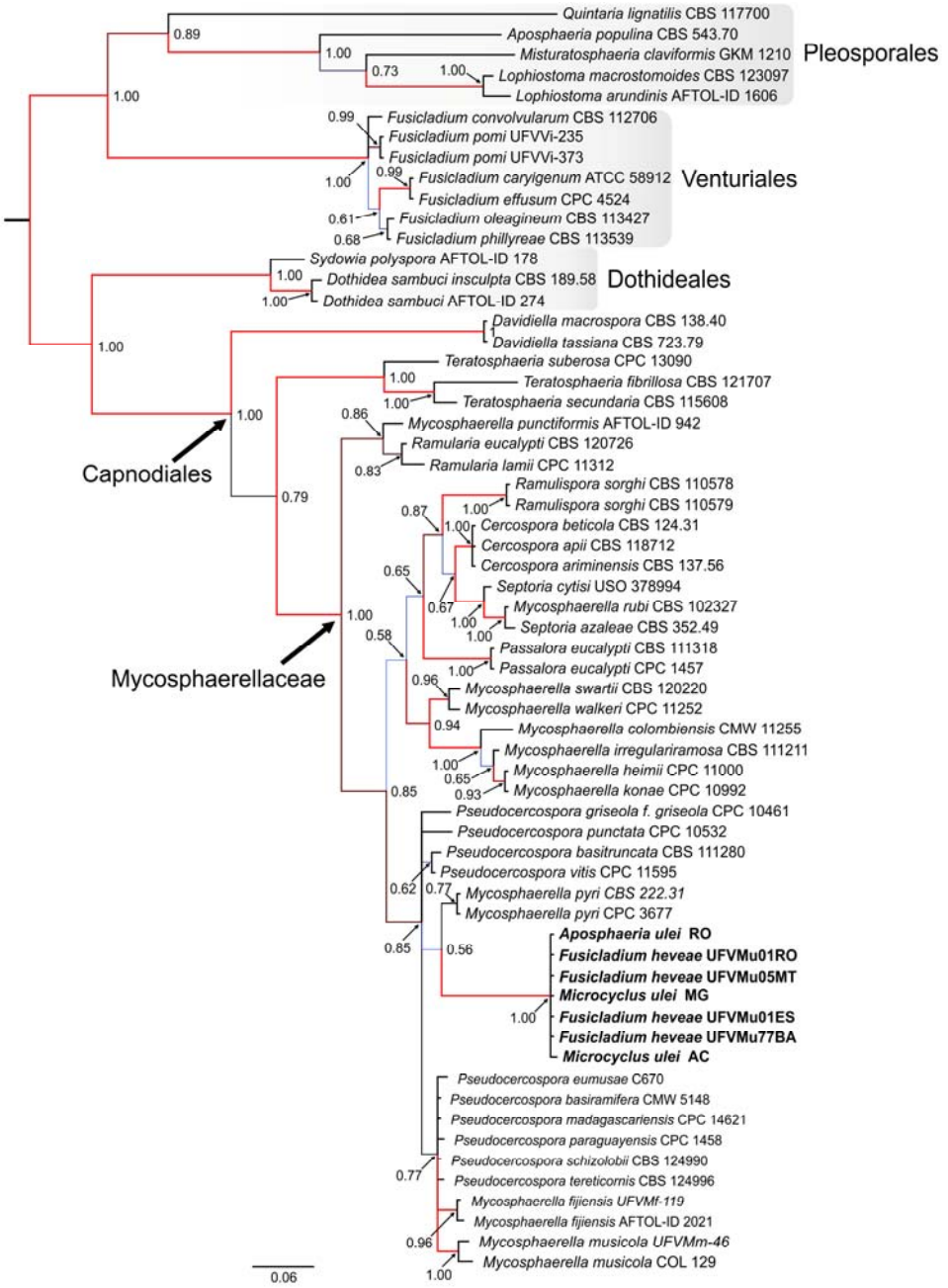

Figure S1. The 50% majority rule tree of 375,000 trees obtained from a Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the LSU sequence alignment. Bayesian posterior probabilities are given at the nodes and values ≥ 0.5 and ≥ 0.7 are shown above as blue and red lines, respectively. Scale bar shows 0.06 expected changes per site. The tree was rooted with members of Pleosporales. Bayesian posterior probability values ≥ 0.5 are shown above

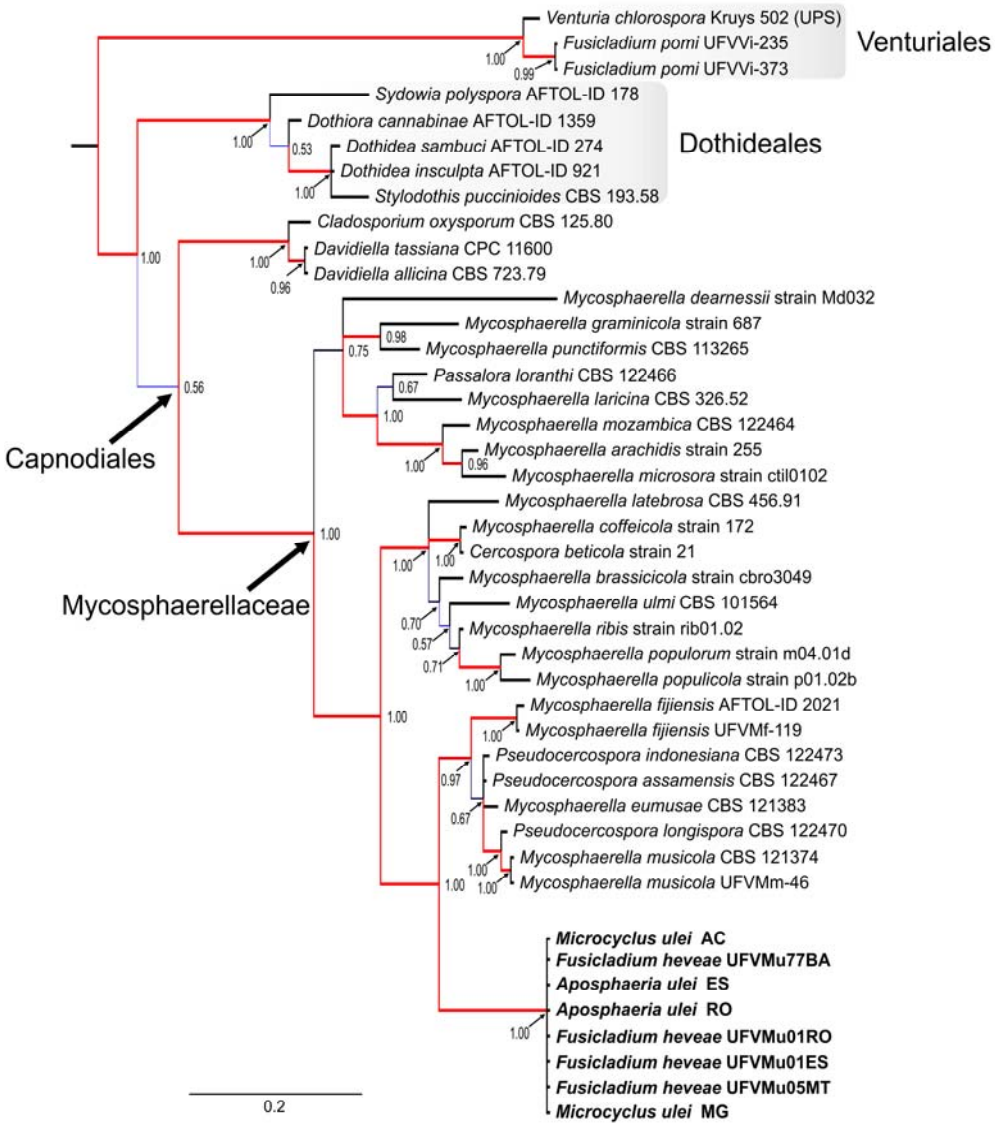

Figure S2. The 50% majority rule tree of 375,000 trees obtained from a Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the mtSSU sequence alignment. Bayesian posterior probabilities are given at the nodes and values ≥ 0.5 and ≥ 0.7 are shown above as blue and red lines, respectively. Scale bar shows 0.2 expected changes per site. The tree was rooted with members of Venturiales.

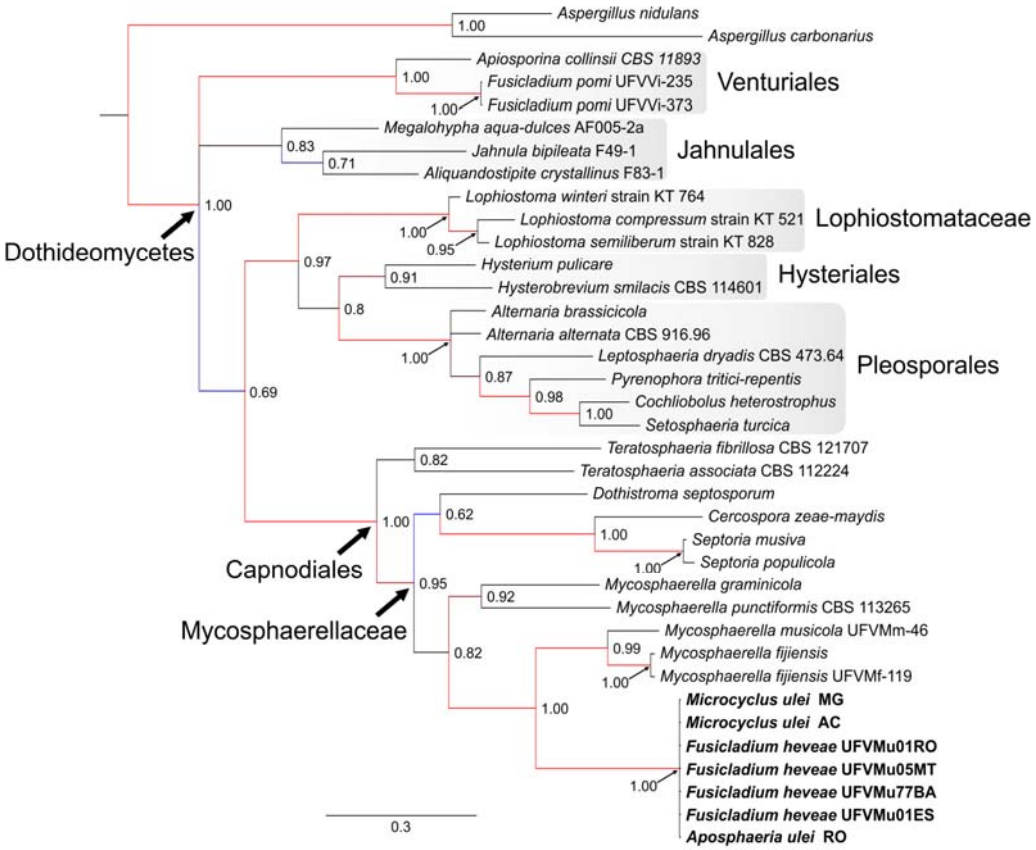

Figure S3. The 50% majority rule tree of 375,000 trees obtained from a Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the MCM7 sequence alignment. Bayesian posterior probabilities are given at the nodes and values ≥ 0.5 and ≥ 0.7 are shown above as blue and red lines, respectively. Scale bar shows 0.3 expected changes per site. The tree was rooted with *Aspergillus nidulans* and *A. carbonarius*.

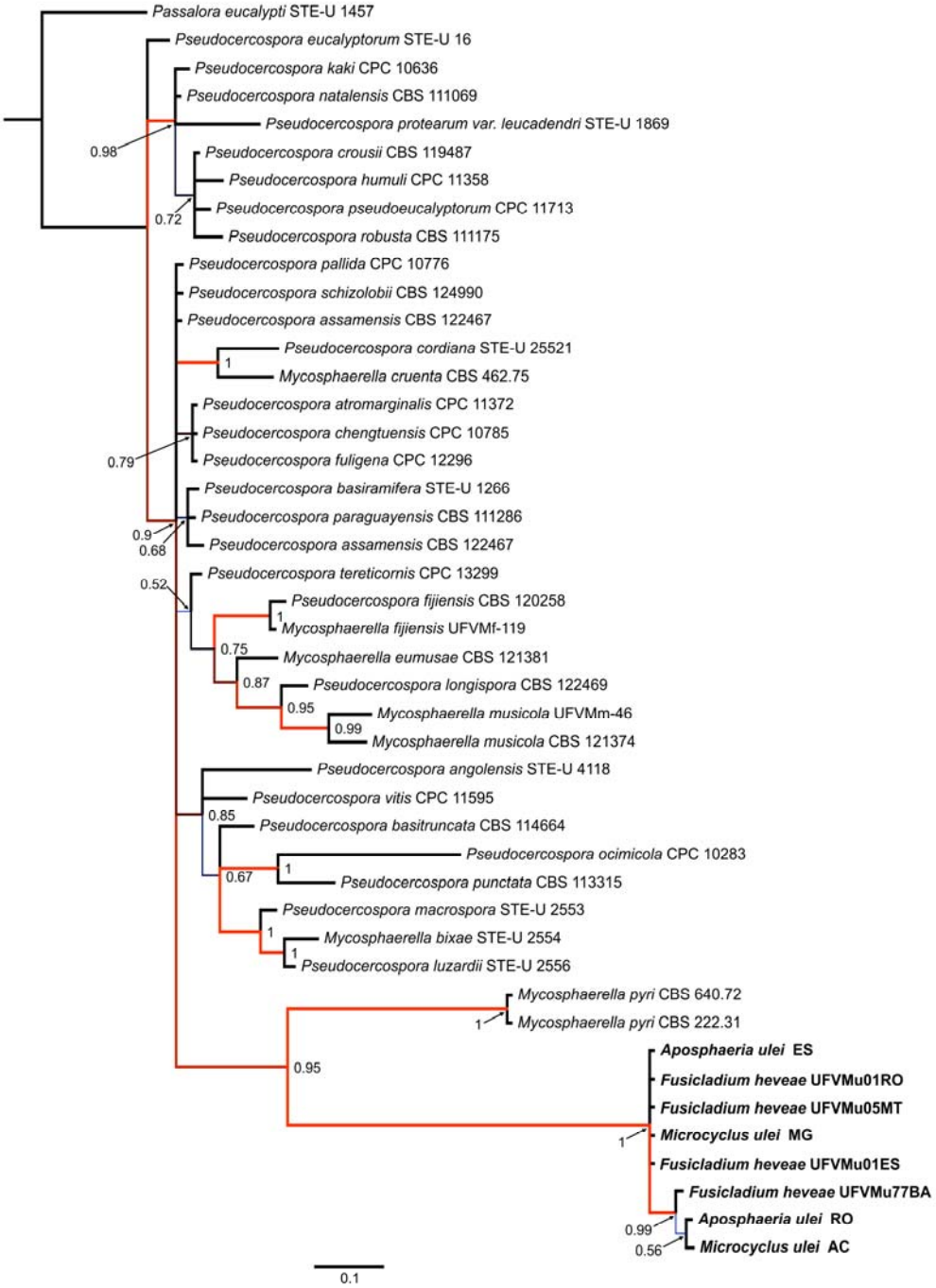

Figure S4. The 50% majority rule tree of 375,000 trees obtained from a Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the ITS sequence alignment. . Bayesian posterior probabilities are given at the nodes and values ≥ 0.5 and ≥ 0.7 are shown above as blue and red lines, respectively. Scale bar shows 0.3 expected changes per site. The tree was rooted with *Passalora eucalypti*.

Figure S4. Reactions of rubber tree leaves to inoculations of pycnospores of *Aposphaeria ulei* (A) and conidia of *Fusicladium heveae* (B). The assessments were made 12 days after inoculation. Water with 0.05% Tween 80 was used as negative control (C).

