TACIANA FERREIRA DOS SANTOS

GENETIC STRUCTURE OF THE POPULATION OF *Pseudocercospora ulei* IN THE WATERSHED OF THE AMAZON RIVER

Dissertação apresentada a Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae.*

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Gleiber Quintão Furtado

Mateus Ferreira Santana

Camila Geovana Ferro

Eduardo Seiti Gomide Mizubuti
(Orientador)

Aos meus amados pais, Silvio e Telma
A mãe Marinalva *(in memoriam)*
E ao inesquecível tio Ismael *(in memoriam)*

dedico.

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SUMÁRIO

ABSTRACT

SANTOS, Taciana Ferreira, M.Sc., Universidade Federal de Viçosa, February, 2018. **Genetic structure of the population of *Pseudocercospora ulei* in the watershed of the Amazon river.** Adviser: Eduardo Seiti Gomide Mizubuti.

South American leaf blight (SALB) is a destructive disease caused by the fungus *Pseudocercospora ulei* that can severely affect rubber tree in monoculture in tropical America. The causal agent of the disease is a hemibiotrophic pathogen, that grows slowly under *in vitro* conditions. Isolation of good quality DNA in proper quantities is not an easy task due to the difficulties in the handling of *P. ulei* under laboratory conditions and the lack of a standard protocol. Although SALB has been known for nearly a century, information on the genetic structure of *P. ulei* in the Amazon region, its center of origin, remains unknown. In this work, adequate protocols for DNA extraction were assessed and the population genetic structure of *P. ulei* in the Amazon region was determined using 12 microsatellite loci (SSR). Six extraction protocols were compared regarding yield and purity of the DNA. A total of 61 isolates were sampled along the Madeira, Purus and Juruá watersheds located in the Amazon region and SSR genotyped. The Doyle and Doyle DNA extraction protocol was the one that presented consistent and better quality extracted DNA. There was linkage disequilibrium between alleles and genetic differentiation between geographically distant populations was detected. Principal component analysis revealed clustering of the isolates according to the watershed boundaries. Two groups were formed, one comprised by individuals from the Madeira river and the other comprised of individuals from the Purus and Juruá rivers.

RESUMO

SANTOS, Taciana Ferreira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Estrutura genética da população de *Pseudocercospora ulei* nas bacias hidrográficas do rio Amazonas.** Orientador: Eduardo Seiti Gomide Mizubuti.

O mal das folhas da seringueira (MDF) é uma doença destrutiva causada pelo fungo *Pseudocercospora ulei* que pode afetar severamente a seringueira em monocultura na América tropical. O agente causal da doença é um patógeno hemibiotrófico que apresenta crescimento lento *in vitro*. O isolamento de DNA de boa qualidade em quantidades adequadas não é uma tarefa fácil devido à falta de um protocolo padrão e a dificuldade de cultivar *P. ulei* em condições de laboratório. Embora o MDF seja conhecido por mais de um século, informações sobre a estrutura genética na região amazônica, seu centro de origem, permanecem desconhecidas. Neste trabalho foram avaliados protocolos adequados para a extração de DNA e a estrutura genética da população de *P. ulei* na região amazônica. Foram comparados seis protocolos de extração em relação a pureza e ao rendimento do DNA. Um total de 61 isolados foram amostrados ao longo das bacias hidrográficas Madeira, Purus e Juruá localizadas na região amazônica e genotipados para 12 locos SSR. O protocolo de extração de DNA de Doyle e Doyle foi o único que apresentou concentração consistente e melhor qualidade do DNA extraído. Houve desequilíbrio de ligação entre os alelos e diferenciação genética entre populações geograficamente distantes foi detectada. A análise de componentes principais revelou agrupamento dos isolados de acordo com os limites das bacias hidrográficas. Foram formados dois grupos, um constituído pelos indivíduos da bacia do rio Madeira e o outro composto por indivíduos dos rios Purus e Juruá.

Introdução geral

A seringueira (*Hevea brasiliensis*) é a principal fonte de borracha natural utilizada nos setores médico/farmacêutico, construção civil, calçados, móveis, autopeças, maquinário industrial e agrícola (Pereira et al., 2000; Bega, 2004; van Beilen e Poirier, 2007). Apesar da composição química da borracha sintética assemelhar-se à da borracha natural, suas propriedades físicas são inferiores e impossibilitam seu uso na produção de vários produtos, como por exemplo, a fabricação de pneus para aviões, automóveis, caminhões e ônibus (Mera, 1977; Santos e Mothé, 2007). Dentre os fatores físico-químicos que fazem da borracha natural um produto insubstituível para diversas aplicações, estão a elasticidade, resiliência, resistência à ruptura, isolamento e impermeabilidade a líquidos e gases (Lieberei, 2007; Mooney, 2009).

Os maiores produtores mundiais de borracha natural são a Tailândia, Indonésia e Malásia, países localizados no sudeste asiático (FAO, 2018). Ásia e África são continentes livres do fungo *Pseudocercospora ulei*, agente etiológico do mal das folhas da seringueira, também chamada de SALB (*South American leaf blight*), a principal doença de *H. brasiliensis*. Existe uma preocupação de pesquisadores com relação à possível introdução dessa doença em plantações da África e, principalmente, da Ásia (van Beilen e Poirier, 2007), uma vez que este continente é responsável por aproximadamente 90% da produção mundial de borracha natural (FAO, 2018) e a maioria dos clones cultivados é considerada suscetível ao SALB (Le Guen et al., 2002). Na América Latina, o fungo devastou cultivos de seringueira estabelecidos no estado do Pará, Brasil, no século XX (Dean, 1987), contribuindo para que o país passasse de exportador para importador. Embora o Brasil ainda apresente uma extensa área para a instalação de seringais e condições edafoclimáticas altamente favoráveis (Gasparotto et al., 1997), a presença de *P. ulei* em todas as regiões produtoras (Gasparotto et al., 1997; 2012) é um dos fatores responsáveis pelo baixo desenvolvimento da heveicultura no país. Além de diminuir a produção do látex, o mal das folhas pode levar a planta à morte quando ocorrem infecções sucessivas (Gasparotto et al., 1989).

O agente causal de SALB pertence ao filo Ascomycota, classe Ascomycetes, ordem Capnodiales e família Mycosphaerellaceae (Hora Júnior et al., 2014). É um fungo hemibiotrófico que provoca doença apenas nas plantas do gênero Hevea

(Junqueira et al., 1986). O cultivo deste patógeno *in vitro* é difícil e, ao longo dos anos, diversos autores têm tentado melhorar metodologias para o crescimento em condições de laboratório (Chee, 1978; Lieberei et al., 1983; Junqueira et al., 1984; 1985; 1986; Mattos, 1999). A melhor estratégia para a obtenção do patógeno é o isolamento realizado de forma direta, a partir da transferência de conídios presentes em lesões foliares recentes, devido a curta viabilidade dos esporos (Junqueira et al., 1984; Gasparotto et al., 1997). Em meio de cultura batata-sacarose-ágar (BSA), a germinação do conídio se inicia após 3h de incubação a 24°C, seguido da rápida emissão do tubo germinativo e crescimento das hifas primárias após 24h (Junqueira et al., 1984). Entretanto, o patógeno apresenta crescimento lento em diversos meios de cultura (Chee, 1978; Lieberei et al., 1983; Junqueira et al., 1984; 1985; 1986; Mattos, 1999) e comumente observa-se apenas a formação de um micélio incipiente com a produção predominante de tecido estromático negro após dois meses (Junqueira et al., 1984). Fungos que produzem estruturas de coloração escura normalmente produzem melanina, que são compostos associados à patogênese e sobrevivência (Kwon-Chung e Rhodes, 1986). No entanto, durante a extração de ácido nucléico, esses compostos constituem um desafio para o isolamento de DNA de boa qualidade e em quantidade adequada (Isla-Flores et al., 2006). A obtenção de DNA puro é o primeiro passo para a realização de estudos baseados em PCR (Reação em Cadeia da Polimerase) (Costa e Moura, 2001), dentre os quais está a utilização de marcadores moleculares para a genotipagem de patógenos biotróficos ou que crescem lentamente (Milgroom, 2015).