Table S1. GenBank accession numbers of sequences derived from strains used in the phylogenetic analysis

Species	Strain	LSU	mtSSU	MCM7	ITS	References
Aliquandostipite crystallinus	F83-1			JN672963		RAJA et al., 2011
Alternaria alternata	CBS 916.96			JN672967		RAJA et al., 2011
Alternaria brassicicola				Genome[a]		GRIGORIEV et al., 2011
Apiosporina collinsii	CBS 11893			JN672970		RAJA et al., 2011
Aposphaeria populina	CBS 543.70	EU754130				de GRUYTER, et al., 2009
Aspergillus carbonarius				Genome		GRIGORIEV et al., 2011
Aspergillus nidulans				Genome		GRIGORIEV et al., 2011
Cercospora apii	CBS 118712	GQ852583				CROUS et al., 2009b
Cercospora ariminensis	CBS 137.56	JF700933				QUAEDVLIEG et al., 2011
Cercospora beticola	CBS 124.31, 21	JF700934	DQ028504			QUAEDVLIEG et al., 2011; FEAU et al., 2006
Cercospora zeae-maydis				Genome		GRIGORIEV et al., 2011
Cladosporium oxysporum	CBS 125.80		DQ089646			
Cochliobolus heterostrophus				Genome		GRIGORIEV et al., 2011
Davidiella allicina				x		This study
Davidiella macrospora	CBS 138.40	EU167591				SIMON et al., 2009
Davidiella tassiana	CBS 723.79, CPC 11600	EU167558	EU514455			SIMON et al., 2009; ARZANLOU et al., 2008
Dothidea insculpta	CBS 189.58, AFTOL-ID 921	DQ247802	FJ190602			SCHOCH et al., 2006b; AFTOL[*]
Dothidea sambuci	AFTOL-ID 274	AY544681	AY544739			AFTOL[b]
Dothiora cannabinae	AFTOL-ID 178		FJ190636			AFTOL
Dothistroma septosporum				Genome		GRIGORIEV et al., 2011
Fusicladium caryigenum	ATCC 58912	AY352591				
Fusicladium convolvularum	CBS 112706	EU035428				CROUS et al., 2007b
Fusicladium effusum	CPC 4524	EU035429				CROUS et al., 2007b
Fusicladium oleagineum	CBS 113427	EU035434				CROUS et al., 2007b
Fusicladium phillyreae	CBS 113539	EU035435				CROUS et al., 2007b

Table S1. Continued

Species	Strain	LSU	mtSSU	MCM7	ITS	References
Fusicladium pomi	UFVVi 235	x	x	x		This study
Fusicladium pomi	UFVVi 373	x	x	x		This study
Hysterium pulicare				Genome		GRIGORIEV et al., 2011
Hysterobrevium smilacis	CBS 114601			JN672992		RAJA et al., 2011
Jahnula bipileata	F49-1			JQ036219		
Letosphaeria dryadis	CBS 473.64			JN673010		RAJA et al., 2011
Lophiostoma arundinis	AFTOL-ID 1606	DQ782384				SCHOCH et al., 2006a
Lophiostoma compressum	KT 521			JN993408		SCHOCH et al., 2012
Lophiostoma macrostomoides	CBS 123097	FJ795439				ZHANG et al., 2009b
Lophiostoma semiliberum	KT 828			JN993400		SCHOCH et al., 2012
Lophiostoma winteri	KT 764			JN993401		SCHOCH et al., 2012
Megalohypha aqua-dulces	AF005-2a			JN673011		RAJA et al., 2011
Misturatosphaeria claviformis	GKM 1210	GU385212				MUGAMBI and HUHNDORF, 2009
Mycosphaerella arachidis	255		DQ028570			FEAU et al., 2006
Mycosphaerella bixae	STE-U 2554				AF362056	CROUS et al., 2001a
Mycosphaerella brassicicola	cbro 3049		DQ028481			FEAU et al., 2006
Mycosphaerella caffeicola	172		DQ028515			FEAU et al., 2006
Mycosphaerella colombiensis	CMW 11255	DQ204745				HUNTER et al., 2006a
Mycosphaerella cruenta	CBS 462.75				AF362065	CROUS et al., 2001a
Mycosphaerella dearnessi	Md032		DQ028485			FEAU et al., 2006
Mycosphaerella eumusae	C670; CBS 121383; CBS 121381	AY875400	EU514416		EU514244	ARZANLOU et al., 2008
Mycosphaerella fijiensis	UFVMf119	x	x	x	x	This study
Mycosphaerella fijiensis	AFTOL-ID 2021; CBS 120258	DQ678098	FJ190656	Genome	EU514248	SCHOCH et al., 2006b,. 2009b GRIGORIEV et al., 2011; ARZANLOU et al., 2008
Mycosphaerella graminicola	strain 687		DQ028492	Genome		FEAU et al., 2006; GRIGORIEV et al., 2011

Table S1. Continued

Species	Strain	LSU	mtSSU	MCM7	ITS	References
Mycosphaerella heimii	CPC 11000	GQ852605				CROUS et al., 2009b
Mycosphaerella irregulariramosa	CBS 111211	GQ852609				CROUS et al., 2009b
Mycosphaerella konae	CPC 10992	GQ852611				CROUS et al., 2009b
Mycosphaerella laricina	CBS 326.52		DQ028496			FEAU et al., 2006
Mycosphaerella latebrosa	CBS 456.91		DQ028569			FEAU et al., 2006
Mycosphaerella microsora	ctil0102		DQ028568			FEAU et al., 2006
Mycosphaerella mozambica	CBS 122464		EU514424			ARZANLOU et al., 2008
Mycosphaerella musicola	COL 129, CBS 121374	AY875380	EU514438		EU514274	ARZANLOU et al., 2008
Mycosphaerella musicola	UFVMm46	x	x	x	x	This study
Mycosphaerella populicola	p01.02b		DQ028553			FEAU et al., 2006
Mycosphaerella populorum	m04.01d		DQ028562			FEAU et al., 2006
Mycosphaerella punctiformis	AFTOL-ID 942, CBS 113265	DQ470968	FJ190611	JN673014		SPATAFORA et al., 2006; AFTOL; RAJA et al., 2011
Mycosphaerella pyri	CBS 222.31; CPC 3677; CBS 640.72	GQ852617 GU214495			AY152591 AY152592	CROUS et al., 2009a, 2009b; VERKLEY et al., 2004;
Mycosphaerella ribis	rib01.02		DQ028512			FEAU et al., 2006
Mycosphaerella rubi	CBS 102327	JF700958				QUAEDVLIEG et al., 2011
Mycosphaerella swartii	CBS 120220	DQ923536				CROUS et al., 2009c
Mycosphaerella ulmi	CBS 101564		DQ028539			FEAU et al., 2006
Mycosphaerella walkeri	CPC 11252	GQ852679				CROUS et al., 2009b
Passalora eucalypti	CBS 111318; CPC 1457; STE-U 1457	GQ852620 GU214458			AF309617	CROUS et al., 2001b, 2009b;
Passalora loranthi	CBS 122466		EU514444			ARZANLOU et al., 2008
Pseudocercospora angolensis	STE-U 4118				AY260063	
Pseudocercospora assamensis	CBS 122467		EU514445		GU269656	ARZANLOU et al., 2008; CROUS et al., 2012
Pseudocercospora atromarginalis	CPC 11372				GU269657	CROUS et al., 2012

Table S1. Continued

Species	Strain	LSU	mtSSU	MCM7	ITS	References
Pseudocercospora basiramifera	CMW 5148; STE-U-1266	DQ204761			AF309595	HUNTER et al., 2006a; CROUS et al., 2001b
Pseudocercospora basitruncata	CBS 111280; CBS 114664	DQ204760			GU269662	HUNTER et al., 2006; CROUS et al., 2012
Pseudocercospora chengtuensis	CPC 10785				GU214672	CROUS et al., 2009a
Pseudocercospora cordiana	STE-U 2552				AF362054	CROUS et al., 2001a
Pseudocercospora crousii	CBS 119487				GU269686	CROUS et al., 2012
Pseudocercospora eucalyptorum	STE-U-16				AF309598	CROUS et al., 2001b
Pseudocercospora fuligena	CPC 12296				GU269711	CROUS et al., 2012
Pseudocercospora griseola f. griseola	CPC 10461	GU348997				SCHOCH et al., 2009a
Pseudocercospora humuli	CPC 11358				GU269723	CROUS et al., 2012
Pseudocercospora indonesiana	CBS 122473		EU514446			ARZANLOU et al., 2008
Pseudocercospora kaki	CPC 10636				GU269728	CROUS et al., 2012
Pseudocercospora longispora	CBS 122470; CBS 122469		EU514448		EU514284	ARZANLOU et al., 2008
Pseudocercospora luzardii	STE-U 2556				AF362057	CROUS et al., 2001a
Pseudocercospora macrospora	STE-U 2553				AF362055	CROUS et al., 2001a
Pseudocercospora madagascariensis	CPC 14621	GQ852651				CROUS et al., 2009b
Pseudocercospora natalensis	CBS 111069				DQ303077	CROUS et al., 2006
Pseudocercospora ocimicola	CPC 10283				GU214678	CROUS et al., 2009a
Pseudocercospora pallida	CPC 10776				GU269758	CROUS et al., 2012
Pseudocercospora paraguayensis	CPC 1458; CBS 111286	GU214479			DQ267602	CROUS et al., 2009a; HUNTER et al., 2006b
Pseudocercospora protearum var.leucadendri	STE-U 1869				AY260089	TAYLOR et al., 2003
Pseudocercospora pseudoeucalyptorum	CPC 11713				GU269811	CROUS et al., 2012
Pseudocercospora punctata	CPC 10532; CBS 113315	GQ852645				CROUS et al., 2009b; SIMON et al., 2009
Pseudocercospora robusta	CBS 111175				DQ303081	Crous et al., 2006
Pseudocercospora schizolobii	CBS 124990; CBS 124990	GQ852646			GQ852765	CROUS et al., 2009b., 2009c
Pseudocercospora tereticornis	CBS 124996; CPC 13299	GQ852647			GQ852770	CROUS et al., 2009b, 2009c

Table S1. Continued

Species	Strain	LSU	mtSSU	MCM7	ITS	References
Pseudocercospora vitis	CPC 11595	GU214483			GU269829	CROUS et al., 2009a, 2012
Pyrenophora tritici-repentis				Genome		GRIGORIEV et al., 2011
Quintaria lignatilis	CBS 117700	GU301865				SCHOCH et al., 2009a
Ramularia eucalypti	CBS 120726	JF700949				QUAEDVLIEG et al., 2011
Ramularia lamii	CPC 11312	JF700950				QUAEDVLIEG et al., 2011
Ramulispora sorghi	CBS 110578; CBS 110579	GQ852653 GQ852654				CROUS et al., 2009b
Septoria azaleae	CBS 352.49	JF700952				QUAEDVLIEG et al., 2011
Septoria cytisi	USO 378994	JF700954				QUAEDVLIEG et al., 2011
Septoria musiva				Genome		GRIGORIEV et al., 2011
Septoria populicola				Genome		GRIGORIEV et al., 2011
Setosphaeria turcica				Genome		GRIGORIEV et al., 2011
Stylodothis puccinioides	CBS 193.58		AF346428			LINDEMUTH et al., 2001
Sydowia polyspora	AFTOL-ID 178	AY544675	AY544756			AFTOL
Teratosphaeria associata	CBS 112224			JN673021		RAJA et al., 2011
Teratosphaeria fibrillosa	CBS 121707	GU323213		x		SCHOCH et al., 2009a; This study
Teratosphaeria secundaria	CBS 115608	JF700962				QUAEDVLIEG et al., 2011
Teratosphaeria suberosa	CPC 13090	JF700963				QUAEDVLIEG et al., 2011
Venturia chlorospora	Kruys 502 (UPS)		DQ384084			KRUYS et al., 2006

[a] Genome portal of the Department of Energy Joint Genome Institute. [b] AFTOL: Assembling the Fungal Tree of Life (AFTOL) project (Lutzoni et al. 2004).

Table S2: Percentages of scores of sporulation intensity, presence of spermogonia and ascostromata obtained by RO38 *Hevea* clone when infected by *Microcyclus ulei* under natural conditions of inoculation in the period of December 15, 2011 to February 24, 2012.

		Sporulation intensity (%)						Presence of spermogonia (%)				Presence of stromata (%)			
Date	Leaf Stage	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
15/12/2011	B2														
19/12/2011	B2		2.15	2.15	3.22										
	B2/C	1.07	21.50	16.12	20.43										
	C		7.52	5.37	20.43										
22/12/2011	B2/C			3.22	3.22										
	C			2.15	66.66	1.07									
	C/D				22.58	1.07									
26/12/2011	C/D		1.16		4.65	3.48	51.16	2.32	3.48	4.65	13.95				
	D					6.97	32.55		13.95		13.95				
29/12/2011	C/D						22.66		4	9.33	9.33				
	D		1.33				76		6.66	21.33	44				
02/01/2012	D				4.76		95.23		9.52	31.74	58.73				
05/01/2012	D				4.83		95.16		6.45	22.58	70.96				
09/01/2012	D				4.83		95.16		6.45	22.58	70.96				
12/01/2012	D				4.83		83.87		6.45	22.58	70.96	50			
16/01/2012	D				3.22		41.93		6.45	22.58	70.96	50			
19/01/2012	D				3.22		41.93		6.45	22.58	70.96	50			
23/01/2012	D							5.55	38.88	42.59	12.96	62.96	25.92	5.55	1.85
26/01/2012	D							5.55	38.88	42.59	12.96	62.96	25.92	5.55	1.85
30/01/2012	D							11.11	37.03	46.29	5.55	46.29	33.33	18.51	1.85
02/02/2012	D							11.11	37.03	46.29	5.55	46.29	33.33	18.51	1.85
06/02/2012	D							11.11	48.14	35.18	5.55	44.44	35.18	18.51	1.85
09/02/2012	D							11.11	48.14	35.18	5.55	44.44	35.18	18.51	1.85
13/02/2012	D							11.11	48.14	35.18	5.55	44.44	35.18	18.51	1.85
17/02/2012	D							11.11	48.14	35.18	5.55	44.44	35.18	18.51	1.85
20/02/2012	D							24.07	50	14.81		27.77	42.59	25.92	3.70
24/02/2012	D							24.07	50	14.81		27.77	42.59	25.92	3.70

CAPÍTULO 2

Spatial pattern and population biology of *Microcyclus ulei* in Hevea agricultural landscapes in Brazil

ABSTRACT

An extensive sampling was conducted to assess the genetic variability of Microcyclus ulei, the causal agent of South American leaf blight (SALB), in commercial plantations of rubber trees. Fifteen local populations from the Amazon and from the main producing areas in Brazil were analyzed using 17 microsatellite loci. A total of 263 multilocus genotypes were identified among 264 isolates sampled and some populations showed random association of alleles across loci. There was high gene diversity in populations collected from commercial plantations, even in areas distant from the Amazon region, the putative center of origin of the pathogen. The occurrence of a bottleneck effect was detected in some populations from the Amazon region and from the Brazilian coast. Some geographically distant populations were genetically related and four main clusters of local populations were detected. Isolation by distance was evident only for populations in the Amazon region. The spatial pattern of the genetic variation of *M. ulei* is the result of historical gene flow probably affected by anthropogenic action and genetic drift caused by the fail of connectivity between rubber tree plantations from the East and West regions of Brazil. *M. ulei* is a pathogen with high evolutionary potential and the genetic variability of the populations can favor adaptation to different biophysical environment. Breeding rubber tree clones with partial resistance is likely to be the most successful control measure and resistant plant material should be cultivated even in areas of marginal environmental conditions for SALB epidemics.

Key words: South American leaf blight, *Hevea brasiliensis,* population genetics, tropical plant pathogens, molecular epidemiology.

INTRODUCTION

Fungal plant pathogens have long been known to constitute a widespread threat to many cultivated species. Plant disease epidemics caused by fungi and fungal-like organisms, the Oomycetes, have altered the course of human history (FISHER et al., 2012). South American leaf blight (SALB) is the fungal disease responsible for the failure of rubber tree (*Hevea* sp.) cultivation in managed landscapes in tropical America (DEAN, 1989). Currently, it is considered a biological threat to many countries in Southeast Asia and in Africa where natural rubber production is a vital economic activity providing 92% of the world production. These regions are SALB-free zones planted with susceptible clones and distant from the Amazon, the center of origin of both the host and the pathogen (CHEE, 1980; CHEE, 1985; MADDEN and WHEELIS, 2003). The introduction of the pathogen into these regions would have profound consequences for the natural rubber industry worldwide.

The disease is caused by the fungus *Microcyclus ulei* (Henn.) von Arx (Ascomycota, Dothideomycetes, Mycosphaerellaceae), which under favorable environmental conditions can induce defoliation, dieback of the canopy and death of susceptible clones (HOLLIDAY, 1970; CHEE and HOLLIDAY, 1986; GASPAROTTO et al., 1997; LIEBEREI, 2007). It is the most destructive disease of the rubber tree and one of the most important yield limiting factor of natural rubber production in the Neotropics (LIEBEREI, 2007).