Existem diversos marcadores genéticos baseados em PCR, os que são loco não específico e os loco-específico, como os microssatélites. Estes marcadores são sequências simples repetidas em tandem de até seis nucleotídeos em tamanho, localizados ao longo do genoma dos organismos (Milgroom et al., 2015). Em virtude do seu alto polimorfismo e seletividade neutra têm sido muito utilizados para estudos de genética de populações (Ellegren, 2004; Schlotterer, 2004). A genética de populações compreende questões referentes aos processos evolutivos e demográficos inferidos a partir da estrutura populacional, que consiste no padrão de diversidade genética dentro e entre populações (Milgroom, 2015). É preciso conhecer a estrutura genética e os mecanismos que determinam a quantidade e distribuição da variabilidade genética na população de fitopatógenos para entender o desenvolvimento da doença, prever a sua evolução e estabelecer estratégias para o melhoramento visando à resistência e à durabilidade dos clones resistentes selecionados (Milgroom e Fry,

1997). Trabalhos sobre a variabilidade de *P. ulei* foram recentemente conduzidos (Le Guen et al., 2004; Barrès et al., 2012; Hora Júnior, 2012), mas até o momento não existem estudos sobre a genética molecular de populações do patógeno no seu provável centro de origem, a região amazônica. Assim, os objetivos do presente trabalho foram: i. Identificar um protocolo adequado para a extração de DNA de *P. ulei* e, ii. Determinar a estrutura genética populacional de *P. ulei* nas bacias hidrográficas do rio Amazonas.

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ARTICLE 1

Comparison of DNA extraction protocols of *Pseudocercospora ulei*

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Abstract

In vitro cultivation and maintenance of *Pseudocercospora ulei* has limited research about South American leaf blight, a highly destructive fungal disease of rubber tree. The slow vegetative growth of the fungus associated with the predominant stromal formation constitutes a challenge for the extraction of DNA of good quality and in adequate quantity required for different purposes. Thus, the objective of the present work was to assess methods for the extraction of *P. ulei* DNA. Six methods of DNA extraction were compared: 1. High quality DNA extraction; 2. High quality DNA extraction plus melanin removal; 3. Modified high quality DNA extraction; 4. Doyle and Doyle protocol; 5. Modified Doyle and Doyle protocol plus melanin removal and 6. Modified Doyle and Doyle protocol. The concentration of the DNA obtained was measured using fluorescence-based technology, the quality estimated by 260/280 and 260/230 ratios using spectrophotometer and the integrity verified by agarose gel electrophoresis. Additionally, the DNA was amplified with the ITS1 and ITS4 primers. There was variation among the protocols tested, but all yielded good amounts of DNA. Protocol 4 was consistently adequate when comparing concentration and quality, in both runs of the experiment. Thus, the original Doyle and Doyle (1990) protocol can be used for extraction of *P. ulei* DNA.

Key words: South American leaf blight, *Microcyclus ulei*, CTAB, method, purification.

Introduction

South American leaf blight (SALB) is the most destructive disease of rubber tree (*Hevea brasiliensis*) in humid areas of South and Central Americas (Lieberei, 2007). The disease is caused by the fungus *Pseudocercospora ulei*, which infects young leaves inducing repeated defoliation, dieback of the canopy, and death of susceptible clones under favorable environmental condition (Holliday, 1970; Chee and Holliday, 1986; Lieberei, 2007; Gasparotto et al, 2012).

The pathogen can form distinct structures when developing on rubber tree plants (Chee and Holiday, 1986). When deposited in new leaflets, the ascospores absorb moisture, germinate, form the germ tube and produce appresoria, from which the infecting hyphae develop. The pathogen penetrates host tissue directly, colonizing the foliar tissue intercellularly through the parenchyma (Liberei, 2007) and after five or six days, leaflets can have lesions with poorly developed stroma covered by conidia (asexual reproduction). One to five months after infection, ascospores are produced inside pseudothecia immersed in well developed black stroma on the surface of the remaining leaflets (sexual reproduction) (Gasparotto et al., 2012; Hora Júnior et al., 2014). Under controlled conditions, in laboratory and growth chamber, sexual structures arenot formed and the development of asexual structures is not as fast as *in vivo* (Holliday, 1970; Chee, 1978; Junqueira et al., 1984).

The slow development of *P. ulei* has made difficult the research on the biology, epidemiology, and physiology of the pathogen under controlled conditions, particularly in the laboratory (Holliday, 1970; Chee, 1978; Junqueira et al., 1984; Sousa, 2010). In old cultures (30 days or older) stromata are commonly formed (Junqueira et al., 1984). Stromata are a mass of hyphae that are commonly rounded and with a firm consistency that harbor reproductive structures (Krugner and Bachi, 2012). However, *in vitro*, it is common to form a compact mass of hyphae, firm, dark, very slow growing, often developing either downwards (buried) or upwards ("aerial"-like) in the culture medium (Holliday, 1970; Chee, 1978; Chee and Holliday, 1986). Variation in growth pattern with little mycelium and production of more freely conidia can also be observed (Holliday, 1970). Transfers of pieces of stromata older than 20 days may delay the development of subsequent cultures and even suppress the production of mycelia and spores (Junqueira et al., 1984). In 15-day-old cultures or

older there is a decrease in the production of conidia by auto-inhibition. Younger cultures (up to 12-days-old) are more likely to present mycelial growth and a large number of spores can be formed in M4 medium (Junqueira et al., 1984).

The dark color of stromata tissue may indicate the presence of melanin, a pigment commonly observed in many fungi (Beltrán-García et al., 2014). *P. ulei* is currently classified in the Mycosphaerellaceae family (Hora Júnior et al., 2014) together with *Mycosphaerella fijiensis* which also forms stromata rich in 1,8-dihydroxynaphthalene (DHN)–melanin (Beltrán-García et al., 2014). Melanin is negatively charged (anionic), which implies the presence of a large amount of free carboxylic acid residues in its polymer, and as a result they bind to the nucleic acid during the homogenization of mycelium (Hearing, 1999). This binding generally results in the co-precipitation with DNA and RNA and absorption of ultraviolet light. This can interfere with the polymerase chain reaction, cDNA synthesis and induce errors in the interpretations of nucleic acid quantification (Eckhart et al., 2000; Satyamoorthy et al., 2002).

Good quality DNA in proper amounts are key features for many applications in genomics. Thus, methods for efficient DNA extraction and capable of producing pure molecules, free of inhibitors, are in high demand (Fulton et al., 1995; Romano and Brasileiro, 1999; Costa and Moura, 2011). Some molecular analysis require good DNA quantity and quality (Oliveira et al., 2013). For the analysis of short sequences repeats (SSR) or microsatellites and sequencing, for example, the requirements for quantity and quality are not so stringent: small amounts of relatively low quality DNA can be used. On the other hand, for other tasks such as whole genome sequencing, restriction site-associated DNA sequencing (RADseq), genotyping by sequencing (GBS) and restriction fragment length polymorphism (RFLP), high amounts of high quality DNA must be provided (Liesack et al., 1991; Wintzingerode et al., 1997).

Different DNA isolation protocols, which vary according to the organism and tissue of interest, can be promptly found in the literature (Dellaporta et al., 1983; Wilson et al., 1992; He et al., 1995; Prakash et al., 1996). Most of these protocols are based on the use of the cationic detergent cetyl trimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1990; Ferreira and Grattapaglia, 1998; Mercado et al., 1999). CTAB solubilizes membranes and forms a complex with DNA facilitating precipitation (Weising et al., 1995) and removal of polysaccharides and proteins (Carninci and Hayashizaki, 1999; Knapp and Chandlee, 1996; Lagonigro et al., 2004).

Mixed alkyl trimethyl ammonium bromide (MATAB) is another detergent used for DNA extraction, usually added to the lysis buffer (Wilson et al., 1992; He et al., 1995; Prakash et al., 1996), but has also been used in the purification of the aqueous phase (Rodrigues et al., 2007). The use of 2% MATAB in the aqueous phase purification of *Bixa orellana* RNA was efficient to avoid co-precipitation of polysaccharides and polyphenols, resulting in the precipitation of nucleic acids free of these contaminants (Rodrigues et al., 2007). Protocols with similar components have been used to extract DNA of *P. ulei* (Richards et al., 1994; Rivas et al. 2004; Bourassa et al., 2005).

Multiple criteria are used to evaluate the efficiency of DNA extraction methods, including, but not restricted to, yield, reproducibility and representativeness of the isolated DNA (Yuan et al., 2012). Due to the inherent characteristics of each fungus such as cell wall composition it is necessary to consider a specific protocol that allows obtaining DNA of quality and, if possible, with high yield (Manian et al., 2001; Selitrennikoff 2001; Yeo and Wong, 2002). According to Manian et al. (2001), each species may require a particular protocol that is efficient for DNA isolation since there is no adequate method of cell lysis for all fungi. Selecting an appropriate DNA extraction protocol for the species of interest may be difficult in view of the variety of methods available. To date, there is no standardized DNA isolation protocol for *P. ulei*, which may be linked to the difficulty for *in vitro* cultivation and maintenance of this fungus due to its slow growing nature and short viability. Therefore, the aim of this study was to identify DNA extraction protocols suitable for the isolation of DNA from *P. ulei.*

Material and methods

Sampling and isolation of *Pseudocercospora ulei*

Samples of leaves with typical SALB symptoms were collected according to a W- walking pattern in rubber tree plantations in the state of Amazonas, Brazil. Conidia were transferred from the lesions to M4 culture medium (Junqueira et al., 1985) under the stereoscopic microscope and plates were kept in the dark for 30 days at 25 ± 1°C. Approximately 100 mg of stromata were removed from the culture medium and macerated in 500 µl of water, spread on Petri dishes containing M4 medium and cultivated for 12 days at 25 ± 1°C under a specific regime of daily alternation of two

cycles of 1h light and 3h of dark, 1h light, 3h of dark (totaling 2h light and 6h dark), followed by 1h light and 15h of dark to stimulate the conidial production (Junqueira et al., 1985). Spores were used to prepare a suspension which was then plated in water-agar medium and incubated for 5h for conidia germination. Single conidia was transferred to M4 medium and incubated at 25 ± 1°C with 12h photoperiod. Among the monosporic isolates available, three were randomly selected for the experiments: AM18, RPE2 and MAN3P4.

Cultivation conditions and sample preparation

Stroma fragment, approximately 100 mg, of each isolate was macerated and transferred to erlenmeyers containing M4 liquid medium, which were kept in an orbital shaker at 120 rpm for 15 days in the dark at 24°C. Fungal biomass produced was dried at room temperature for 24 h, ground in liquid nitrogen until it became a fine powder and frozen at -80°C. Twenty five milligrams of macerated biomass from each sample were used for each repetition in all DNA extraction protocols.

Methods of DNA extraction

Six DNA extraction protocols described in the literature were modified as necessary and evaluated for *P. ulei* (Table 1). All experiments were conducted with five replicates (one erlenmeyer with culture = one replicate) of each sample. The experiment was conducted in two different occasions.

Protocol 1- High quality DNA extraction (Spanu et al., 1995). Fungal tissue (25 mg) and 1.168 ml of lysis buffer (430 µl of buffer A – Sorbitol, Tris-HCl pH 9, EDTA, pH 8; 430 µl of buffer B – Tris-HCl pH 9, EDTA pH 8, NaCl, 2% CTAB; 170 µl of buffer C - N- lauroylsarcosine sodium salt; 130 µl of PVP; 8 µl of Proteinase K) were added to a microtube, mixed by vortexing and incubated at 65°C for 30 min. After incubation, 385 µl of KAc (5M, pH 7.5) was added and the tubes were immediately placed on ice for 30 min. The mixture was homogenized and centrifuged at 5,000 g for 20 min at 4°C. The supernatant was removed and transferred to another microtube containing 1 ml of chloroform-isoamyl alcohol (24:1 v/v). The mixture was stirred for 10-15 s by vortexing and centrifuged at 4,000 g for 10 min at 4°C. The samples were treated with RNAse A (100 µg/ml) and incubated in dry bath for 2 h at 37°C. DNA was precipitated with 90 µl of sodium acetate and 900 µl of isopropanol at room temperature and

incubated for 5 min at room temperature. The samples were centrifuged at 10,000 g for 30 min at 4°C. The "pellet" was washed with 300 µl of 70 % ethanol and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was carefully removed and discarded. The "pellet" was kept at room temperature to dry and resuspended in 30 µL of TE (Tris-EDTA) buffer at 65°C for 2 h and immediately stored at −80°C.

Protocol 2- High quality DNA extraction (Spanu et al., 1995) *plus melanin removal* (Lagonigro et al. 2004): Genomic DNA was extracted following the procedure described in protocol 1, with additional steps for melanin removal as described by Lagonigro et al. (2004). An aliquot of 200 µl of water, 65 µl of NaCl and 800 µl of CTAB-Urea solution (50 mM Tris-HCl, pH 7.0, 1% CTAB, 4 M urea, 1 mM EDTA) were added to DNA previously extracted. The solution was mixed gently and incubated at 4°C overnight. The mixture was centrifuged at 12,000 g for 15 min at 4 °C for DNA precipitation. The "pellet" was resuspended with 200 µl of guanidine hydrochloride and 400 µl of 100% ethanol. The mixture was incubated for 1 h on ice and centrifuged at 12,000 g for 15 min. After centrifugation, the supernatant was discarded and the DNA "pellet" was washed twice with 70% ethanol, centrifuged and dried at room temperature. DNA was resuspended in 30 µl of TE buffer at 65°C for 2 h and stored at −80°C.

Protocol 3- Modified high quality DNA extraction. A modified version of protocol 1. Lysis buffer (1.168 ml) was added to 25 mg of fungal tissue and the mixture was kept for 30 min at 65°C, inverting the tubes at 5 min intervals. Then, the polysaccharides were precipitated with 385 µl of KAc on ice for 30 min and centrifuged at 5,000 g for 15 min at 4°C. After centrifugation, 300 µl of 2% MATAB and 300 µl of chloroform-isoamyl alcohol (24:1) mixture were added to the microtube and centrifuged at 4,000 g for 10 min at 4°C for the separation of phases. This step was repeated if the supernatant had dark color (light to dark brown). The top aqueous phase was transferred to a clean 2 ml microtube containing 10 µl of RNAse A (final concentration of 100 µg/ml) and incubated at 37°C for 2 h. DNA was precipitated with 100 µl of NaAc and 1000 µl of cold isopropanol and the mixture was centrifuged at 10,000 g for 15 min at 4°C. The DNA samples were washed twice with 500 µl of 70% ice-cold ethanol, centrifuged at 10,000 g for 10 min at 4°C and resuspended in 30 µl of TE buffer at 65°C for 2 h.