Natural rubber is a high-value commodity and projections by the

specialized market point to an increasing demand for this product in the coming years. The physicochemical properties of natural rubber such as elasticity, plasticity, resistance to wear, isolator of electricity and imperviousness to liquids and gases, make it an irreplaceable product (LIEBEREI, 2007; MOONEY, 2009). Additionally, for several purposes, synthetic rubber from petroleum-compounds does not constitute a suitable alternative. The greatest challenge for natural rubber production is to meet the increasing global demand for the product. In 2010, 10.5 million tons of natural rubber were produced worldwide and until 2020 the demand is projected to rise to 16.4 million tons (IRSG, 2012). Despite the governmental programs PROBOR I, II and III, conducted between 1966 and 1989, aimed at increasing the supply of natural rubber, Brazil produced only about 220,000 tons (~ 2% of the total world production) and imported 260,000 tons to support its internal market demand (FAO, 2012; MDIC, 2012). On the other hand, Brazil has the largest area available for planting rubber trees and the constantly growing demand for natural rubber provides a good opportunity for the expansion of rubber tree cultivation in the country which was the world's largest natural rubber producer in the first two decades of the 20th century. The disease ruined commercial rubber tree plantations in the Amazon and the lack of effective control measures prevented the development of initiatives aimed at establishing the self-sufficient pneumatic industry in Brazil (DEAN, 1989; GRANDIN, 2009).

The strategies available to control SALB are limited. The deployment of resistant clones is considered to be the best option to manage the disease. Planting high-yielding resistant clones would allow cultivation of rubber trees

in zones where the pathogen occurs. Breeding programs for SALB resistance were developed in the tropical Americas, Africa and Asia, but few clones were recommended for planting in areas affected by SALB because of the instability of the resistance and low yields. Most likely, selection for qualitative resistance, and the lack of knowledge about the genetic variability of the pathogen population, were the main reasons for the reduced efficacy of the resistant clones planted in several areas (JUNQUEIRA et al., 1990; PERALTA et al., 1990; GARCIA et al., 2002a, LE GUEN et al., 2011).

The breeding program of a perennial species cultivated in large acreage will be successful only if it takes the genetic structure of the pathogen population into account. The key features of the pathogen population that should be determined are: (i) the amount of genetic variability; (ii) the geographic distribution of pathogen genotypes; (iii) the degree of representativeness of the genetic variation of the pathogen population at a given screening site; (iv) the temporal dynamics of the frequency of genotypes in an area and the factors that contribute to this process (PEEVER et al., 2000).

Currently, high-yielding resistant clones are being developed in a breeding program denominated CIRAD-Michelin-Brazil SALB resistance. The program is based on the concept of partial or incomplete resistance and the clones released so far are as competitive as the high-yield Asian clones and can be used to manage SALB in suitable areas of tropical Americas (RIVANO et al., 2010). Furthermore, resistant clones are also interesting as a risk reducing strategy of economic impact associated with a potential introduction of SALB in Asia and Africa (GARCIA et al., 2004; LE GUEN et al., 2011).

Despite the achievements of the program, most breeding activities were conducted without proper knowledge of the genetic variability of the pathogen population in a large scale. Selectively neutral genetic markers allow researchers to make inferences about the role of evolutionary mechanisms: mutation, gene flow, recombination, and random genetic drift, in shaping the genetic variation of populations of *M*. *ulei*. Estimating parameters related to these mechanisms is the first step for studying the population biology of plant pathogens (McDONALD, 1997).

The genetic information of the pathogen population can be useful for better understanding disease outbreaks, predicting future disease development, accessing key information on the ecology of the pathogen and its evolutionary potential, and for developing effective strategies in breeding for disease resistance (MILGROOM and FRY, 1997; MEEÛS et al., 2007). The information on population genetics of a causal agent of disease can also reveal the mode and history of spread of the pathogen (McDONALD, 1997). Studies of the epidemiology of SALB were recently concluded (GUYOT et al., 2008; HONORATO JÚNIOR, 2010) but to date, no detailed study of molecular population genetics of *M*. *ulei* has been conducted in Brazil. The aims of this study were to determine the genetic structure and to understand important aspects of the population biology of *M. ulei* in commercial rubber tree plantations in Brazil.

MATERIAL AND METHODS

Sample collection and DNA extraction

Samples were collected in commercial rubber tree plantations located in areas of high incidence of SALB in Acre, Rondônia, Mato Grosso, Bahia, Minas Gerais, Espírito Santo, Rio de Janeiro and São Paulo State, Brazil (Figure 01 and Table 01). In each field, samples of leaves with typical SALB symptoms were collected according to a W-walking pattern. Single germinated conidia in water agar were collected under the microscope and plated on potato sucrose agar (PSA) routinely used in research from Plantações Michelin da Bahia in Bahia, containing sucrose (30mM), potassium phosphate monobasic (14.7mM), pH 5.0 ± 0.2 and supplemented with cysteine (10000 ppm), tryptophan (2500 ppm), threonine (2500 ppm) and chloramphenicol (50µg/mL) for 20 days at 24 ± 1 ºC in the dark.

For DNA extraction, isolates were cultivated on M4 culture medium (JUNQUEIRA et al., 1984) in the dark for 2 months at 24 ± 1 ºC. Total DNA was extracted following standard cetyltrimethyl ammonium bromide extraction procedures (DOYLE and DOYLE, 1990) from mycelia macerated in liquid nitrogen. The DNA integrity was analyzed in agarose gel electrophoresis and the concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

Microsatellite genotyping

All individuals were genotyped using 9 microsatellite markers developed by Le GUEN et al. (2004) and 8 markers developed by the Centre

de Coopération International en Recherche Agronomique pour le Développement (CIRAD) (BENOIT BARRÈS, personal communication). The markers were combined in six multiplex panels (Table S1). We performed multiplex PCR reactions with Type-it Microsatellite PCR Kit as described by the manufacturer (Qiagen). PCR products were separated on a ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All samples were migrated together with fluorescent molecular size markers. The data were processed by GeneMarker Software V1.91 (SoftGenetics). Haplotypes were created after allele numbers were assigned to each locus of each individual. Each allele was numbered according to the number of repeat units relative to the smallest allele at each locus (LE GUEN et al., 2004).

Analysis of genetic data

Genotypic diversity at each sampling location was estimated by the Stoddard and Taylor's G index (STODDARD and TAYLOR, 1988) and bootstrapping was conducted using 1000 pseudoreplicates to construct a 95% confidence interval. The index was scaled according to sample size and rarefaction curves were used to estimate the number of expected genotypes for the smallest sample size being compared (GRÜNWALD, et al., 2003). The diversity indices and the rarefaction calculations were performed with the vegan and vegetarian packages of the R program version 2.14.0 (R Development Core Team 2011). To analyze allelic diversity for samples of variable sizes, allelic richness (Ar) and private allelic richness (pAr) were computed using the rarefaction method implemented in HP-Rare (KALINOWSKI, 2005). Gene diversity H_E was estimated following NEI

(1978), using the Genepop 4.1 software (ROUSSET, 2008). Tests for linkage disequilibrium (LD) were used to detect the nonrandom association of alleles at different loci. LD was assessed using Multilocus 1.3 (AGAPOW and BURT, 2001) with the unbiased estimate of multilocus linkage disequilibrium (r_d). Significance was assessed using 1000 randomizations of the data set to create a null distribution. The program Bottleneck (CORNUET and LUIKART, 1997) was used to detect potential signals of recent bottlenecks.

The coefficient of gene differentiation (G_{ST}) was calculated using FSTAT version 2.9.3 program (GOUDET, 1995). The genetic differentiation between populations was tested using the theta (θ) estimator of WEIR and COCKERHAM (1984), with the generation of 1000 randomized data sets as described in the program Multilocus 1.3. The relationship between genetic diversity and geographic distance was evaluated by the Mantel's test (MANTEL 1967) implemented in Arlequin version 3.11 program (EXCOFFIER et al., 2005) using 1000 random permutation for significance test.

Population subdivision was also assessed with the model-based Bayesian clustering algorithm implemented in GENELAND 3.1.4 (GUILLOT et al., 2005; GUILLOT, 2008) and Structure 2.2 packages (PRITCHARD et al., 2000; FALUSH et al., 2003). GENELAND simulations were conducted using spatial information of the populations and the uncorrelated allele frequency model. The best number of groups, K, was given by the dimension value (here the value of K) in which the Markov chain spent most iterations (GUILLOT et al., 2005). The value of K was allowed to vary from 1 to 20 in ten independent simulations. Iteration parameters were set to 1,000,000 iterations with a burning-in period of 100,000 iterations. The Structure was

used with the admixture model with correlated allele frequency and default settings. Iteration parameters were set to a burning-in period of 10,000 iterations followed by 100,000 iterations. The likelihood of the assignments [Ln P(K)] was evaluated for K varying from 1 to 20 in ten independent simulations. To assess the value of K that best explains the partitioning of the genetic data, the ΔK estimator was computed as described by EVANNO et al. (2005).

The analysis of molecular variance (AMOVA) was used to estimate the variance components due to within and among population genetic variation and for estimating the degree of population differentiation. This analysis was conducted using Arlequin version 3.11 program (EXCOFFIER et al., 2005). Two estimates of the degree of population structure were assessed in the AMOVA: i. populations were structured as defined by sampling locations, and ii. the populations were structured as defined after the analysis with Geneland.

RESULTS

The *M. ulei* population from commercial rubber tree plantations in Brazil has high genetic variability. In total, 263 multilocus genotypes were identified among 264 isolates with a number of alleles ranging from 3 to 10 (Table S1). Initially, the 264 individuals analyzed were grouped in 15 local populations. The Acre state sub-populations AC1 (Low Acre State region) and AC2 (High Acre State region) and the Rondônia state sub-population RO1 (Midwest of Rondônia State) were comprised of, at least, 2 areas (Table 01). The same multilocus genotype was found in the population AC1.

A high percentage of polymorphic loci were estimated in the local populations. The polymorphism of 10 populations (AC1, MT1, MT2, SP1, RJ1, MG1, ES1, BA1, BA2 and BA3) was above 80% (Table 02). The least polymorphic population was AC2 with 64.7% whereas both BA1 and BA3 populations were completely polymorphic. The scaled Stoddart and Taylor genotypic index (G) had similar values for all populations and the genotypic richness estimated for the smallest size sample ($E(g_n)$) was 5.6 individuals for AC1 and it was maximum (6) for all other populations. Highest levels of variability were observed for MT1, where gene diversity (H_E: 0.51) and allelic richness (Ar: 2.4) were high. However, private allelic richness was not the highest value (pAr: 0.1). The 'composite populations' RO1 presented the second highest diversity (H_E: 0.49 and Ar: 2.16), but it was higher in private allelic richness (pAr: 0.21) than MT1, while AC2 tended to display the lowest amount of genetic diversity (H_E: 0.3, Ar: 1.71) with no private alleles. In general, populations with high heterozygosity (AC1, MT2, SP1, RJ1, BA2 and

BA3) had high allelic richness, while the populations AC1 and BA2 also showed high private allelic richness.

Random association of alleles among loci was detected in eight populations: AC2, RO1, MT2, MG1, MG2, ES2, ES3 and BA1. Strong gametic disequilibrium was found only in the RJ1 population. Significant signatures of bottlenecks on the distribution of allele frequencies were detected in several populations (Table 03). Notably, the populations AC2, RO1, MG2 and ES2 displayed highly significant signals.

There was evidence of genetic differentiation between the local populations. Gene differentiation estimated by the average G_{ST} was high (0.23) as was the estimated θ value (0.23; P<0.001). The 95% confidence interval for the G_{ST} estimate ranged from 0.21 to 0.26. All F_{ST} values were significant (P<0.001), except for populations ES1 and ES3 (Table 04). Mantel's tests comparing the matrix of genetic distances (F_{ST}) with the matrix of geographic distances of all populations resulted in a low, but significant, correlation coefficient (r = 0.23; P= 0.03). There was a marginally significant correlation between genetic and geographic distances only when populations from the Amazon region (AC1, AC2, RO1, MT1 and MT2) were analyzed (r = 0.6; P= 0.09).

The Bayesian clustering method used in Geneland inferred four clusters (Figure 02 and Figure S1). In all ten runs, the composite populations AC1 and AC2 and the local populations SP1, MG2, ES1, ES2 and ES3 were assigned to the first cluster (Figure 02A and E). The composite population RO1 formed the second cluster (Figure 02B and E); the local populations MT1, MT2, MG1 and RJ1 were assigned to the third cluster (Figure 02C and

E); and the local populations from Bahia State (BA1, BA2 and BA3) formed the fourth cluster (Figure 02D and E). Based on Structure, three distinct genetic groups (K=3) could be devised (Figure 03 and Figure S2). The admixture and correlated allele frequencies models in Structure defined the first cluster as comprised of the local populations AC1, AC2, MT1, SP1, MG2, ES1, ES2 and ES3; the second cluster was made of RO1, MT2, RJ1 and MG1 local populations and the third cluster was comprised of the BA1, BA2 and BA3 local populations.

The results of the AMOVA (Table 05) for the local populations showed that 76.5% of the variation was found within and 23.5% was due to variation among populations. When the AMOVA was conducted assuming the population structure inferred by the Geneland as *a priori* hypothesis the greatest amount of variation, 73.6%, was distributed within populations, 13.9% of the variation was attributed to differences between clusters and 12.5% between populations within clusters. The level of differentiation was high within population (Φ_{st}=0.26; P=0.000), but was moderate for both populations among clusters (Φ_{ct}=0.13; P=0.000) and populations within clusters (Φ_{sc}=0.14; P=0.000). Considering each cluster individually, the percentage of variation within populations was 81.8, 83.5 and 91.9% for the clusters 1 (AC1, AC2, SP1, MG2, ES1, ES2 and ES3), 3 (MT1, MT2, MG2 and RJ1) and 4 (BA1, BA2 and BA3), respectively.

DISCUSSION

The Brazilian populations of *M. ulei* in commercial plantations of *Hevea* spp. are structured, have high genotypic diversity, and there is evidence of dispersal mediated by anthropogenic actions in the spatial distribution of the pathogen genotypes. The genotypic diversity index was maximum in all sampled populations, except in population AC1. High genotypic diversity supports the idea that sexual reproduction takes place and ascospores play an important role in the development of SALB epidemic wherever rubber trees are grown in Brazil.

Sexual structures of *M*. *ulei* were observed in the early 1900s, before the first serious epidemics that led to the failure of rubber commercial plantations in the Amazon region (HENNINGS 1904a, CHEE and WASTIE, 1980). Two types of spores, conidia and ascospores, are postulated to be of epidemiological relevance. However, for a long time only conidia were initially attributed to be able to cause infection. Ascospores are usually found in small quantities and evidence for the participation of ascospores in the disease cycle was not demonstrated until 1945 when Koch's postulates were fulfilled (LANGFORD, 1945). Only recently, the disease life cycle was re-examined to investigate the role of ascospores and it was confirmed that these propagules are infective (GUYOT and DOARÉ, 2010). Indirect evidence of the importance of ascospores in SALB epidemics was also inferred by its detection in aerobiology studies conducted under field conditions throughout the year (CHEE, 1976; HONORATO JÚNIOR, 2010). The genetic evidence gathered from the current study provides additional support for the role of

ascospores in SALB epidemics in all commercial rubber trees plantations in Brazil.

In addition to ascospores and conidia, the spores (pycnospores) produced in pycnidia-like structures were initially thought to participate as an intermediate stage in the life cycle of *M*. *ulei*. However, there was no convincing evidence that pycnospores are infective. In classical heterothallic fungi, the fusion of cytoplasms, plasmogamy, must occur between two genetically distinct individuals in order for sexual reproduction to take place. To date, there is no information on the genetic regulation of mating in *M*. *ulei* and very little is known about the involvement of gametes. Although not yet fully demonstrated, the pycnospores may act as male gametes and be involved in plasmogamy (LANGFORD, 1945; CHEE and HOLLIDAY, 1986). Nevertheless, more detailed investigation on this issue is needed.

Sexual recombination seems to be common and plays an important role in the evolution of populations of *M*. *ulei*. High genotypic diversity, low frequencies of multilocus genotypes, and the presence of sexual structures (“spermatia” and ascospores) suggest that sexual reproduction is widespread. Additionally, the formal test of linkage disequilibrium revealed that eight populations showed random association of alleles across loci. Thus, there is evidence of the occurrence of recombination in all populations sampled and the importance of ascospores in the disease epidemiology. This finding must be carefully considered when establishing management strategies to control SALB epidemics.

Evidence of recombination is an important knowledge about the reproductive biology of a pathogen (MILGROOM, 1996). High gene diversity

was detected in local populations of *M*. *ulei* sampled from commercial plantations, even in areas distant from the center of origin of both host and pathogen. Populations from the Atlantic coast, BA2, BA3, SP1 and RJ1, showed as high heterozigosity as populations from the Amazon area, such as AC1, RO1, MT1 and MT2. The same observations were made for allelic richness and private allelic richness for the populations AC1, RO1 and BA2. South American leaf blight is considered innocuous in natural systems but a serious problem in monocultures (EVANS, 2002).The high genetic diversity of the pathogen population can explain, at least in part, the severe infection rate of *M*. *ulei* in genetically uniform plantings due to the density and proximity of the host.

Detailed knowledge about the geographic pathways followed by spores from their source to the new location can provide information about the history of migration, the origin of the genetic composition of the new populations, the ecological and evolutionary processes that affected the populations and the efficacy of control measures adopted to manage plant diseases (HOLDENRIEDER et al., 2004; ESTOUP and GUILLEMAUD, 2010). The role of migration in structuring *M*. *ulei* populations is supported by bayesian clustering methods based on the ancestrality information. The local populations ES1, ES2, ES3, MG2 and SP1 were genetically related to the same source population that founded populations AC1 and AC2. Similarly, local populations MG1 and RJ1 shared the same source population as MT1 and MT2. Interestingly, the local populations in Bahia state did not originate from those located in the Upper Amazon Basin (Acre, Rondônia and Mato Grosso), most likely from areas of Pará state, near Belém. In the early 20th

century the Ford Motor Company tried to establish a rubber tree breeding program in the state of Pará and released high-yielding clones of the Fx series (Ford cross series). The first rubber tree plantations in Bahia state were established with Fx clones (MEDEIROS and BAHIA, 1971), and the pathogen could have been carried with the germplasm.

Although the number of microsatellites markers used in this study were not high, enough polymorphisms were detected that support the occurrence of founder events: these events contributed to the establishment of the local populations MG2 and ES2 both located in the Southeastern Brazilian coastal area, and AC2 and RO1 in the Amazon area. The partition of “new” populations founded in the Brazilian coast from three independent sources located in the Amazon region led to genetically structured population of *M*. *ulei* in Brazil, but with no isolation by distance. The lack of continuous areas cultivated to rubber tree between the North and the Southeast regions of Brazil may have contributed to the strong degree of population structuring. These populations probably were founded by long distance dispersal (LDD) events. For plant pathogenic fungi, long distance dispersal events can either result from passive transport by wind or from human activity (BROWN and HOVMØLLER, 2002). Passive airborne LDD events have been reported to occur with rust fungi such as *Hemileia vastatrix* on *Coffea* (BOWDEN et al., 1971), *Puccinia melanocephala* on sugarcane (PURDY et al., 1985), *Melampsora larici-populina* on poplar (BARRÉS et al., 2008) and *Puccinia striiformis f. sp. tritici* on wheat (HOVMØLLER et al., 2008; WANG et al., 2010). Two hypotheses are put forward about the gene flow and founder effect of *M. ulei* to rubber trees in commercial planting: (i) inoculum dispersal

naturally occurred from foci in wild trees in Amazon rainforest; or (ii) human activities inadvertently mediated by transport of infected leaves in budded stumps or budwood (HOLLIDAY, 1970; CHEE and HOLLIDAY, 1986). The wind dispersal hypothesis seems less realistic for explaining the introduction of *M. ulei* in the Brazilian coastal region considering the spatial pattern of three independent genetic groups from the Amazonian endemic area.

The genetic structure and the spatial distribution of the local populations of *M. ulei* of the Amazon region (AC1, AC2, RO1, MT1 and MT2) are influenced by the degree of isolation by distance, suggesting a regular and gradual dispersal over the native range of the pathogen (BARRÉS et al., 2008). In the Amazon region, the genetic structure of wild rubber trees populations is explained by both geographic location and isolation by distance within the watersheds of the Purus and Juruá rivers in the cluster Acre–Madre de Dios (Peru), the watersheds of the Madeira river for the population from Rondônia state, and of the Tapajós river for the populations in Mato Grosso state (LE GUEN et al., 2009). The natural populations of *M. ulei* in the North region are apparently subjected to the same genetic discontinuity as that of its host. Therefore, structured pathogen populations in commercial rubber plantations in the Amazon region were founded probably from different inoculum sources from wild rubber trees at each watershed.

The high genetic variability observed in the sampled populations and the occurrence of the pathogen in all regions where rubber trees are grown can favor adaptation to the different biophysical environment increasing the epidemics severity, as already demonstrated by studies of physiological variability in large and small scale studies (JUNQUEIRA et al., 1986;

MATTOS et al., 2003). This variability also explains the lack of durability of qualitative resistance in the *Hevea*/*M. ulei* interaction (CHEE and WASTIE, 1980; GARCIA et al., 2002b; PLOETZ, 2007; LE GUEN et al., 2007). Additionally, the present study provides information that allows us to classify *M*. *ulei* as a pathogen of “high evolutionary potential” (McDONALD and LINDE, 2002). Therefore, given the capacity of microevolutionary changes of *M. ulei* in agricultural environment, the increase in the diversity of breeding clones with horizontal resistance to SALB is the best strategy manage SALB in both favorable and marginally favorable environments.

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FIGURE LEGENDS

Figure 01. Sampled areas (dots) located rubber producing regions in Brazil. States from which samples were collected are shaded.

Figure 02. Maps of posterior probability to belong to the clusters 1 (A), 2 (B), 3 (C) and 4 (D) and mode of the posterior probability (E) to belong to of each 15 populations of *Microcyclus ulei* in commercial areas of rubber of Brazil. Results obtained with GENELAND considering a spatial and uncorrelated allele frequencies model. Values are represented by a white-to-red graded scale; black dots represent sampled sites.

Figure 03. Bayesian clustering of 264 multilocus microsatellite haplotypes of *Microcyclus ulei* in commercial areas of rubber of Brazil using Structure (model with admixture).

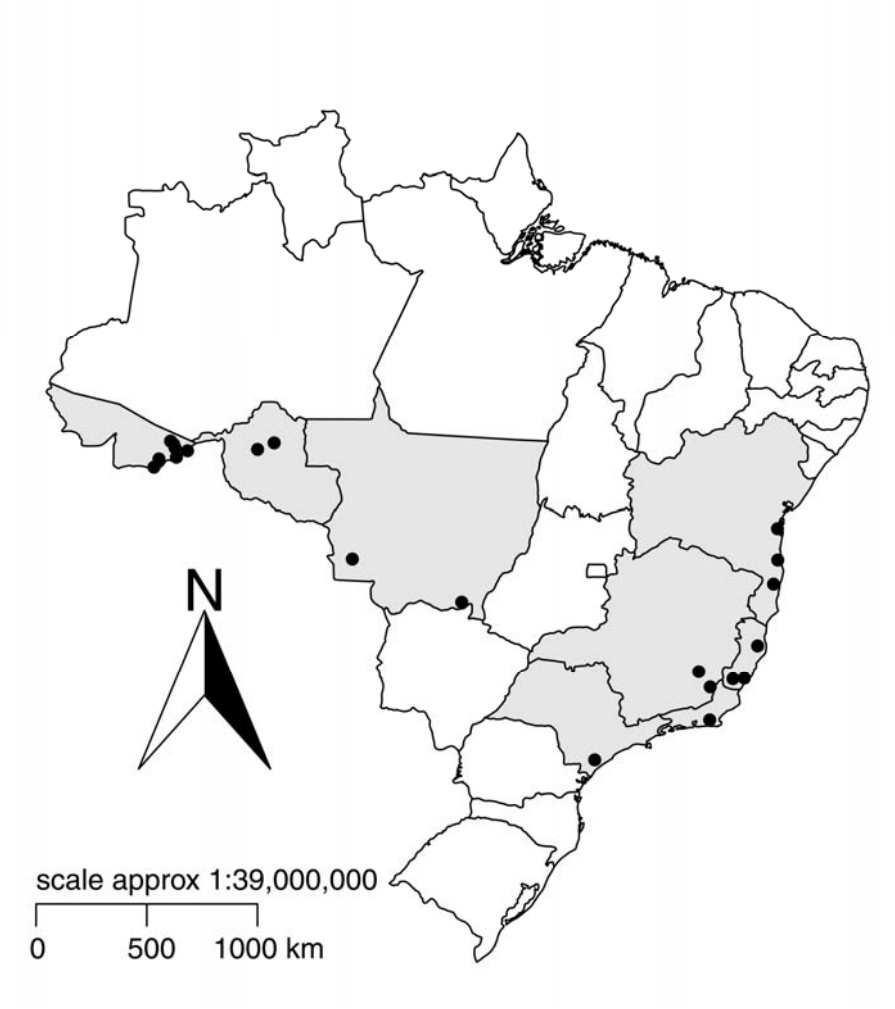

Figure 01. Hora Júnior et al

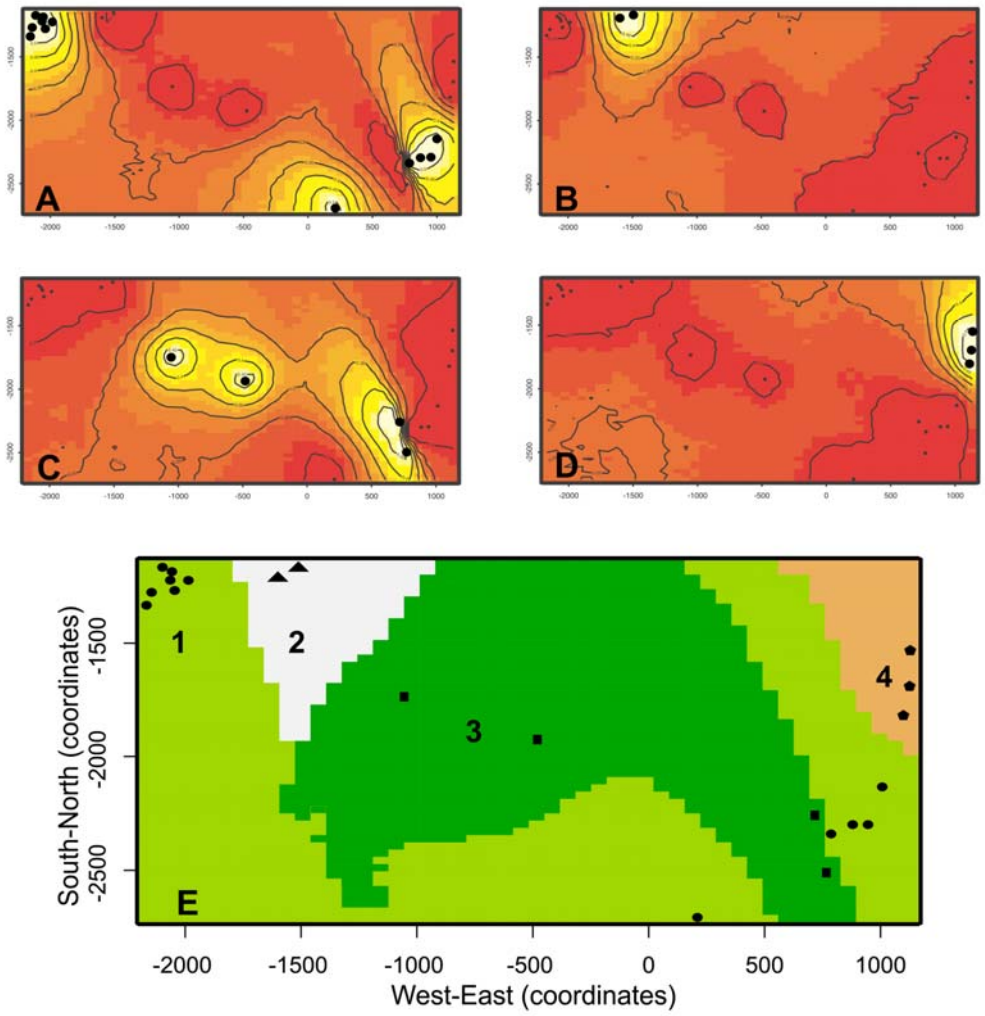

Figure 02. Hora Júnior et al

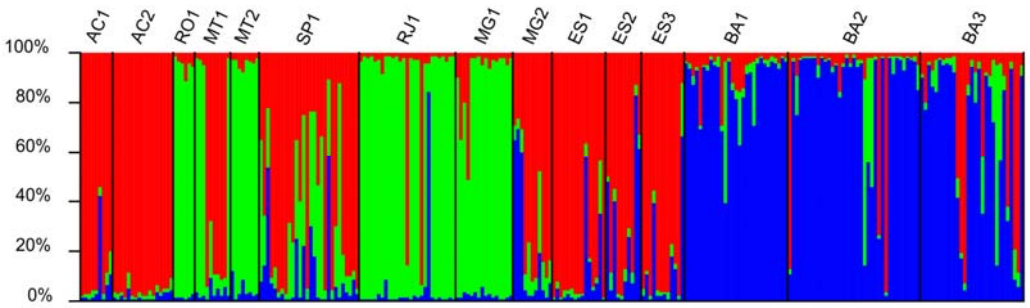

Figure 03. Hora Júnior et al

Table 01. Origin and sampling date of the 264 *Microcyclus ulei* isolates in commercial areas of rubber of Brazil.

Location	No. of indivíduos	Populations*	Collector	Sampling Date
Acre				
Epitaciolândia	4	AC1	B.T. Hora Júnior	2010
Xapuri	5	AC1	B.T. Hora Júnior	2010
Capixaba	3	AC2	B.T. Hora Júnior	2010
Plácido de Castro	3	AC2	B.T. Hora Júnior	2010
Senador Guiomard	2	AC2	B.T. Hora Júnior	2010
Rio Branco	3	AC2	B.T. Hora Júnior	2010
Bujari	6	AC2	B.T. Hora Júnior	2010
Rondônia				
Ariquemes	3	RO1	J. Honorato Júnior	2010
Buritis	3	RO1	J. Honorato Júnior	2010
Mato Grosso				
Pontes e Lacerda	10	MT1	B.T. Hora Júnior	2010
Itiquira	8	MT2	B.T. Hora Júnior	2009
São Paulo				
Registro	28	SP1	B.T. Hora Júnior	2010
Rio de Janeiro				
Silva Jardim	27	RJ1	B.T. Hora Júnior	2010
Minas Gerais				
Oratórios	16	MG1	B.T. Hora Júnior	2010
Muriaé	11	MG2	B.T. Hora Júnior	2010
Espírito Santo				
Cachoeiro do Itapemirim	15	ES1	B.T. Hora Júnior	2010
Guarapari	10	ES2	B.T. Hora Júnior	2010
Sooretama	12	ES3	B.T. Hora Júnior	2009
Bahia				
Porto Seguro	29	BA1	B.T. Hora Júnior	2008
Una	37	BA2	B.T. Hora Júnior	2008
Igrapiúna	29	BA3	B.T. Hora Júnior	2008

*Abbreviations of local populations in capital letters that will be used hereafter.

Table 02. Genetic diversity indices and linkage disequilibrium estimated for microsatellites data sets of 15 local populations of *Microcyclus ulei* in commercial areas of rubber of Brazil.

Population (Sample Size)	% of polymorphic loci	G	$E(g_n)$	Ar	pAr	H_E	r_d
AC1 (9)	82.3 (14/17)	1.28 (-0.6 – 3.16)[a]	5.58	2.05	0.14	0.39	0.096**
AC2 (17)	64.7 (11/17)	2.83 (0.19 – 5.46)	6	1.71	0.00	0.30	0.025
RO1 (6)	76.4 (13/17)	1 (-0.59 – 2.59)	6	2.16	0.21	0.49	0.0076
MT1 (10)	88.2 (15/17)	1.66 (-0.41 – 3.73)	6	2.40	0.10	0.51	0.053**
MT2 (8)	82.3 (14/17)	1.33 (-0.49 – 3.15)	6	2.15	0.00	0.44	0.019
SP1 (28)	94.1 (16/17)	4.66 (1.35 – 7.96)	6	2.12	0.06	0.44	0.030**
RJ1 (27)	82.3 (14/17)	4.5 (1.04 – 7.95)	6	2.14	0.09	0.45	0.0375***
MG1 (16)	88.2 (15/17)	2.66 (0.14 – 5.17)	6	1.87	0.07	0.35	0.033
MG2 (11)	70.5 (12/17)	1.83 (-0.29 – 3.95)	6	1.72	0.00	0.32	-0,010
ES1 (15)	82.3 (14/17)	2.5 (0.06 – 4.93)	6	1.85	0.00	0.35	0.048**
ES2 (10)	70.5 (12/17)	1.66 (-0.42 – 3.74)	6	1.76	0.03	0.32	0.005
ES3 (12)	76.4 (13/17)	2 (-0.23 – 4.23)	6	1.83	0.06	0.32	0.022
BA1 (29)	100 (17/17)	4.83 (1.33 – 8.32)	6	1.83	0.01	0.33	0.005
BA2 (37)	94.1 (16/17)	6.16 (2.19 – 10.12)	6	2.20	0.13	0.45	0.021**
BA3 (29)	100 (17/17)	4.83 (1.41 – 8.24)	6	2.16	0.05	0.44	0.016*

G, Stoddart and Taylor scaled index of multilocus genotypic diversity; $E(g_n)$, Genotypic richness estimated for the smallest sample size; Ar, allelic richness corrected for sample size; pAr, private allelic richness corrected for sample size; H_E, unbiased estimate of gene diversity (Nei 1978); r_d, multilocus estimate of test of linkage disequilibrium.

p-value: significant = p<0.5 *, highly significant = p<0.01 **, very highly significant = p<0.001 ***. [a] Number in parentheses indicate the lower and upper limits of 95% confidence intervals of the indices estimates.