Protocol 4- Doyle and Doyle extraction (1990). Fungal material was ground in liquid nitrogen and incubated in 800 µl of the isolation buffer (100 mM Tris-HCl, pH 8; 1,4

M NaCl, 20 mM EDTA; 2% CTAB; 0,2% 2-mercaptoethanol) at 60°C for 60 min. After incubation, 800 µl of chloroform-isoamyl alcohol (24:1 v/v) was added to the mixture to separate the phases, and centrifuged at 7,000 g for 10 min at 4°C. DNA precipitation was obtained after adding 600 µl of cold isopropanol and centrifugation at 3,000 g for 2 min at 4°C. The DNA "pellet" was kept in the wash buffer (76% ethanol, 10 mM ammonium acetate) for 20 min, centrifuged at 4000 g for 2 min and resuspended in 50 µl of resuspension buffer (10 mM ammonium acetate; 0,25 mM EDTA). For RNA removing, 6 µl of RNAse A (final concentration of 10 $\mu g \cdot mL^{-1}$) were added to the solution and kept in dry bath for 30 min at 37°C. DNA was precipitated with a mix of 200 µl of water, 150 µl of ammonium acetate and 1000 µl of ethanol, centrifuged at 10,000 g at 4° C, dry at room temperature, resuspended in 30 µl of TE buffer at 65°C for 2 h and stored at -80°C.

Protocol 5- Modified Doyle and Doyle protocol (1990) *plus melanin removal* (Lagonigro et al., 2004). Protocol 4 was used with an additional procedure for melanin removal. Briefly, 200 µl of water, 65 µl of NaCl and 800 µl of CTAB-Urea solution were added to DNA extracted and the mixture was incubated overnight at 4°C. DNA was precipitated and centrifuged at 12,000 g for 15 min at 4°C. The pellet was resuspended in 200 µl of guanidine hydrochloride and 400 µl of 100% ethanol. This mixture was kept for 1 h on ice bath and centrifuged at 12,000 g for 15 min. The supernatant was discarded and the DNA "pellet" was washed twice with 70% ethanol, centrifuged at 12,000 g for 10 min at 4º C and dried at room temperature. DNA was resuspended in 30 µl of TE buffer at 65°C for 2 h and stored at -80°C.

Protocol 6- Modified Doyle and Doyle protocol. Protocol 4 was used with minor modifications. Each sample containing 2X CTAB buffer was incubated at 60°C for 60 min. After incubation, 300 µl of 2% MATAB and 300 µl of chloroform-isoamyl alcohol (24:1 v/v) was added to the mixture and centrifuged at 7,000 g for 10 min at 4° C. The aqueous phase was transferred to a new microtube containing 800 µl of cold isopropanol and centrifuged at 3000 g for 2 min at 4°C for "pellet" formation, which was kept in the wash buffer for 20 min in room temperature. The suspension was centrifuged at 4000 g for 2 min at 4° C. The top phase was discarded and DNA "pellet" resuspended in 50 µl of resuspension buffer (same buffer described in protocol 4) with addition of 8 µl of RNAse A (final concentration 100$\mu g \cdot mL^{-1}$). This solution was incubated at 37° C for 30 min and 200 µl of water, 150 µl of ammonium acetate and

1000 µl of ethanol were added. The mix was centrifuged at 10,000 g at 4° C for DNA precipitation, dried at room temperature, resuspended in 30 µl of TE buffer at 65°C for 2 h and stored at -80° C. DNA integrity and quantification.

The integrity of the DNA was verified by horizontal electrophoresis at 80 V for 2 h, on 0.8% agarose gel followed by staining with Bromophenol Blue dye and the GelRED® intercalating agent, in 1X TBE buffer (0.089M Tris base, 0.089M Boric acid, 0.002M EDTA pH 8.0). The band patterns were compared with λ-phage DNA marker (100 ηg.µL^{-1}). The purity of the DNA was evaluated using the 260/280 and 260/230 ratios in NanoDrop 2000 spectrophotometer (Thermo Scientific®). Additionally, the quantity of genomic DNA was determined by using the fluorescence-based Qubit technology as described by the manufacturer (Thermo Scientific®).

Amplification through PCR

To confirm the integrity and quality of the extracted DNA, a sample of each combination isolate-protocol of extraction (treatment) was tested by PCR amplification with the ITS1 and ITS4 primers that amplify the region around ITS1, 5.8S, and ITS2. PCR reactions were conducted at a final volume of 12.5 µl, containing 1 µl DNA (20 ng.µL-1), 6.2 µl of Milli-Q H_2O, 0.5 µl DMSO (Dimethyl sulfoxide), 0.5 µl dNTP's (2.5 mM each), 0.5 µl Forward Primer (5 pmoles.mL^{-1}), 0.5 µl Reverse Primer (5 pmoles.mL^{-1}), 0.75 µl $MgCl_2$ (50 mM), 2.5 µl 5X Colorless GoTaq® Reaction Buffer and 0.05 µl Taq DNA polymerase (5 U.µL^{-1}). The conditions were optimized according to the recommendations for the Promega GoTaq® enzyme. The annealing temperature was set at 58°C.

The resulting amplification products were stained with GelRED® intercalating agent and separated by electrophoresis using 1X TBE buffer on a 2% agarose gel at 100 V for 60 min. The 100bp DNA ladder (Invitrogen) was used as molecular weight pattern.

Data analysis

Kruskal-Wallis test was conduced to evaluate the effect of extraction protocols on DNA concentration in the experiments 1 and 2. All analyses were processed with R version 3.4.3 program (Boettiger and Eddelbuettel, 2017; R Core Team, 2017).

Results

There was variation regarding the extraction protocol that provided higher yield of DNA between the two experiments repeated in time. In the experiment 1, the highest amount of DNA was obtained with the modified high quality DNA extraction protocol (protocol 3) and the least efficient was the DNA extraction protocol of Doyle and Doyle (protocol 5). In the second experiment, the best protocol was 5 and the methods of high quality DNA extraction (protocol 1) and protocol 3 extracted the least amounts of nucleic acid (Figure 1). Although protocols 3 and 5 showed a higher concentration of extracted DNA, they did not differ significantly from high quality DNA extraction plus melanin removal (protocol 2) and Doyle and Doyle extraction methods (protocol 4), for which consistent behavior was observed in both the experiments 1 and 2 (Figure 1).

In the two experiments conducted, the best 260/280 nm ratio was reached by protocols 4 and 6. High absorbance was detected for DNA extracted with protocol 1 (> 2.0) and a very low ratio with protocol 5 (<1.8). The results for protocol 3 varied between experiments (Figure 2). The 260/230 nm ratio was satisfactory for protocol 4 and it was very low for protocol 5 (<2.0). Protocols 1 and 3 also had a 260/230 nm ratio < 2.0, unlike the values found for 2 and 6 (> 2.2) (Figure 3).

The integrity of the extracted DNA samples was analyzed on agarose gel by electrophoresis and all the isolation methods had a DNA band. The intensity of the bands varied between the extraction protocols and between the isolates. RNA bands were detected in samples extracted with protocols 1, 2, and 3 (Figure 4).

In relation to the PCR amplification with the ITS1 and ITS4 primers, all samples had PCR amplicons. The PCR products were of approximately 600 bp. All appeared as a well defined band on gel, consistent and free of impurities (Figure 5).