Table 03. Results of several tests run on the 15 *Microcyclus ulei* populations in the software Bottleneck, in order to test the existence of an heterozygosity excess observed in the samples (calculated as Nei's gene diversity, Nei 1987) compared to the heterozygosity expected under mutation-drift equilibrium and termed the allelic richness. Only p-values of significative results are represented: significant = p<0.5 *, highly significant = p<0.01 **, very highly significant = p<0.001 ***

Populations	% of polymorphic loci	Sign test, IAM	Sign test, SMM	Standardized difference test, IAM	Standardized difference test, SMM	H excess Wilcoxon test, IAM	H excess Wilcoxon test, SMM
AC1	82.3 (14/17)	*				*	
AC2	64.7 (11/17)	**		***	*	***	***
RO1	76.4 (13/17)	***	**	***	***	***	***
MT1	88.2 (15/17)	***		***		***	*
MT2	82.3 (14/17)			**		**	*
SP1	94.1 (16/17)			*		*	
RJ1	82.3 (14/17)	**		**		**	
MG1	88.2 (15/17)		*				
MG2	70.5 (12/17)	**	**	***	*	***	**
ES1	82.3 (14/17)			*		*	
ES2	70.5 (12/17)	**	**	**	*	***	**
ES3	76.4 (13/17)						
BA1	100 (17/17)						
BA2	94.1 (16/17)	*		**		**	
BA3	100 (17/17)	**		**		**	

Table 04. Pairwise comparisons of genetic differentiation (F_{st}) between local populations of *Microcyclus ulei* in commercial areas of rubber tree in Brazil.

	Differentiation													
Populations	AC1	AC2	RO1	MT1	MT2	SP1	RJ1	MG1	MG2	ES1	ES2	ES3	BA1	BA2
AC1														
AC2	0.10													
RO1	0.25	0.36												
MT1	0.23	0.26	0.22											
MT2	0.31	0.36	0.21	0.11										
SP1	0.15	0.13	0.27	0.12	0.19									
RJ1	0.27	0.31	0.17	0.16	0.15	0.19								
MG1	0.38	0.41	0.33	0.18	0.15	0.23	0.17							
MG2	0.25	0.36	0.32	0.22	0.33	0.26	0.27	0.33						
ES1	0.17	0.12	0.36	0.16	0.33	0.11	0.28	0.31	0.25					
ES2	0.22	0.28	0.35	0.20	0.36	0.15	0.27	0.32	0.24	0.16				
ES3	0.18	0.20	0.40	0.18	0.37	0.11	0.30	0.32	0.24	0.03^{ns}	0.15			
BA1	0.30	0.37	0.40	0.27	0.32	0.25	0.32	0.37	0.32	0.29	0.34	0.28		
BA2	0.26	0.31	0.32	0.20	0.25	0.21	0.28	0.30	0.27	0.23	0.21	0.21	0.11	
BA3	0.18	0.25	0.28	0.13	0.18	0.13	0.20	0.21	0.14	0.16	0.12	0.14	0.08	0.06

^{ns} non-significant in grey.

Table 05. Hierarchical analysis of molecular variance (AMOVA) of 15 local populations of *Microcyclus ulei* in commercial areas of rubber tree in Brazil.

		Percentage of variation populations			
Dataset	Number of populations	Among groups	Among populations within groups	Within populations	Φ statistics
Local Populations	15	–	23.53	76.47	Φ_{st} 0.23*
Cluster 1	7	–	18.18	81.82	Φ_{st} 0.18*
Cluster 3	4	–	16.48	83.52	Φ_{st} 0.16*
Cluster 4	3	–	8.90	91.90	Φ_{st} 0.08*
Global	15	13.92	12.48	73.59	Φ_{ct} 0.13*
					Φ_{sc} 0.14*
					Φ_{st} 0.26*

* P < 0.001

SUPPORTING INFORMATION

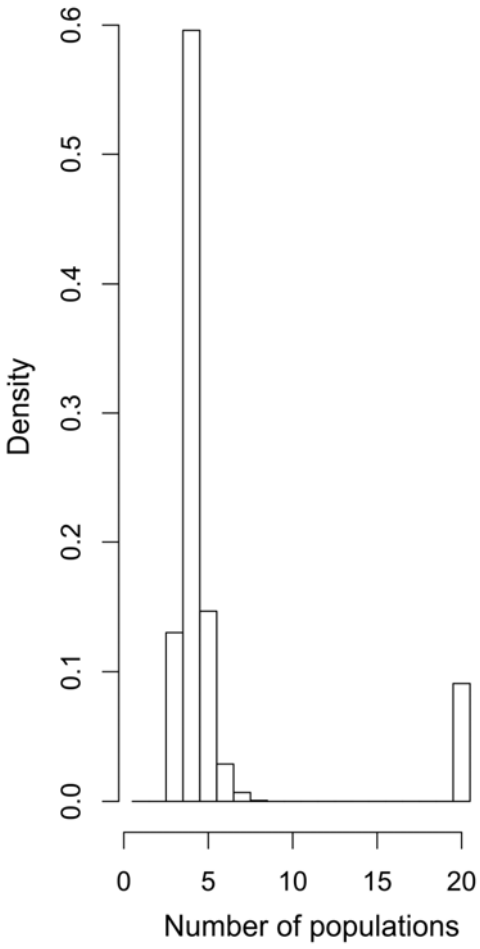

Figure S1. Posterior distribution of inference of the number of cluster(s) (K) by Geneland software of populations of *Microcyclus ulei* in commercial areas of rubber of Brazil.

Table S1. Multiplexing information and genetic variability of the 17 microsatellite markers used in this study. Diversity indices have been evaluated on the global sample of 264 *Microcyclus ulei* isolates. NA: Number of alleles; H_E: gene diversity calculated following Nei (1978) in Genepop.

Multiplex pool	Locus name	Genbank Number	Labeling dye	Motif repeat	Allele range	NA	H_E
1	Mu03	GQ420365	HEX	$(GT)_5GC(GT)_7$	89 – 105	4	0.1542
	Mu08	AY228718	6-FAM	$(CA)_8$	112 – 122	5	0.4005
	Mu11	GQ420358	NED	$(CT)_9(GT)7TT(GT)_4$	195 – 207	6	0.5481
	Mu24	GQ420360	6-FAM	$(GA)_{31}$	240 – 250	6	0.4507
2	Mu05	GQ420355	HEX	$(CGC)_5(\ldots)(TGGA)_5$	99 – 107	5	0.199
	µMu09	GQ420357	6-FAM	$(GT)_6$	140 – 146	4	0.5046
	µMu13	GQ420359	NED	$TG)_3((TG)_3CG)_9\ldots(TG)_{14}$	137 – 155	8	0.5861
3	Mu01	GQ420364	HEX	$(TG)_{13}$	136 – 152	7	0.639
	Mu14	GQ420366	6-FAM	$(CA)_5(\ldots)(CA)_5$	206 – 220	6	0.4536
	Mu35	GQ420368	NED	$(GT)_{30}$	329 – 339	6	0.5389
4	Mu37	GQ420361	6-FAM	$(AC)_7CTCC(CT)_9$	133 – 137	3	0.1573
	Mu16	AY228713	NED	$(TG)_{11}$	186 – 192	4	0.357
	Mu28	GQ420367	6-FAM	$(CA)_{14}$	326 – 336	6	0.2876
5	Mu38	GQ420362	NED	$CG(CA)_3(\ldots)CG(CA)_4(\ldots)CG(CA)_6$	197 – 221	6	0.4286
	Mu41	GQ420363	6-FAM	$(CA)_7$	254 – 270	5	0.4377
6	Mu06	GQ420356	6-FAM	$(CA)_{15}$	106 – 124	7	0.3864
	Mu09	AY228717	NED	$(TC)_{31}$	164 – 192	10	0.3108

Table S2. Inference of the number of cluster(s) (K) by Structure software that best explain the genetic structure among the 15 populations of *Microcyclus ulei* in commercial areas of rubber of Brazil. The ΔK was computed according to Evanno et al. (2005).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-4515.48	0.31	—	—	—
2	10	-4246.78	162.84	268.7	104.07	0.63
3	10	-3874.01	1.29	372.77	245.12	189.18
4	10	-3746.36	1.39	127.65	114.84	82.15
5	10	-3733.55	2.12	12.81	31.4	14.75
6	10	-3689.34	33.06	44.21	189.51	5.73
7	10	-3834.64	266.83	-145.3	143.74	0.53
8	10	-3836.2	162.73	-1.56	186.23	1.14
9	10	-3651.53	125.5	184.67	511.73	4.07
10	10	-3978.59	493.17	-327.06	547.38	1.1
11	10	-3758.27	118.26	220.32	204.9	1.73
12	10	-3742.85	123.02	15.42	246.94	2
13	10	-3974.37	574.68	-231.52	411.76	0.71
14	10	-3794.13	172.61	180.24	436.62	2.52
15	10	-4050.51	527.64	-256.38	259.95	0.49
16	10	-4046.94	297.49	3.57	94.55	0.31
17	10	-4137.92	414.58	-90.98	183.81	0.44
18	10	-4045.09	180.48	92.83	414.25	2.29
19	10	-4366.51	485.48	-321.42	5297.99	10.91
20	10	-9985.92	17333.33	- 5619.41	—	—

CAPÍTULO 3

Influence of hosts with partial resistance on the genetic structure of the pathogen *Microcyclus ulei* in Hevea spp.

ABSTRACT

The control strategies to manage South American leaf blight (SALB), the most destructive disease of rubber trees, are limited and the main resource has been planting partially resistant clones. Estimating whether resistance will remain effective is crucial to expand natural rubber production in areas where SALB is endemic. Therefore, proper knowledge about how the deployment of resistant and susceptible *Hevea* clones affect the genetic structure of populations of the causal fungal pathogen *Microcyclus ulei* needs to be generated. We analyzed 39 isolates of *M. ulei* collected in susceptible (Fx 3864 and IAN 717) and 29 from resistant (CDC 312, FDR 5788, MDF 180 and PMB1) rubber clones of *Hevea brasiliensis* planted in the Northeast region of Brazil. All individuals were genotyped for 17 microsatellites loci and 67 multilocus genotypes were identified. Bayesian cluster analysis revealed that *M. ulei* individuals could be clustered into four genetically distinct groups that coexist locally. Groups 1 and 4 were formed by individuals collected in susceptible clone (Fx 3864 and IAN 717); group 2 is a mix of individuals from IAN 717 and Fx 3864 and from the partially resistant clones CDC 312, MDF 180 and PMB1; and group 3 has individuals sampled from the partially resistant clones CDC 312, FDR 5788, MDF 180 and PMB1. There was strong genetic differentiation between isolates sampled from susceptible and resistant clones. The null hypothesis of random association of alleles among loci was rejected only for group 2 due to a high degree of admixture. The type of resistance and the genetic background influence the levels of heterozygosity of the population of *M*. *ulei*. Higher heterozygosity and allele

richness were recorded for groups 2 and 3 compared to groups 1 and 4. The virulence spectrum of 77 isolates collected from the susceptible clone Fx 3864 and from the partially resistant clones CDC 312, FDR 5788, MDF 180 and PMB1 was determined and compared to historical data. No gradual increase in race complexity over time (2004 to 2009) was detected, thus there is no evidence of selection for virulence in the studied population of *M. ulei*.

Key words: South American leaf blight, disease resistance, *Hevea brasiliensis*, virulence.

INTRODUCTION

Natural rubber is a high-value commodity of strategic importance because it cannot be replaced by the petroleum-derived synthetic alternatives in many of its most significant applications (LIEBEREI, 2007). Currently, rubber tree (*Hevea brasiliensis*) is the main commercial source of natural rubber (van BEILEN and POIRIER, 2007). However, despite the fact that the center of origin of *H*. *brasiliensis* is the Amazon region of South America, in 2010, 96% of the world production of 10.4 million tons of natural rubber was obtained from rubber plantations located in Asia and Africa (FAO, 2012).

The main cause of failure of large-scale cultivation of *H. brasileinsis* in tropical America is the occurrence of epidemics of South American leaf blight (SALB), an endemic fungal disease caused by *Microcyclus ulei* (P. Henn.) v. Arx. (Ascomycota) (LANGFORD 1945; HOLLIDAY, 1970; CHEE and HOLLIDAY, 1986). Under favorable environmental conditions SALB can induce defoliation, dieback of the canopy and death of susceptible clones (GASPAROTTO et al., 1997). The risk of introduction of SALB to Asia and Africa makes *M. ulei* a biological threat that could influence the global market of many rubber products given the high risks of substantial reduction in the yield of natural rubber (CHEE, 1980; CHEE, 1985; MADDEN and WHEELIS, 2003). Therefore, quarantine measures for SALB-free zone are strictly enforced for any transcontinental connection that could directly link the tropical regions of South America to those in Southeast Asia (FAO, 2011).

The control strategies available for SALB are limited and the main

scientific issue in natural rubber production from *H. brasiliensis* is host resistance because it imposes only minor environmental impact and productive costs. Planting resistant clones would allow cultivation of rubber trees in zones where the pathogen occurs. Additionally, this control measure can also be used in SALB-free areas as a risk reducing strategy of economic impact on the potential introduction of SALB in Asia and Africa (GARCIA et al., 2004; LE GUEN et al., 2011). Breeding programs for SALB resistance were developed in the Tropical Americas, Africa and Asia, but few clones are currently recommended for planting in areas affected by SALB, because of the instability of the resistance and low yield of the selected resistant clones. The breeding programs employed qualitative resistance to SALB and were largely conducted without proper knowledge of pathogen diversity (SIMMONDS, 1990; JUNQUEIRA et al., 1990; PERALTA et al., 1990; LE GUEN et al., 2011).

The cultivar MDF 180, a primary clone derived from Madre de Dios in the Peruvian region, is an example of durable resistance in *H. brasiliensis* to SALB. This cultivar has showed a high level of partial resistance for more than 30 years in Bahia and Mato Grosso states in Brazil. MDF 180 allows only moderate levels of sporulation and inhibits the development of the teleomorph stages of the fungus (LE GUEN et al. 2008). Genetic analyses conducted so far indicate that the resistance of MDF 180 is predominantly conferred by a qualitative resistance gene and a major quantitative resistance factor, as well as by four minor effect QTLs at a secondary level (LE GUEN et al. 2011). Interestingly, the partial resistance of clone RO 38 is conferred by quantitative trait loci (QTLs) and has been effective against

several isolates of *M. ulei* under both field and controlled conditions (LESPINASSE et al., 2000; LE GUEN et al., 2003). However, highly aggressive isolates of *M*. *ulei* are able to overcome its quantitative resistance (LE GUEN et al., 2007).

Theoretical studies indicate that virulence of plant pathogens would evolve slowly in the presence of partially resistant hosts, thus the effectiveness of host resistance is expected to be high (GANDON and MICHALAKIS, 2000; PARLEVLIET, 2002). Pathogen populations must constantly adapt to changes in their environment to survive and in agricultural ecosystems environmental changes may include resistant varieties and gradual adaptation to partial resistance, eventually leading to the erosion of resistance performance (McDONALD and LINDE, 2002). The adaptation to partial resistance was observed in field experiments with the barley scald pathogen *Rhynchosporium secalis* (ABANG et al., 2006), the potato late blight pathogen *Phytophthora infestans* (ANDRIVON et al., 2007) and the wheat *Stagonospora* blotch pathogen *Phaeosphaeria nodorum* (SOMMERHALDER et al., 2011).

Currently, high-yielding resistant clones are being selected by the CIRAD-Michelin-Brazil SALB resistance breeding program, using the concept of partial or quantitative resistance (Le GUEN et al. 2002; GARCIA et al. 2004; GARCIA et al 2011). The resistance evaluation trials are conducted in a multi-site network of large-scale trials in Brazil and Ecuador (RIVANO et al., 2010). However, an important component for evaluating the durability of the resistance has been neglected: the genetic variability of the pathogen population and the evolution of virulence in *M*. *ulei*. Knowledge about these

issues can contribute to the deployment of host resistance and to estimate for how long host resistance will remain effective. Therefore, the aims of this study were to characterize the intraspecific variability of the pathogen population collected from susceptible (S) and resistant (R) rubber tree clones, to estimate the genetic differentiation between contemporary samples of isolates of these populations (S and R), to assess the spectrum of virulence of the isolates from S and R clones, and then to make inferences about selection.