Discussion

Each extraction protocol had its own pros and cons, although all methods of isolation have produced relatively large amounts of DNA. The good performance of a given protocol is probably related to the use of CTAB in lysis buffer since when incorporated into the extraction buffer it removes membranes, lipids and promotes cell lysis even though the tissue is rich in polysaccharides and contaminated by

excess metabolites such as fungal structures (Sambrook and Russell 2001, Michiels et al 2003, Xu et al 2004, Athanasio et al, 2016). In addition, it still removes proteins from DNA (Moore and Dowhan 1995). Although protocols 3 and 5 have been outstanding in terms of DNA concentration, it is difficult to consider them to be the best protocols due to the variation observed between the experiments. The difference in the results obtained for the two experiments on DNA yield is probably associated with the growth of the fungus that, under *in vitro* conditions, produces incipient mycelium, conidia and stromal tissue (Junqueira et al., 1984). Therefore, three hypotheses can be raised about the differences in results: i. The presence of a greater amount of stromal tissue, conidia or mycelium in each repetition can influence the concentration of extracted DNA; ii. The successive repications decreased the mycelial production and sporulation of the pathogen in some repetitions, and stromal material predominated, consequently, there was variation in the amount of extracted DNA; and iii. The stroma is melanized and the variation in the amount of melanin in the different isolates resulted in fluctuations between the experiments.

The first hypothesis seems to be more appropriate because, although the fungal biomass used in each repetition has gone through the same process before the extraction, it is not possible to know exactly what type of structure and how much they contributed as fungal biomass in each repetition. In addition, in some organisms the number of copies of the chromosome may vary according to the growth stages (Cooper and Helmstetter, 1968; Donachie, 2001). Thus, it is necessary to try a standardization of the number of chromosome copies of the in initial tissue to be used for DNA. One way to do this would be to extract DNA from the different structures produced by the pathogen, separately.

Protocols 2 and 4 were consistent in both experiments and, thus, are the most representative regarding DNA concentration. In addition, total genomic DNA extracted with protocol 4 showed good quality considering that values for the 260/280 nm (1.8 - 2.0) and for the 260/230 nm ratio (2.0 - 2.2) were obtained. On the other hand, for protocol 2 values above the expected absorbance range were observed. The values should be between 1.8 and 2.0 and between 2.0 and 2.2 for the 260/280 and 260/230 nm ratios, respectively. Values outside this range indicate the presence of proteins, phenol or other contaminants that are absorbed by the 280 and 230 nm peaks (Teare et al., 1997). The DNA obtained with protocols 1 and 5 did

not have high purity, since 260/280 and 260/230 nm ratios were observed to be lower or slightly higher than the tolerated range. Variations in the 260/280 nm ratio are associated with small changes in the pH of the DNA solution. When the 260/280 nm ratio is underrepresented and overrepresented by 0.2-0.3, the DNA is likely to be found in acid solutions and basic solutions, respectively (Wilfinger et al., 1997). If the 260/280 nm ratio is less than 1.8, protein contamination or the presence of organic contaminants such as phenol or other aromatic compounds is suggested. However, no phenol was used in any of the protocols tested in the present study, so it is likely that the samples are contaminated with some type of protein. The 260/230 nm ratio appreciably less than 1.8 indicates the presence of contaminants having absorbance at 230 nm, such as the TE buffer (Wilfinger et al., 1997) or some type of salt (Dilhari et al., 2017). The potential presence of salts at high concentrations in DNA samples can be explained by the use of sodium acetate during the precipitation phase of nucleic acid in protocols 1 and 2 and the use of sodium chloride in protocol 5.

RNA bands were observed in protocols 1, 2, and 3, even after treating samples for a relatively long time for RNA removal and using a higher concentration of RNAse than in other protocols. The purity of the DNA samples is reduced when RNA is present (Romano and Brasileiro, 1999). The extracted DNA should be free of contaminants including the presence of proteins, carbohydrates, lipids, RNA and any other cellular constituents that may inhibit the activity of restriction enzymes, ligase and thermostatic DNA polymerases (Asadzaheh et al., 2010). It is necessary to adjust the RNAse treatment to improve the evaluation of the different protocols.

Although all protocols resulted in suitable amounts of DNA for amplification, only protocol 4 can be recommended at this time to be used in molecular studies that require high-quality DNA of *P. ulei*. This is a well-known protocol used for plant and fungal materials (Corrêa et al., 2013). The reagents used and the procedures adopted can be easily found or executed in laboratories on a routine basis. The other protocols can be used for other purposes that are not so stringent on DNA quality. However, except for protocol 4, none of the methods tested in this study were consistent. This result resembles studies in which researchers have concluded that even for specific samples, differences in yield and purity of DNA may occur (Artyukhin and Woo, 2012, Maaroufi et al 2004, Mirnomeni et al. 2010.

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Figure legends

Figure 1. Concentration of *Pseudocercospora ulei* DNA using different extraction protocols. 1: High quality DNA extraction; 2: High quality DNA extraction plus melanin removal; 3: Modified high quality DNA extraction; 4: Doyle and Doyle extraction; 5: Modified Doyle and Doyle protocol plus melanin removal; 6: Modified Doyle and Doyle protocol. Means followed by the same letter do not differ statistically by the Kruskal-Wallis test. Bars indicate the standard error.

Figure 2. Distribution of the DNA quality averages of *Pseudocercospora ulei,* evaluated by the 260/280 ratio in experiment 1 (black boxplot) and experiment 2 (gray boxplot). 1: High quality DNA extraction; 2: High quality DNA extraction plus melanin removal; 3: Modified high quality DNA extraction; 4: Doyle and Doyle extraction; 5: Modified Doyle and Doyle protocol plus melanin removal; 6: Modified Doyle and Doyle protocol.

Figure 3. Distribution of the DNA quality averages of *Pseudocercospora ulei,* evaluated by the 260/230 nm ratio in experiment 1 (black boxplot) and experiment 2 (gray boxplot). 1: High quality DNA extraction; 2: High quality DNA extraction plus melanin removal; 3: Modified high quality DNA extraction; 4: Doyle and Doyle extraction; 5: Modified Doyle and Doyle protocol plus melanin removal; 6: Modified Doyle and Doyle protocol.

Figure 4. Integrity of the total DNA extracted from the dry biomass of three isolates of *Pseudocercospora ulei* using six DNA extraction protocols. L: DNA ladder at 100 $ng.\mu L^{-1}$; 1: High quality DNA extraction; 2: High quality DNA extraction plus melanin removal; 3: Modified high quality DNA extraction; 4: Doyle and Doyle extraction; 5: Modified Doyle and Doyle protocol plus melanin removal; 6: Modified Doyle and Doyle protocol. I1: Isolate AM18, I2: Isolate RPE2; I3: Isolate MAN3P4.

Figure 5. PCR amplified product with primers ITS1 and ITS4 using genomic DNA of three isolates of *Pseudocercospora ulei* extracted using different protocols. L: 100 bp DNA marker; C: Negative control; 1: High quality DNA extraction; 2: High quality DNA extraction plus melanin removal; 3: Modified high quality DNA extraction; 4: Doyle and Doyle extraction; 5: Modified Doyle and Doyle protocol plus melanin removal; 6: Modified Doyle and Doyle protocol. I1: Isolate AM18, I2: Isolate RPE2; I3: Isolate MAN3P4.

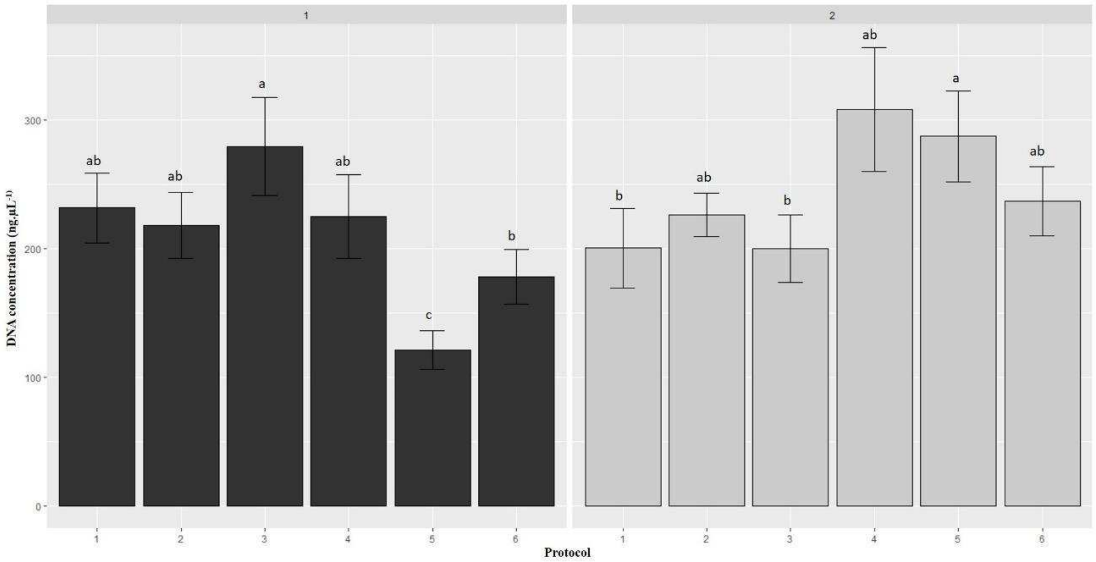

Figure 1. Santos et al

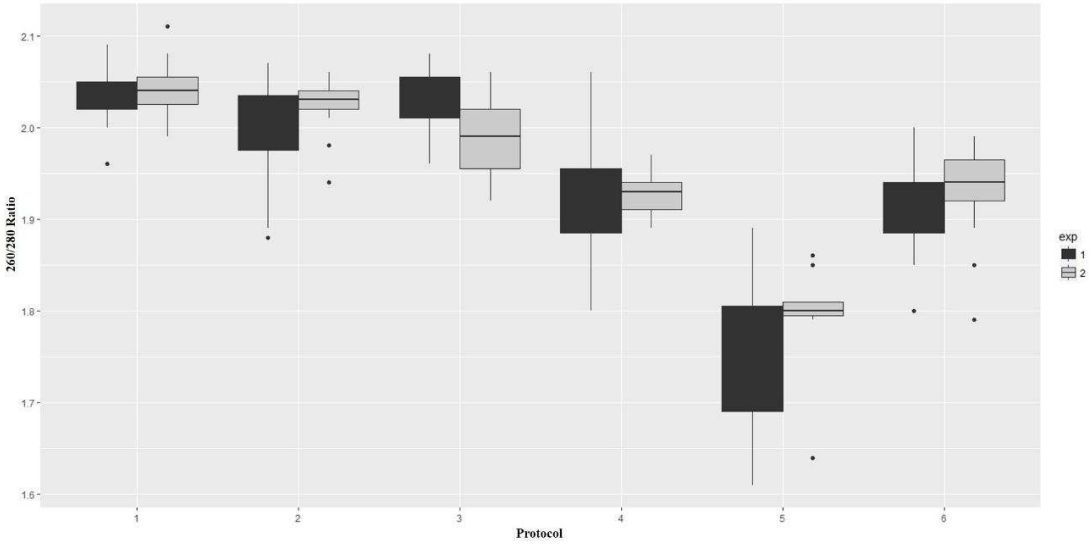

Figure 2. Santos et al.

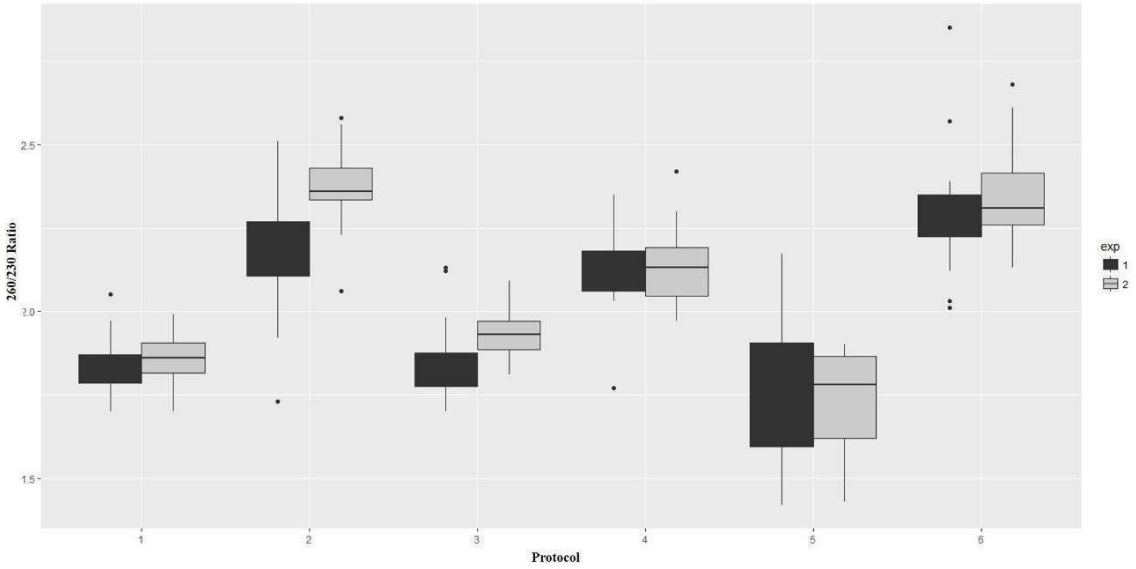

Figure 3. Santos et al.

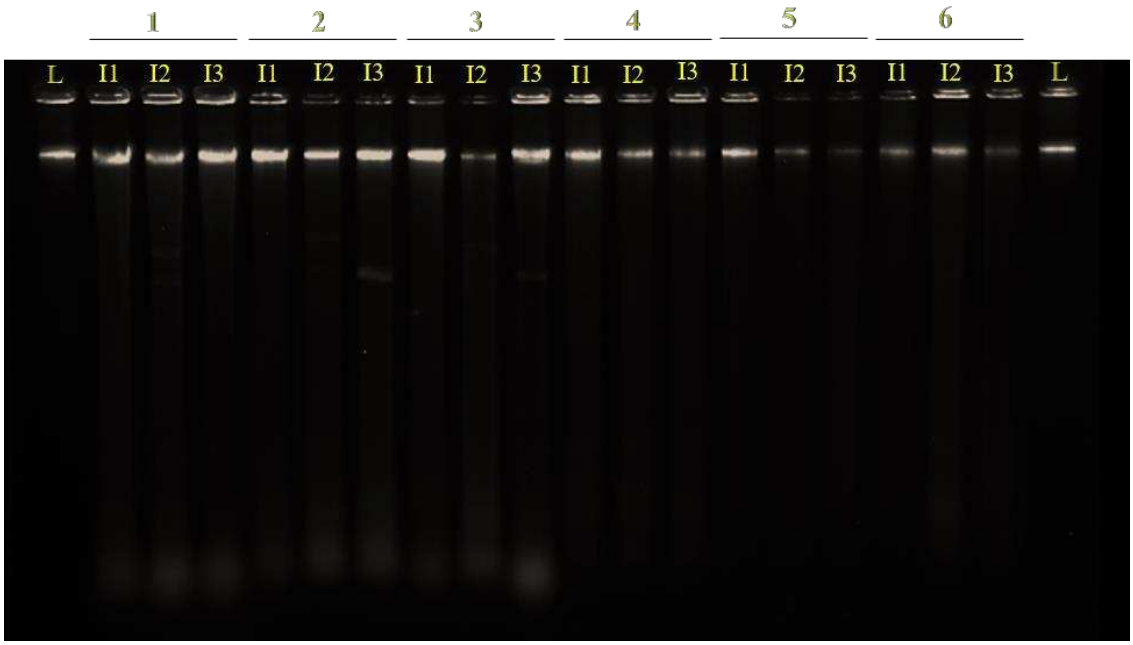

Figure 4. Santos et al.