MATERIAL AND METHODS

Sample collection and DNA extraction

Samples were collected at the research site of the Plantações Michelin da Bahia (PMB), located in the municipality of Ituberá, Bahia State, Brazil. Conducive weather conditions and high availability of inoculum make this an area of high SALB pressure. Sampled plots were located in a north-south transect of susceptible, Fx 3864 and IAN 717, and partially resistant, CDC 312, FDR 5788, MDF 180 and PMB1, rubber clones (Figure 01 and Table 01). Eight isolates were sampled from the susceptible clone Fx 3864. Fifteen isolates were sampled from the partially resistant clones CDC 312, FDR 5788 and PMB1: six isolates from a germplasm collection site and nine from a clonal garden. From the region of Massaranduba and Sucupira farms, 12 and 14 isolates, respectively, were collected from the susceptible clones Fx 3864 (4 isolates from Massaranduba and 8 from Sucupira) and IAN 717 (11 isolates from Massaranduba and 3 from Sucupira) and six isolates from the partially resistant clone CDC 312 (2 isolates from Massaranduba and 4 from Sucupira). Thirteen isolates were obtained from the Biriba farm, 3 from CDC 312, 5 from Fx 3864 and 5 from the partially resistant MDF 180 clone (Table 01). Single germinated conidia on water agar were collected under the microscope and transferred to potato sucrose agar (PSA) culture medium routinely used in research from Plantações Michelin da Bahia in Bahia, containing sucrose (30mM), potassium phosphate monobasic (14.7mM), pH 5.0 ± 0.2 and supplemented with cysteine (10000 ppm), tryptophan (2500 ppm), threonine (2500 ppm) and chloramphenicol (50µg/mL). Petri dishes

were kept for 20 days at 24 ± 1 ºC in the dark.

For DNA extraction isolates were cultivated on M4 culture medium (JUNQUEIRA et al., 1984) in the dark for 2 months at 24 ± 1 ºC. Total DNA was extracted following the standard cetyltrimethyl ammonium bromide extraction procedure (DOYLE and DOYLE, 1990) from mycelia macerated in liquid nitrogen. The DNA integrity was analyzed by agarose gel electrophoresis and the concentrations were checked with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

Microsatellite genotyping

All 68 individuals were genotyped using 9 microsatellite markers developed by Le GUEN et al. (2004) plus 8 markers developed by the Centre de Coopération International en Recherche Agronomique pour le Développement (CIRAD) (BENOIT BARRÈS, personal communication). The markers were combined in six multiplex panels (Table S1). We performed multiplex PCR reactions with Type-it Microsatellite PCR Kit as described by the manufacturer (Qiagen). PCR products were separated on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All samples were analyzed together with fluorescent molecular size markers to score the alleles. Data were processed by GeneMarker Software V1.91 (SoftGenetics). Haplotypes were created after allele numbers were assigned to each locus of each individual. Each allele was numbered according to the number of repeat units relative to the smallest allele at each locus (LE GUEN et al., 2004).

Clustering analysis

Population subdivision was assessed with the model-based Bayesian clustering algorithm implemented in Structure 2.2 (PRITCHARD et al., 2000; FALUSH et al., 2003). The admixture model with correlated allele frequency was implemented and the geographic origin of the isolates was used as prior information to assist clustering. Iteration parameters were set to a burning-in period of 10,000 iterations followed by 100,000 iterations of simulations. The likelihood of the assignments [Ln P(K)] was evaluated for K varying from 1 to 13 in 10 independent simulations. To assess the value of K that best explains the partitioning of the genetic data, the ΔK estimator was computed as described by EVANNO et al. (2005).

In order to identify clusters of genetically related genotypes, without assuming population genetic models, discriminant analysis of principal components (DAPC; Jombart et al. 2010), a multivariate method implemented in the R package adegenet (Jombart 2008), was used to investigate the genetic diversity among isolates.

Genetic data analysis

Genotypic diversity at each group was estimated by the Stoddard and Taylor's G index (STODDARD and TAYLOR, 1988) and bootstrapping was conducted using 1000 pseudoreplicates to construct a 95% confidence interval. The index was scaled according to sample size and rarefaction curves were used to estimate the number of expected genotypes for the smallest sample size being compared (GRÜNWALD, et al., 2003). The diversity index and the rarefaction calculations were performed with the vegan and vegetarian packages of the R program version 2.14.0 (R

Development Core Team 2011). To analyze allelic diversity for groups of variable sizes, allelic richness (Ar) and private allelic richness (pAr) were computed using the rarefaction method implemented in HP-Rare (KALINOWSKI, 2005). Gene diversity H_E was estimated following NEI (1978), using the Genepop 4.1 software (ROUSSET, 2008). Tests for linkage disequilibrium (LD) were used to detect the nonrandom association of alleles at different loci. LD was assessed using MultiLocus 1.3 (AGAPOW and BURT, 2001) with the unbiased estimate of multilocus linkage disequilibrium (r_d). Significance was assessed using 1000 randomizations of the data set to create a null distribution.

The coefficient of gene differentiation (G_{ST}) was calculated using FSTAT program version 2.9.3 (GOUDET 1995). The genetic differentiation between groups was tested using the theta (θ) estimator of WEIR and COCKERHAM (1984), with the generation of 1000 randomized data sets as described in the Multilocus program. Hierarchical analyses of molecular variance (AMOVA) were used to obtain an overview of the distribution of variation within and among groups. AMOVA were carried out using Arlequin version 3.11 program (EXCOFFIER et al., 2005).

Assessment of virulence profiles

Isolates of *M*. *ulei* were collected from plots planted with Fx 3864, CDC 312, FDR 5788, MDF 180 and PMB1 rubber tree clones from 2004 to 2009 (Table S3). The race of each isolate was determined by inoculating a set of 10 rubber tree clones that has been previously characterized for their ability to discriminate *M. ulei* isolates (JUNQUEIRA et al., 1988; MATTOS et

al., 2003; LE GUEN et al., 2007 and LE GUEN et al. 2008). Conidia were suspended in sterilized water with 0.05% Tween 80, and the final concentration was adjusted to $2x10^5$ spores/mL (MATTOS et al., 2003). The lower surfaces of three young leaves of the Fx 3864 rubber tree clone were spray-inoculated with the inoculum suspension using a HS Airbrush Complete set (Paasche Airbrush company) and plants were kept in chambers at 24°C, relative humidity above 85%, artificial daylight of 2000 lux and 12 h photoperiod. Water with 0.05% Tween 80 was used as negative control. Sporulation was scored 12 days after inoculation using a 1–6 scale adapted from JUNQUEIRA et al. (1988), where 1 = necrotic non-sporulating lesions, 2 = chlorotic non-sporulating lesions, 3 = slight sporulation on the lower side of the leaflets, 4 = moderate sporulation on the lower side of the leaflets, 5 = high sporulation on the lower side of the leaflets, and 6 = high sporulation on both sides of the leaflets. Race complexity of an isolate was determined according to the number of differentials on which the isolate caused symptoms (scores 3 to 6) and was summarized by a race complexity index (ANDRIVON and VALLAVIEILLE-POPE, 1995).

RESULTS

Genetic structure

We analyzed 39 isolates of *M. ulei* collected from susceptible (Fx 3864 and IAN 717) and 29 from partially resistant (CDC 312, FDR 5788, MDF 180 and PMB1) rubber clones (Figure 01, Table 01). All 17 microsatellite markers were polymorphic and the number of alleles ranged from two to six (Table S1). In total, 67 multilocus genotypes were identified. Assuming the origin of isolates as prior information (Table 01), four genetic clusters were clearly identified (Figure 02, Figure S1 and Table S2). However, regardless of spatial location, isolates sampled from partially resistant clones differed from those sampled from susceptible ones (Figure 02A). Groups 1 and 4 were formed by isolates collected from susceptible clones Fx 3864 and IAN 717, group 2 is a mix of isolates from Fx 3864 and IAN 717 (susceptible) and the partially resistant clones CDC 312, MDF 180 and PMB1, and group 3 is composed only by isolates sampled from partially resistant clones (CDC 312, FDR 5788, MDF 180 and PMB1) (Figure 02B). Discriminant analysis used on the PCA coordinates of genotypes and assignment of genotypes to these clusters produced two main axes explaining 43.8% and 32.5% of the total variability among clusters. The multivariate method indicated an optimum of four genetic clusters similar to the pattern of differentiation provided by Structure (Figure 03).

Some isolates from the same location belonged to different genetic groups and the spatial distribution of the four genetic groups is illustrated in Figure 4. Group 1 is comprised of 18 isolates which were obtained from all

locations where susceptible clones are cultivated. Thirteen isolates came from Fx 3864: four isolates from each Massaranduba, Sucupira and Biriba farms, and one from Plot 111. Five isolates were obtained from IAN 717 planted in the Massaranduba farm. Group 2 is comprised of 19 isolates: eight isolates were sampled from Fx 3864, of which four came from Plot 111, three from Sucupira and one from the Biriba farm. Four isolates were sampled from IAN 717 and came from the Massaranduba farm. Finally, seven isolates were obtained from partially resistant clones, of which two isolates came from the PMB 1 clone located in the Clonal garden site, two from the CDC 312 clone planted in the Massaranduba and Biriba farms and three from MDF 180 clone in the Biriba farm. All 22 isolates of group 3 were obtained from partially resistant clones. Seven isolates were obtained from the rubber tree collection site, of which five came from CDC 312 and two from FDR 5788. Six isolates were sampled from 03 clones planted in the Clonal garden. Nine isolates were obtained from the three farms: Seven isolates from CDC 312, of which one from Massaranduba, four from Sucupira and two from the Biriba farm; and two isolates from MDF 180 planted in the Biriba farm. The fourth genetic group was comprised of nine isolates. Three isolates were obtained from Fx 3864 planted in Plot 111 and one planted in the Sucupira farm. The other 5 isolates were sampled from IAN 717 planted in the Massaranduba farm.

Genetic characteristics of the four genetic groups identified

A high percentage of polymorphic loci were estimated for groups 2 and 3 (100% and 94%, respectively) against groups 1 (76%) and 4 (58%). The measures of genetic diversity and linkage disequilibrium between the groups

are displayed in Table 02. The Stoddart and Taylor scaled genotypic index (G) had similar values for all populations and the genotypic richness estimated for the smallest sample size (E(gn)) was 8.8 individuals for group 2. Maximum genotypic richness (9) was estimated for the other three groups. Private allele richness (pAr), allelic richness (Ar) and gene diversity (H_E) were highest in group 2, followed by group 3, except for pAr. The null hypothesis of random association of alleles among loci was rejected when all 68 isolates were considered as a single population and also for group 2 which was comprised of isolates obtained from partially resistant clones.

The overview of the distribution of variation within and among groups indicated that 23.4% of the total variance was distributed among groups and 76.6% within groups (Φ_{ST}= 0.23, P < 0.001) (Table 03). There was high evidence of genetic differentiation between groups. Gene differentiation estimated by the average G_{ST} was high (0.20) and the estimated θ value was 0.23 (P < 0.001). Significant F_{ST} values between groups were estimated for all pairwise comparisons (P < 0.001) (Table 04). The lowest F_{ST} value was estimated for groups 2 and 3 and the highest for the differentiation between group 4 and 1 (0.35) and between 3 (0.34).

Virulence profiles

Similar virulence profile of the isolates obtained from the susceptible clone Fx 3864 and from the partially resistant clones CDC 312, FDR 5788, MDF 180 and PMB1 was detected for isolates sampled from 2004 and 2008 (Figure 05). From a total of 77 individuals, 59 pathotypes were found, but none were of common occurrence among the clones and among the years

(Table S3). The isolates from partially resistant clones of 2008 that were genotyped had a low complexity. In the survey using these clones and also MDF 180 and Fx 3864 showed no tendency of selection for complex races.

DISCUSSION

In the present study, we tested the hypothesis that the genetic structure of the population of *M. ulei* can be influenced by the resistance level of *Hevea* clones. Bayesian clustering analyses revealed that isolates could be grouped according to the clone resistance level suggesting selection plays a role in shaping the evolution of *M. ulei*. Additionally, there is high genetic differentiation between sympatric pathogen populations from susceptible and partially resistant hosts. For pathogens, hosts with different levels of resistance can be considered as divergent habitats and host-shift speciation can therefore be regarded as a particular case of ecological speciation wherein the pathogen has to adapt to a novel host (VIENNE et al., 2009; GIRAUD et al., 2010).

High-yielding and partially resistant rubber tree clones with moderate sporulation intensity and low stromata density were recently released (GARCIA et al., 2004). Two of these clones, CDC 312 and FDR 5788, have MDX 40 and MDF 180 as the resistant parental, respectively, which indicate the same genetic background of other resistant materials originally found in the Peruvian region of Madre de Dios (LE GUEN et al., 2004, 2011). The molecular basis of durable partial resistance of the primary clone MDF 180 during the disease progression is probably due to the up-regulation of the transcriptional machinery in association with tissue integrity and to the capacity to limit the activity, growth and spread of the pathogen (GARCIA et al., 2011).

Population genetics analyses revealed that all isolates obtained from

FDR 5788 and the great majority from CDC 312 were grouped (Group 3) together with isolates from MDF 180. Isolates from PMB1, that had unknown parental affinities, are also part of Group 3. Groups 1 and 4 were formed by isolates collected from susceptible clones. The existence of strong differentiation between *M. ulei* from partial resistant vs. susceptible rubber tree clones suggest restricted gene flow and clone specificity. Examples of specificity for resistant varieties/cultivars are available for *Alternaria alternata* causing *Alternaria* brown spot on citrus (PEEVER et al., 2000), *Didymella rabiei* causing *Ascochyta* blight of chickpea (PEEVER et al., 2004), *Venturia inaequalis* causing scab on apples (GUÉRIN et al., 2007; GLADIEUX et al., 2011); and the rust fungi *Cronartium ribicola* causing white pine blister rust (RICHARDSON et al., 2008) and *Melampsora larici-populina* causing poplar rust (XHAARD et al., 2011).

Sufficient reproductive isolation by strong selection against immigrants imposed by resistant varieties is the strongest and probably the most efficient barrier to gene flow between sympatry populations of a plant pathogen (HENDRY et al., 2007; GLADIEUX et al., 2011). In general, for fungi that mate within its host after colonization, cultivar specificity necessarily and automatically strongly restricts gene flow (GIRAUD et al., 2010). Although the mating system of *M. ulei* has not been characterized, ascostromata become mature at about four to six weeks after infection on mature leaves and the development of these structures apparently involve spermatization events (LANGFORD, 1945; HOLLIDAY, 1970; CHEE and HOLLIDAY, 1986). In addition to the development low number of pseudothecia in partially resistant clones, such as CDC 312, FDR 5788 and PMB1 (GARCIA et al., 2004), the

random association among alleles in isolates of Group 3 suggest the regular occurrence of sexual recombination.

The probable source of isolates for the populations in partially resistant clones are the susceptible ones and host shift must occur to allow for infection (DENNEHY et al., 2010; GLADIEUX et al., 2011). Group 2 is formed by individuals obtained from susceptible and partially resistant clones and linkage disequilibrium was detected among isolates of this group and there was high gene and allelic diversity. A recurring change of host environment may thus slow down the fixation process, resulting in the maintenance of greater variation in the population (van PUTTEN et al., 2005).

Partially resistant hosts generally select for higher virulence and more aggressive isolates (AHMED et al., 1996; GANDON and MICHALAKIS, 2000; COWGER and MUNDT, 2002). The pathogen evolves towards an increase in competitiveness, which in turn results in an increase in virulence in order to compensate for the decrease in its growth rate due to host resistance, and directional selection can lead to the erosion of resistance (McDONALD and LINDE, 2002). We did not find a gradual increase in race complexity over time for the isolates collected from 2004 to 2009. Thus, there is no evidence of selection for complex races in *M. ulei* from hosts with partial resistance. The reduction in race complexity can be the result of local adaptation for partially resistant clones. The process of adaptation to new hosts will be facilitated if the flow of maladapted ancestral genes (decrease in virulence to other rubber tree clones) into the population adapting to a resistant host is significantly reduced (HENDRY et al., 2007; GLADIEUX et al., 2011). The reduction of gene flow and possible selection by the host due to local

adaptation has been observed for other pathogens (CAPELLE and NEEMA, 2005), and further studies are required to assess the selection for a higher level of aggressiveness in isolates obtained from partially resistant rubber tree clones.

Our data are consistent with the hypothesis that the widespread adoption of resistant clones has selected for SALB isolates of Group 3 and this research has demonstrated the importance of understanding the population structure of plant-pathogenic fungi for the design and implementation of effective programs for resistance breeding. As the control of SALB is dependent upon the use of resistant cultivars, our findings have important consequences for durable resistance management in rubber.

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FIGURE LEGENDS

Figure 01. Sampled areas (dots) located in rubber tree producing regions in the farm of Plantações Michelin da Bahia, Brazil.