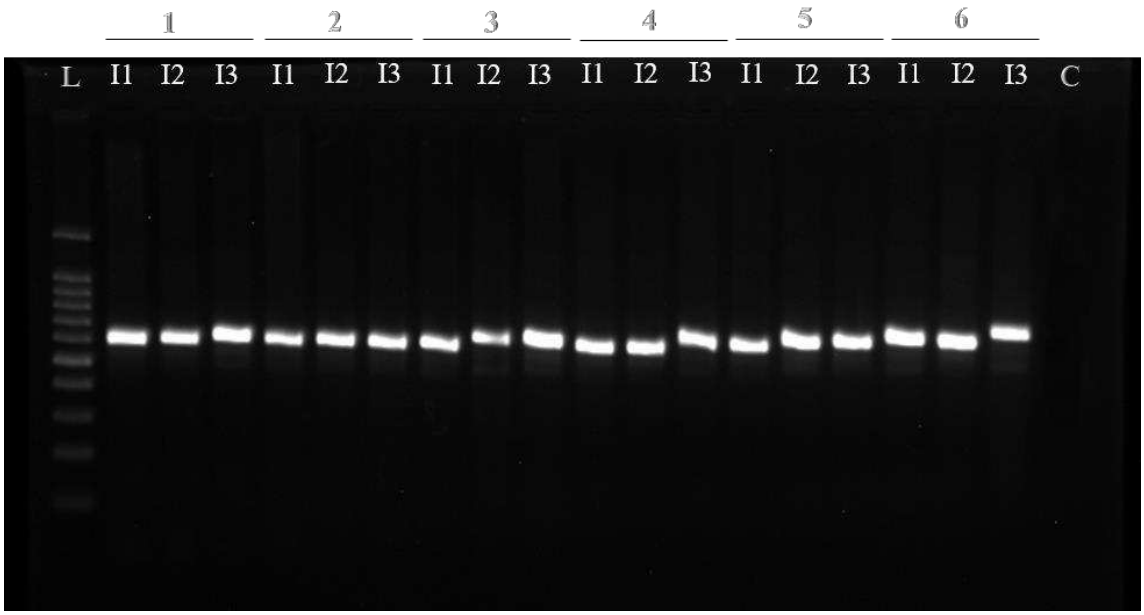

Figure 5. Santos et al.

Table 1. Summary of various DNA extraction protocols.

Protocols	References	Chemical lysis	Purification	Precipitation	Melanin removal
1	Spanu et al., 1995	A	C	E	-
2	Spanu et al., 1995 and Lagonigro et al., 2004	A	C	F	+
3	Spanu et al., 1995 modified	A	D	E	-
4	Doyle and Doyle, 1990	B	C	F	-
5	Doyle and Doyle, 1990 and Lagonigro et al., 2004	B	C	F	+
6	Doyle and Doyle, 1990 modified	B	D	F	-

A - Sorbitol, Tris-HCl pH 9, EDTA, pH 8; Tris-HCl pH 9, EDTA pH 8, NaCl, 2% CTAB; N-lauroylsarcosine sodium salt; PVP; Proteinase K. B - 100 mM Tris-HCl, pH 8; 1,4 M NaCl, 20 mM EDTA; 2% CTAB; 0,2% 2-mercaptoethanol. C - Chloroform-isoamyl alcohol (24:1 v/v). D - 2% MATAB; Chloroform-isoamyl alcohol (24:1 v/v). E - Isopropanol. F- Ethanol.

ARTICLE 2

Population structure of *Pseudocercospora ulei* in the center of origin of the pathogen: the Amazon river basin.

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Abstract

Pseudocercospora ulei is a haploid fungus that causes South American leaf blight (SALB) of rubber tree in the Amazon region and is a potential threat to the global supply of natural rubber if introduced in Asia and Africa. Despite the importance of SALB, there is no information on the genetic variability of the pathogen in its putative center of origin: the Amazon river basin. Therefore, the genetic structure of *P. ulei* populations in the Amazon region was studied using microsatellite markers. A set of 61 monosporic isolates were obtained from planted rubber trees distributed in three tributaries of the Amazon river: Madeira, Purus and Juruá rivers. Isolates were genotyped with a set of 12 microsatellite loci. All loci were polymorphic with a mean of 5.9 alleles per locus. The G''_{ST} suggested that there is a group formed by isolates collected near the Madeira river and a second group with isolates from the Purus and Juruá watersheds. This differentiation between populations can be explained by isolation by distance or due to different native rubber tree species present in each of these regions. The structuring of the *P. ulei* population resembles that of *Hevea* spp.

Key words: South American leaf blight, population genetics, *Microcyclus ulei*, molecular epidemiology, SSR

Introduction

Natural rubber is a commodity of high commercial value used in more than 40 thousand products in the global industry, including medical-pharmaceutical devices, surgical gloves, aircraft tires and several engineering and consumer products (Lieberei et al., 2007; Gasparotto et al., 2012). The main source of natural rubber is *Hevea brasiliensis*, a species native to the Amazon region (Gonçalves et al., 1983; Van Beilen and Poirier, 2007). Due to the great applicability of the raw material for different products, there is an increasing global demand for natural rubber (IRSG, 2017). Asia is responsible for more than 90% of the world's natural rubber production, while Latin America produces only 3%, mainly from Brazil and Guatemala (FAO, 2018). Although Brazil has a large area available for planting rubber trees (Gasparotto et al., 1997), yields are limited due to epidemics of South American leaf blight (SALB), an endemic fungal disease in countries that grow rubber trees in Latin America (Páez et al., 2015).

SALB, also known as leaf blight, is caused by a foliar fungal pathogen previously named *Microcyclus ulei* (Holliday, 1970; Chee and Holliday, 1986; Lieberei, 2007; Van Beilen and Poirier, 2007) and currently known as *Pseudocercospora ulei* (Hora Júnior et al., 2014). The fungus was first observed in the Amazon region, its probable center of origin, in 1900 in the state of Amazonas, Brazil (Hennings, 1904). *P. ulei* is present in all rubber growing regions of the country (Gasparotto et al., 1997; Mattos et al., 2003) and under favorable environmental conditions can induce successive defoliation, dieback of the canopy and death of susceptible *Hevea* clones (Holliday, 1970; Chee and Holliday, 1986; Lieberei, 2007).

Due to the severe restrictions in control strategies, the deployment of resistant clones is considered to be the best option to manage the disease (Garcia et al., 2004). Breeding programs for SALB resistance were developed in tropical America, Africa and Asia, but due to the instability of resistance and the low yield of the selected materials, few commercial clones were recommended. The breeding program CIRAD-Michelin- Brazil (CMB) (Le Guen et al., 2002; Garcia et al., 2004; Garcia et al., 2011), was successful in obtaining resistant clones as productive as the Asian ones (Rivano et al., 2010). These clones can be cultivated in areas conducive to SALB (Garcia et al., 2011) or in escape areas. Growing resistant clones is an interesting preventive strategy to reduce the economic impact anticipated to be generated by the introduction of the pathogen in disease-free areas (Garcia et al., 2004). Although moderately resistant

rubber clones are available (Gasparotto et al., 2012), the success, durability, and large-scale implementation of control measures depend on detailed knowledge of the evolutionary, biological and genetic aspects of the disease causal agent and its populations (McDonald and Linde, 2002).