Figure 02. Bayesian clustering of 68 multilocus microsatellite haplotypes of *Microcyclus ulei* in commercial areas planted to rubber tree (model with admixture). (A) Dataset organized by spatial locations of six sites sampled. Isolates obtained from partially resistant clones are indicated with asterisks. (B) Dataset organized by four genetic groups.

Figure 03. Scatterplot of the discriminant analysis of principal components (DAPC) on *Microcyclus ulei* genotypes. Only the two-first principal components of the DAPC are represented. The first axis is the horizontal axis. At the top left, the Eigenvalues of the 3 axes are represented.

Figure 04. Geographic distribution of membership of the four genetic groups, showing proportion of individuals assigned to each genetic group at each site.

Figure 05. Virulence complexity of isolates of *Microcyclus ulei* collected from susceptible clone (Fx 3864) and partially resistant clones (CDC 312, FDR 5788, MDF 180 and PMB1) from 2004 and 2008 in the area of Plantações Michelin da Bahia, Brazil.

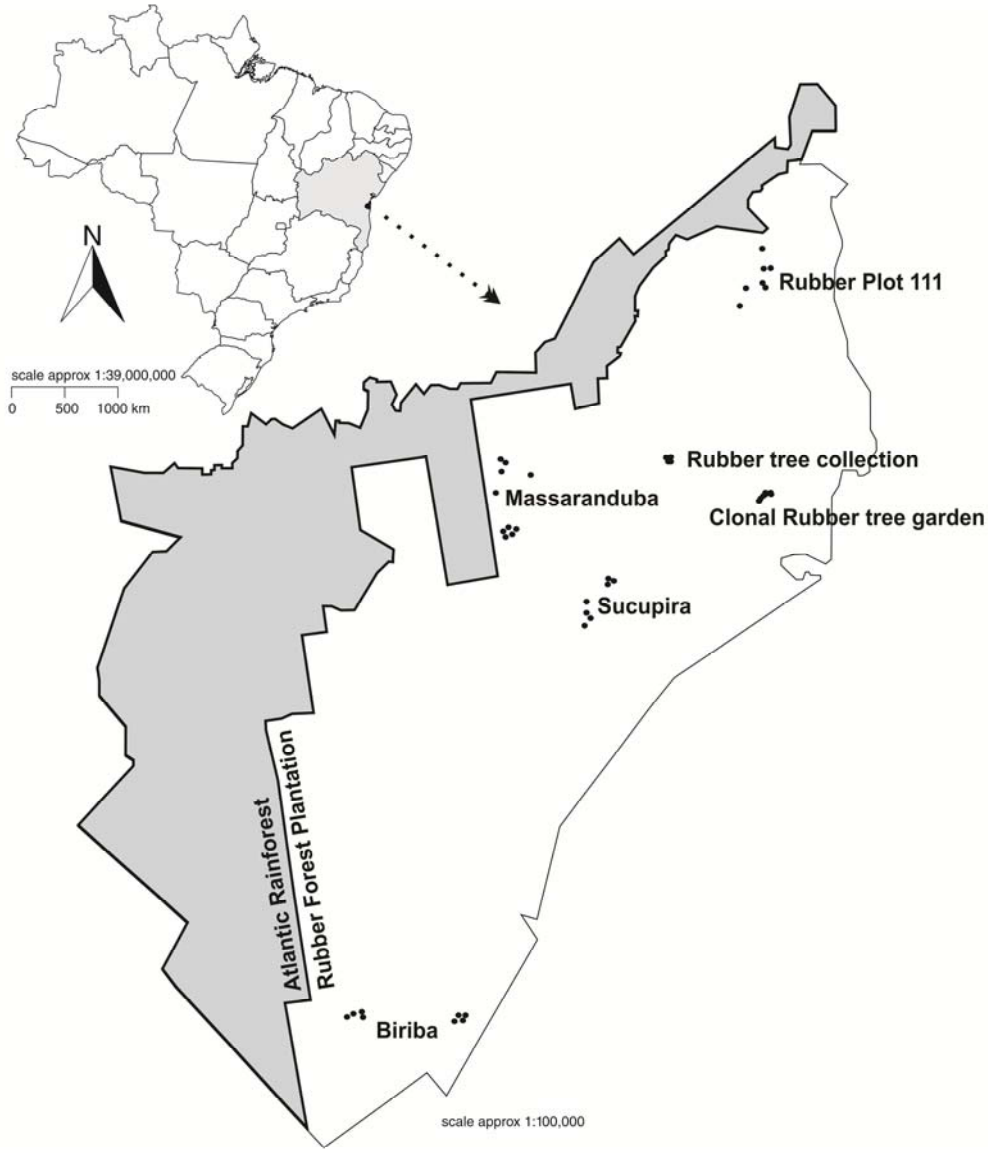

Figure 01. Hora Júnior et al

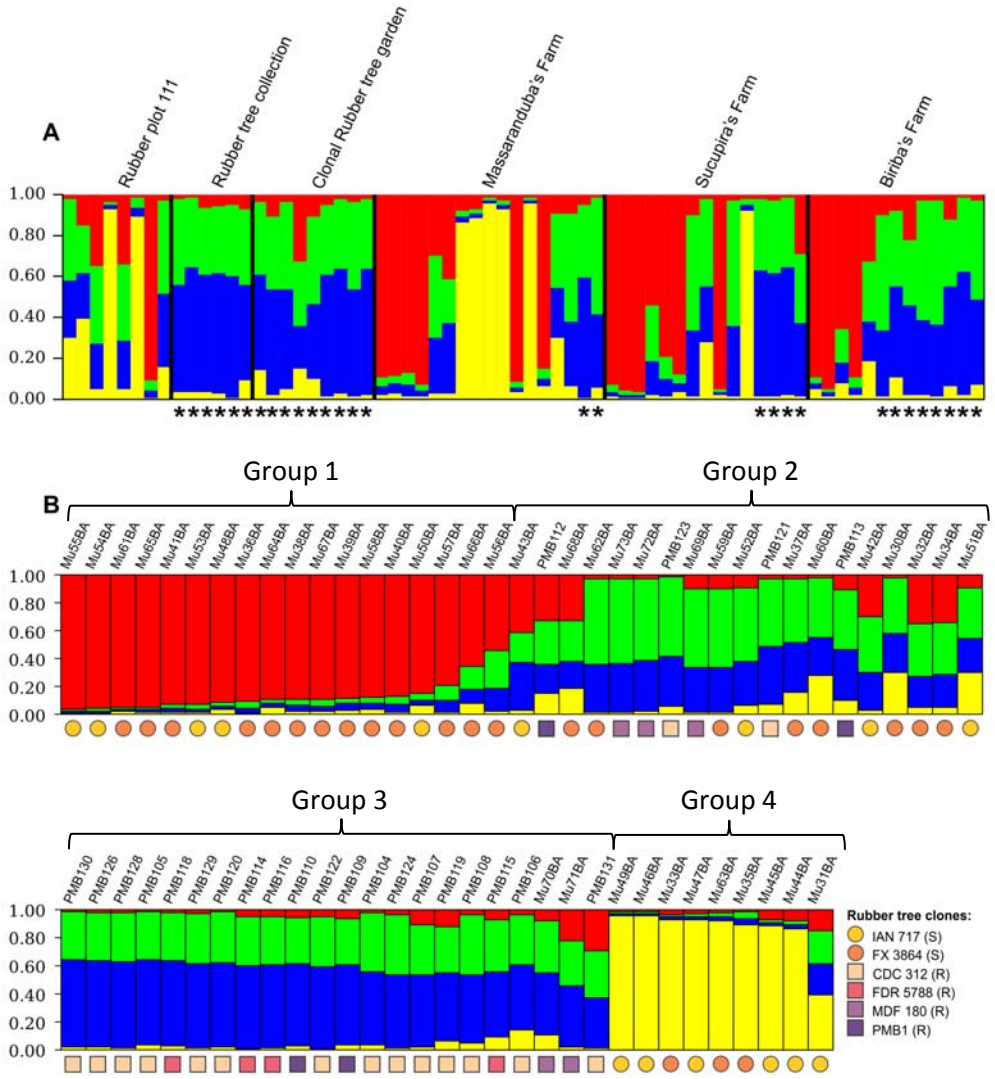

Figure 02. Hora Júnior et al

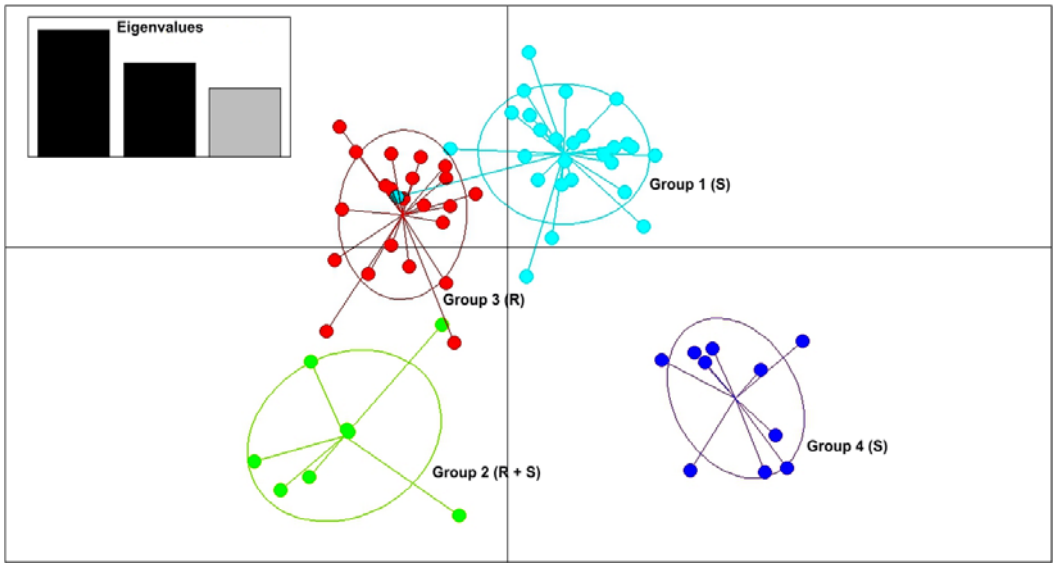

Figure 03. Hora Júnior et al

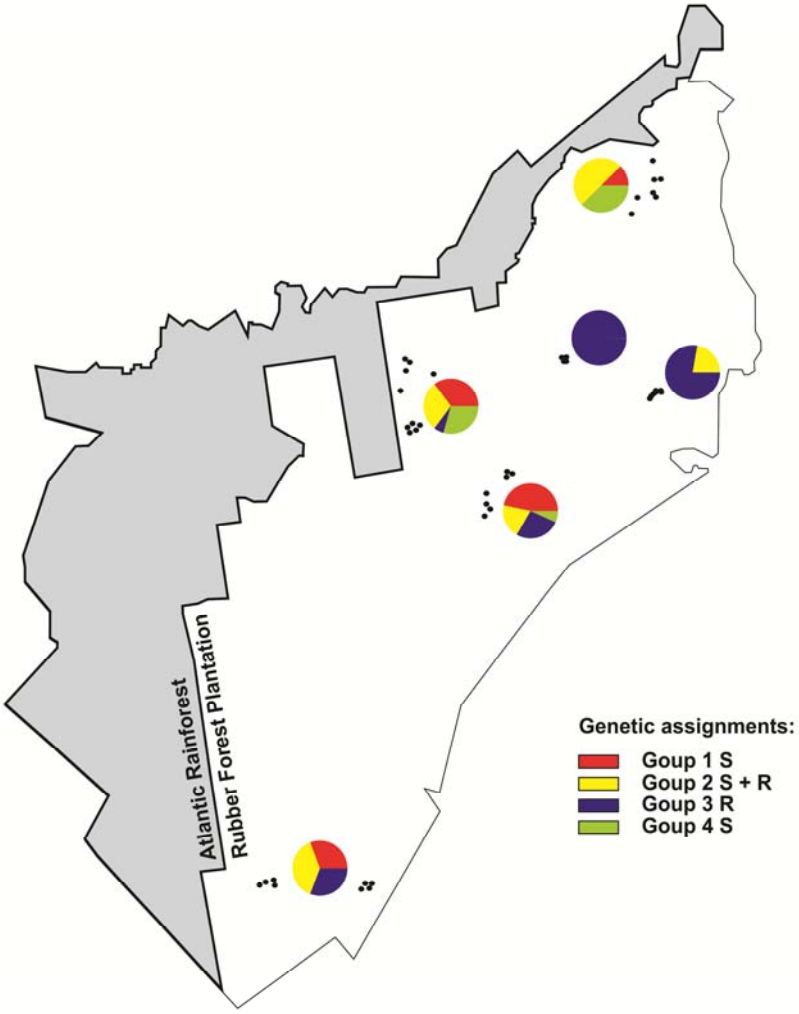

Figure 04. Hora Júnior et al

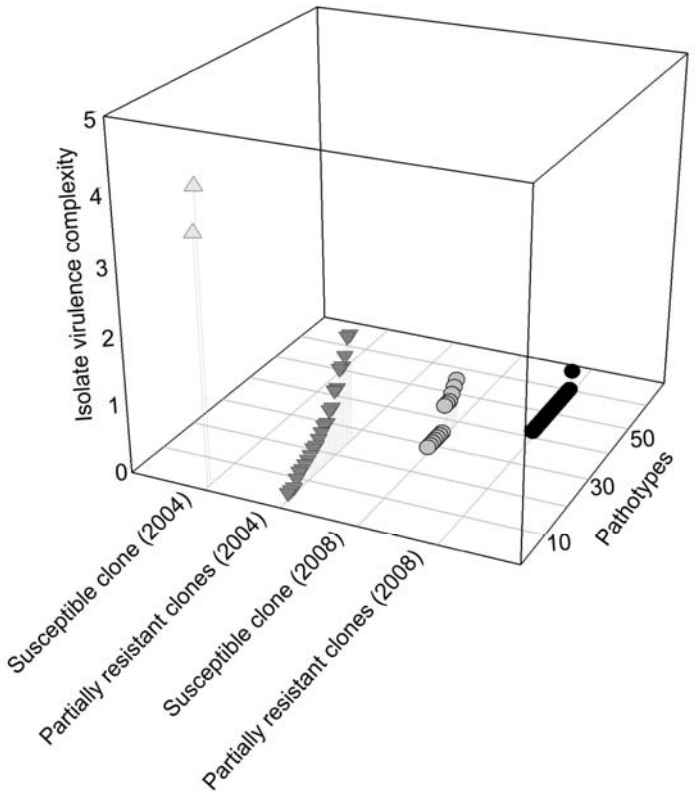

Figure 05. Hora Júnior et al

Table 01. Origin and sampling dates of the 68 isolates of *Microcyclus ulei* obtained from plantations of the Plantações Michelin da Bahia, Brazil.

Locations	Sample size	Clone rubber tree* (number of isolates from each clone)
Rubber Plot 111	8	Fx 3864 (8)
Rubber tree collection	6	CDC 312 (2), FDR 5788 (2), PMB1 (2)
Clonal garden	9	CDC 312 (5), FDR 5788 (2), PMB1 (2)
Massaranduba Farm	17	CDC 312 (2), Fx 3864 (4), IAN 717 (11)
Sucupira Farm	15	CDC 312 (4), Fx 3864 (8), IAN 717 (3)
Biriba Farm	13	CDC 312 (3), Fx 3864 (5), MDF 180 (5)

* CDC 312 (AVROS 308 x MDX 40); FDR 5788 (HAR 8 x MDF 180), Fx 3864 (PB 86 x FB 38); IAN 717 (PB 86 x F 4542); MDF 180 (Primary clone of *H. brasiliensis*); PMB1 (unknown parental). AVROS – Algemene Vereniging Rubberplanters Oostkust Sumatra; CDC – Clavellinas *Dothidella* Cross; F – Ford; FB – Ford Belém, FDR – Firestone *Dothidella* Resistant; Fx – Ford Cross; HAR – Harbel Estate (Firestone); MDF – Madre de Dios Firestone; MDX – Madre de Dios Cross.

Table 02. Genetic diversity indices and linkage disequilibrium estimated for microsatellites data sets of four groups of isolates (sub-populations) of *Microcyclus ulei*.

Population	Sample Size	G	$E(g_n)$	Ar	pAr	H_E	r_d
Group 1	18	2.00 (-0.80 – 4.80)[a]	9.00	1.89	0.21	0.29	0.004
Group 2	19	1.91 (-0.93 – 4.75)	8.78	2.46	0.40	0.44	0.029*
Group 3	22	2.44 (-0.66 – 5.55)	9.00	2.02	0.19	0.35	0.013
Group 4	9	1.00 (-0.94 – 2.94)	9.00	1.71	0.04	0.30	0.027

G, Stoddart and Taylor scaled index of multilocus genotypic diversity; $E(g_n)$, Genotypic richness rarefied for the smallest sample size; Ar, allelic richness corrected for sample size; pAr, private allelic richness corrected for sample size; H_E, unbiased estimate of gene diversity (Nei 1978); r_d, multilocus estimate of test of linkage disequilibrium.

*p-value: significant at α = 0.5. [a] Numbers in parentheses indicate the lower and upper limits of 95% confidence intervals of the estimates.

Table 03. Hierarchical analysis of molecular variance (AMOVA) of four groups (sub-populations) of *Microcyclus ulei*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	55.0	0.92 Va	23.4
Within populations	64	193.5	3.02 Vb	76.6
Total	67	248.5	3.95	

Table 04. Pairwise comparisons of population differentiation among groups of isolates (sub-populations) of *Microcyclus ulei*.

Populations	Differentiation			
	Group 1	Group 2	Group 3	Group 4
Group 1				
Group 2	0.20[a]			
Group 3	0.25[a]	0.13[a]		
Group 4	0.35[a]	0.22[a]	0.34[a]	

[a] Theta value was significant at P < 0.001;

SUPPORTING INFORMATION

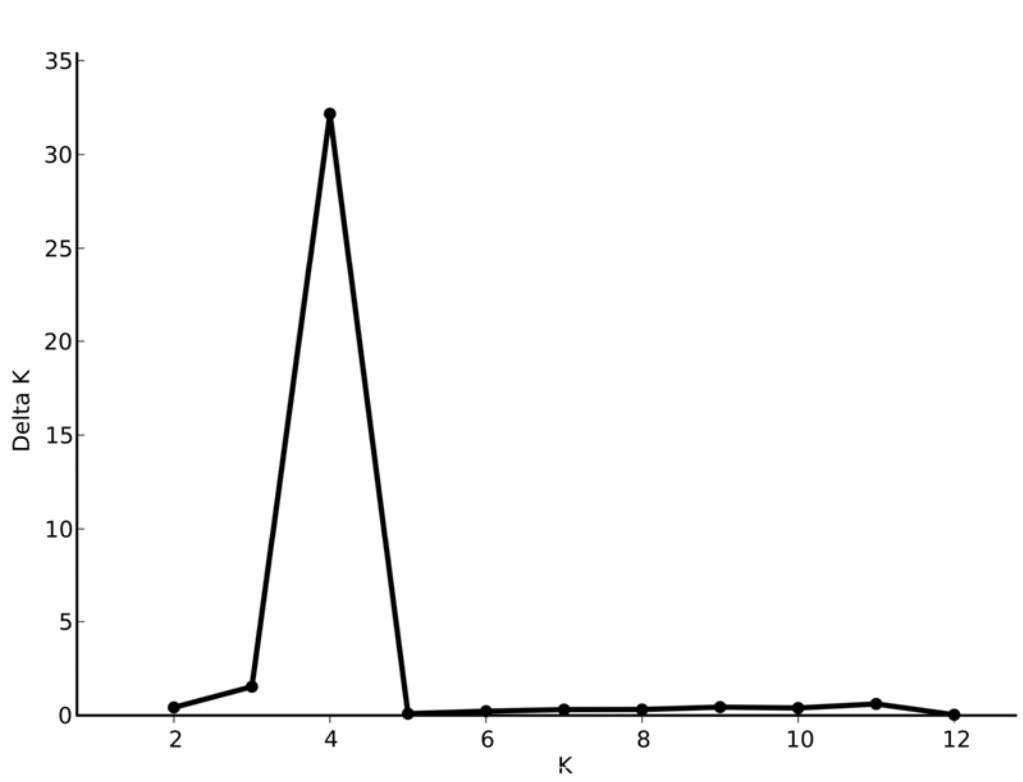

Figure S1. ΔK calculated of posterior distribution of the number of populations of *Microcyclus ulei* in commercial areas of rubber of PMB.

Table S1. Multiplexing information and genetic variability of the 17 microsatellite markers used in this study. Diversity indices have been evaluated on the global sample of 68 *Microcyclus ulei* isolates. NA: Number of alleles; H_E: gene diversity calculated following Nei (1978) in Genepop.

Multiplex pool	Locus name	Genbank Number	Labeling dye	Motif repeat	Allele range	NA	H_E
1	Mu03	GQ420365	HEX	$(GT)_5GC(GT)_7$	89 – 97	3	0.2498
	Mu08	AY228718	6-FAM	$(CA)_8$	112 – 116	3	0.2897
	Mu11	GQ420358	NED	$(CT)_9(GT)7TT(GT)_4$	195 – 207	6	0.6387
	Mu24	GQ420360	6-FAM	$(GA)_{31}$	240 – 244	3	0.3061
2	Mu05	GQ420355	HEX	$(CGC)_5(\ldots)(TGGA)_5$	101 – 107	4	0.1646
	µMu09	GQ420357	6-FAM	$(GT)_6$	140 – 146	4	0.5239
	µMu13	GQ420359	NED	$TG)_3((TG)_3CG)_9\ldots(TG)_{14}$	141 – 147	4	0.4416
3	Mu01	GQ420364	HEX	$(TG)_{13}$	136 – 144	5	0.4563
	Mu14	GQ420366	6-FAM	$(CA)_5(\ldots)(CA)_5$	210 – 212	2	0.3638
	Mu35	GQ420368	NED	$(GT)_{30}$	331 – 339	4	0.2141
4	Mu37	GQ420361	6-FAM	$(AC)_7CTCC(CT)_9$	135 – 137	2	0.1857
	Mu16	AY228713	NED	$(TG)_{11}$	188 – 190	2	0.222
	Mu28	GQ420367	6-FAM	$(CA)_{14}$	330 – 334	3	0.2603
5	Mu38	GQ420362	NED	$CG(CA)_3(\ldots)CG(CA)_4(\ldots)CG(CA)_6$	197 – 203	4	0.5333
	Mu41	GQ420363	6-FAM	$(CA)_7$	264 – 270	4	0.5172
6	Mu06	GQ420356	6-FAM	$(CA)_{15}$	108 – 126	6	0.4942
	Mu09	AY228717	NED	$(TC)_{31}$	174 – 178	3	0.1916

Table S2. Inference of the number of cluster(s) (K) by Structure software that best explain the genetic structure among the 4 populations of *Microcyclus ulei* in commercial areas of rubber of PMB. The ΔK was computed according to Evanno et al. (2005).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-907.28	0.61	NA	NA	NA
2	10	-923.43	79.35	-16.15	32.67	0.41
3	10	-906.91	20.75	16.52	31.59	1.52
4	10	-858.80	12.46	48.11	400.95	32.16
5	10	-1211.64	1017.87	-352.84	79.42	0.07
6	10	-1643.90	1496.08	-432.26	315.14	0.21
7	10	-1761.02	1845.70	-117.12	547.12	0.29
8	10	-1331.02	898.05	430.00	270.83	0.30
9	10	-1171.85	846.96	159.17	362.96	0.42
10	10	-1375.64	1455.73	-203.79	556.57	0.38
11	10	-2136.00	1884.54	-760.36	1130.26	0.59
12	10	-1766.10	1295.85	369.90	25.61	0.01
13	10	-1370.59	964.88	395.51	NA	NA

Table S3. Virulence profiles of isolates of Microcyclus ulei collected in the field at Michelin Plantation of Bahia from Fx 3864, CDC 312, FDR 5788, MDF 180 and PMB1 rubber tree clones in the period of 2004-2009.

Clone	Isolates/Year	F 4542[a]	Fx 2261	Fx 2784	Fx 2804	Fx 3899	Fx 4098	Fx 985	IAN 6158	MDF 180	PA 31	Ci[c]
CDC 312	PMB 51 and 52/04	+ 3	+ 4	- 2	+ 5	+ 6	+ 4	- 1	+ 4	+ 5	+ 3	1,54
	PMB 53/04	+ 4	+ 4	- 2	+ 5	+ 5	+ 5	- 1	+ 3	+ 4	- 2	0,67
	PMB 54/04	+ 3	+ 4	- 2	+ 4	+ 5	+ 4	- 1	+ 3	+ 4	- 2	0,22
	PMB 55/04	+ 3	+ 3	- 2	+ 5	+ 6	+ 5	- 1	+ 3	+ 5	- 2	0,90
	PMB 56/04	+ 4	+ 4	- 2	+ 4	+ 5	+ 5	- 1	+ 3	+ 4	- 2	0,45
	PMB 63 and 64/04	+ 3	+ 3	- 2	+ 5	+ 6	+ 4	- 2	+ 5	+ 4	+ 4	1,54
	PMB 75/04	+3	-2	-2	+5	-2	+5	+4	-2	+4	+3	0,38
	PMB 78/04	+4	+4	-2	+5	-2	+5	+6	-2	+4	+4	0,67
	PMB 79/04	+4	+5	-2	+4	-2	+5	+5	-2	+5	+3	0,90
	PMB 104/08*	-1	-2	+4	+3	+5	+3	+3	-2	+3	-2	0,26
	PMB 105 and 107/08*	-1	-2	+3	+3	-2	+4	+4	-2	+4	-2	0
	PMB 106/08*	-2	-2	-2	+4	-2	+3	+4	-2	+4	-2	0
	PMB 108/08*	-1	-2	+3	-1	+4	+4	+4	-2	+4	-2	0
	PMB 119/08*	+4	-1	-2	-2	+3	-2	+3	-2	+3	-2	0
	PMB 120/08*	-2	-2	-2	-2	-2	-2	+3	-2	+3	-2	0
	PMB 122/08*	+3	-2	-2	-2	-1	-2	+3	+3	+3	-2	0
	PMB 125/08*	-2	-2	-2	-2	+3	+3	+3	+3	+3	-2	0
	PMB 126/08*	-2	-2	-2	-2	+3	+3	+3	-2	+3	-2	0
	PMB 127/08*	+4	-2	+3	+3	+4	+3	+4	-2	+4	-2	0
	PMB 129/08*	-2	-2	-2	+3	-2	-2	+3	-2	+3	-1	0
	PMB 130/08*	+3	-2	-2	-2	-1	+3	+4	-2	+3	-2	0

Table S3. Continued

Clone	Isolates/Year	F 4542	Fx 2261	Fx 2784	Fx 2804	Fx 3899	Fx 4098	Fx 985	IAN 6158	MDF 180	PA 31	Ci
FDR 5788	PMB 26/04	-2	+ 3	- 2	+ 3	+ 3	+ 3	+ 3	+ 3	+ 5	+ 3	0,25
	PMB 49 and 50/04	+ 4	- 2	- 2	- 1	+ 3	+ 4	- 1	+ 3	+ 4	- 1	0
	PMB 59/04	+ 3	- 2	- 2	- 1	+ 4	+ 4	- 2	+ 5	+ 5	- 1	0,32
	PMB 60/04	+ 3	- 2	- 2	- 1	+ 4	+ 4	- 2	+ 5	+ 4	- 1	0,16
	PMB 76/04	+5	+4	-2	+4	+4	+3	+3	+4	+5	-2	0,51
	PMB 77/04	+3	+3	-2	+4	+5	+5	+4	+4	+4	-2	0,51
	PMB 83/04	+5	+5	-2	+4	+5	+3	+5	+4	+5	-2	1,29
	PMB 114/08*	+3	+3	-2	+3	+3	+3	+4	-2	+4	+3	0
	PMB 115 and 118/08*	-2	+3	-2	+3	+3	+3	+3	-2	+3	+3	0
	PMB 116/08*	-2	+3	-2	+3	-2	+3	+3	-2	+3	-2	0
	PMB 117/08*	-2	+4	-2	+3	+4	+3	+4	+3	+4	-2	0
MDF 180	PMB 65/04	+ 3	- 2	- 2	- 2	- 2	+ 5	- 1	+ 5	+ 4	+ 4	0,32
	PMB 66/04	+ 4	- 2	- 2	- 2	- 2	+ 4	- 1	+ 4	+ 4	+ 4	0
	PMB 67/04	+ 4	- 1	- 2	- 2	- 2	+ 4	- 2	+ 5	+ 4	+ 4	0,16
PMB 1	PMB 28 and 48/04	+ 4	- 1	- 2	- 2	+ 6	+ 6	- 1	+ 4	+ 5	+ 4	1,16
	PMB 61 and 62/04	+ 4	- 2	- 2	- 1	+ 5	+ 5	- 2	+ 5	+ 4	+ 4	1,16
	PMB 70/04	-2	-2	-2	+4	+3	+4	+3	+3	+5	-2	0,19
	PMB 71, 80 and 81/04	-2	-2	-2	+4	+3	+4	+3	+3	+4	-2	0
	PMB 82/04	-2	-2	-2	+4	-2	+4	-2	-2	+5	-2	0,09
	PMB 109/08*	-2	-2	-2	+3	-2	+3	+3	+3	+4	-2	0
	PMB 110/08*	-2	-2	+4	+3	-2	+3	-2	-1	+4	-2	0
	PMB 111 and 112/08*	-2	-2	-2	-2	-2	+3	-1	+3	+4	-2	0

Table S3. Continued

Clone	Isolates	F 4542	Fx 2261	Fx 2784	Fx 2804	Fx 3899	Fx 4098	Fx 985	IAN 6158	MDF 180	PA 31	Ci
FX 3864	PMB 113/08*	-1	-2	+4	-2	-1	+3	-1	+3	+4	-2	0
	PMB 36, 37, 38, 41, 42 and 43/04	- 1	- 2	- 2	+ 4	+ 4	+ 4	+ 6	+ 3	+ 4	- 2	4,2
	PMB 40, 44 and 45/04	- 2	- 2	- 2	+ 5	+ 3	+ 4	+ 6	+ 4	+ 3	- 2	3,6
	PMB 87/08	-2	+3	-2	+3	-1	+3	+3	-2	+4	+3	0
	PMB 88/08	-2	+3	-2	-1	-2	+3	+4	+3	+3	-1	0
	PMB 89/08	-2	-2	-2	-2	-2	+4	+4	-2	+4	-2	0
	PMB 90/08	-2	+3	-2	-2	-2	+4	+4	-2	+4	-1	0
	PMB 91/08	+3	+3	-2	+3	+3	+4	+5	+3	+4	-2	0,61
	PMB 92/08	-2	+3	-2	-1	+3	-2	+5	+4	+4	-2	0,38
	PMB 93/08	+3	+3	-2	-1	+3	+4	+5	+3	+3	-2	0,53
	PMB 94/08	-1	-2	-2	+3	+3	-2	+4	+3	+4	-2	0
	PMB 95/08	-1	+3	-2	-2	+3	-2	+5	-2	+3	+3	0,38
	PMB 96/08	+3	+4	-2	-2	-2	+4	+4	+4	+4	+3	0
	PMB 97/08	-2	-2	-2	+3	-1	+4	+4	-2	+5	+3	0,38
	PMB 98/08	+3	+3	-2	-2	-2	+4	+4	-2	+4	-2	0
	PMB 99/08	+3	-2	-2	+3	+3	-2	+5	-2	+4	+3	0,46
	PMB 87/08	+ 3	+ 4	- 2	+ 5	+ 6	+ 4	- 1	+ 4	+ 5	+ 3	0

[a] F 4542 (primary clone of *H. benthamiana*); Fx 2261 (F 1619 x Avros 1883); Fx 2784 (F 4542 x Avros 363); Fx 2804 (F 4542 x TJIR 1); Fx 3899 (F 4542 x Avros 363); Fx 4098 (PB 86 x B 110); Fx 985 (F 315 x Avros 183); IAN 6158 (Fx 43-655 x PB 86); MDF 180 (primary clone of *H. brasiliensis*); PA 31 (primary clone of *H. pauciflora*). [b] -1 = necrotic non-sporulating lesions,- 2 = chlorotic non-sporulating lesions, + 3 = slight sporulation on lower side of the leaflets, + 4 = moderate sporulation on lower side of the leaflets, + 5

= high sporulation on lower side of the leaflets, and + 6 = high sporulation on both sides of the leaflets. [c] Race complexity indice of according with ANDRIVON and VALLAVIEILLE-POPE (1995). [*] Individuals collected in 2008 from partially resistant clone were genotyped.

CONCLUSÃO GERAL

1. A filogenia molecular do teleomorfo suporta a classificação de *Microcyclus ulei* na família Mycosphaerellaceae. s str, ordem Capnodiales, em Ascomycota, relacionado a espécies com morfologia sexual similar ao gênero *Mycosphaerella*.

2. A partir da perspectiva evolutiva, o ciclo sexual de *M. ulei* é proposto iniciando com espermogônias em folhas próximas a maturidade levando a ascósporos maduros em pseudotécios dentro de estroma.

3. O patógeno tem alta variabilidade genética e associação aleatória de alelos.

5. Populações geograficamente distantes são geneticamente relacionadas a partir de três grupos genéticos da região amazônica.

6. Clones de seringueira com resistência parcial causam forte diferenciação genética em populações do patógeno.