Genetic structure comprises the quantification of genetic variation and its distribution within and between populations (Milgroom and Peever, 2003). Knowledge of the genetic structure is important to infer the evolutionary potential of a population, since it can provide an insight into the risk of plant pathogens developing resistance to fungicides and/or antibiotics, or overcome the resistance of cultivars (McDonald and Linde, 2002). Breeding programs for the development and strategic use of resistant varieties will have higher chances to succeed if the geographical distribution of pathogen genotypes is carefully evaluated; when host varieties are challenged with representative genotypes; when there is information about movement of genotypes and the risk of introduction into an area; and the dynamics of genotype frequencies over time(Peever et al., 2000).

Although research on obtaining SALB resistant clones has progressed over time (Peralta et al., 1990; Garcia et al., 2002), most activities were performed without knowledge of the genetic variability of the population. Parameterization of the evolutionary mechanisms responsible for shaping genetic variation in the population is the first step in the study of the population biology of the pathogen. The use of genetic markers allows inference on the evolutionary mechanisms: mutation, selection, migration (gene flow), recombination, and genetic drift (McDonald, 1997).

There are several molecular markers that are used for genetic studies of fungal populations, but the microsatellites or simple sequence repeats (SSR), have been intensively used due to the high level of polymorphism, locus-specificity and codominant nature (Dutech et al., 2007). These markers can detect high levels of variation, which contributes to increase the resolution of genealogical and genetic variability studies (Caixeta et al., 2009). In recent years, microsatellite markers have been used to study the genetic variability of populations of several plant pathogenic fungi, such as *Rhizoctonia solani* (Zala et al., 2008), *Mycosphaerella fijiensis* (Yang and Zhong, 2008), *Sclerotinia sclerotiorum* (Gomes et al., 2011; Lehner et al., 2015), *Zymoseptoria tritici* (Suffert et al., 2015), among others (Tucker et al., 2015; Das et al., 2016; Kolmer et al., 2017).

For a long time, *P. ulei* isolates from different geographic regions were characterized by inoculation into a range of *Hevea* spp. clones to examine pathogenic variation ("races") (Junqueira et al., 1986; Rivano, 1997; Mattos et al., 2003; Bevenuto et al., 2017). However, in recent years the genetic variability of the populations of the pathogen has been investigated using microsatellite markers (Le Guen et al., 2004; Barrès et al., 2012; Hora Júnior et al., 2012). Fifteen populations of *P. ulei* were sampled in the main Brazilian rubber producing regions. Using small and large spatial scale analytical tools, high genetic variability was found among spatially structured populations and rapid genetic differentiation in sympatric populations of the pathogen due to selection imposed by partially resistant hosts (Hora Júnior, 2012). Barrès et al. (2012) using a set of 16 microsatellite markers, inferred that the most probable scenario for the establishment of *P. ulei* populations sampled in Ecuador, Costa Rica, French Guiana and Brazil (a single population from south of Mato Grosso state) was from a non-sampled area in the Amazon. Therefore, despite being an interesting study, the results were not conclusive due to sampling limitations in potentially informative areas.

To date, no molecular analysis to uncover the diversity and evolutionary potential of the causal agent of SALB in its center of origin, has been performed. As *P. ulei* is supposedly originated from the Amazon, a wider sampling of this region will enable the diversity of its (sub) populations to be better understood, to determine how the pathogen populations evolved in the center of origin, and the implications of these changes for disease management. Therefore, the aims of this study were to determine the genetic structure of the *P. ulei* population in the Amazon river basin.

Material and methods

Sample collection, isolation and extraction of DNA

Leaves with typical symptoms of SALB were collected from active or remaining commercial plantations of rubber tree located in Acre (AC) and Amazonas (AM) states, between 2010 and 2017. Thirty-four isolates from AC were obtained from: Bujari (n = 5), Mâncio Lima (n = 4), Rio Branco (n = 8), Rodrigues Alves (n = 1) and Xapuri (n = 16). Twenty-seven isolates from AM were sampled from: Beruri (n = 2), Boca do Acre (n = 7), Eirunepé (n = 5), Manaus (n = 3), Manicoré (n = 7) and Rio Preto da Eva (n = 3) (Table 1). The number of individuals obtained per municipality

varied according to disease incidence and field size. Although several cultures were attempted from each field, the difficulties to grow the pathogen *in vitro* prevented the standardization of the same number of isolate for each location. In dense plantations, the sampling was accomplished according to a W-walking pattern and in each area the latitude and longitude information for each sample were recorded for geographic distribution analysis of the isolates.

Conidia were collected from lesions under the microscope. Conidia were plated on M4 culture medium (Junqueira et al., 1984) and grown for 30 days at 25 ± 1°C in the dark. The resulting stroma was macerated in 500 µL of mili-Q sterilized water to promote the release of fungal structures and to obtain a suspension that was poured on solid M4 medium and kept for 12 days at 25 ± 1°C under a regime of two daily cycles of light. The first cycle was: 1h of light and 3h of dark, 1h of light, and 3h of dark. The first cycle totaled 2h of light and 6h of dark, followed by the second cycle: 1h light and 15h dark to stimulate the production of conidia of the pathogen (Junqueira et al., 1985). To obtain monosporic isolates, 5 ml of sterilized distilled water plus one drop of Tween 20% were added to the conidia produced and an aliquot of the suspension was transferred to water-agar. After 5h of incubation, a block of culture medium containing only one germinated conidium was cut, placed on M4 medium, and incubated at 25 ± 1°C with photoperiod of 12h until colony development.

The monosporic isolates were transferred to liquid M4 medium and flasks were placed on an orbital shaker at 120 rpm in the dark at 24°C during 15 days. The fungal biomass was then dried at room temperature for 24 h, ground in liquid nitrogen and kept at -80 °C until DNA extraction, which was performed as described for Spanu et al. (1995). The DNA quality and quantity were checked with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), while DNA integrity was analyzed using electrophoresis on a 0.8% agarose gel in TBE (0.089M Tris base, 0.089M Boric acid, 0.002M EDTA pH 8.0). Bromophenol blue dye and the GelRED® intercalating agent were added to each well. For subsequent analysis, DNA concentration of each isolate was adjusted to 20 ng/µL.

Genotyping

Primer pairs flanking 12 SSR loci were used for genotyping 61 isolates obtained in this study. Six markers were described by Le Guen et al. (2004) and the others six

developed by Barrès et al. (2012). The forward sequences of the primers were labeled with the fluorophores dyes NED, 6-FAM or Hex to determine the allelic composition of more than one locus at a time. Multiplex PCR reactions were performed (Hora Júnior, 2012) using Multiplex PCR 5X Master Mix kit as described by the manufacturer (New England BioLabs, Inc.). The PCR products were sent to Macrogen for fragment analysis using 400 HD^{TM} size standard marker to score alleles with GeneMarker^{TM} software V2.7.0 (Soft Genetics). The size of DNA fragments were manually binned into alleles according to the number of repeat units at each locus.

Data analysis

All analyses were performed with the R program version 3.4.3 (R Core Team, 2017). Initially, a genotype accumulation curve was generated with the poppr package version 2.6.1 to evaluate evidence of saturation and thus indicate whether the loci used were sufficiently sampled to adequately represent the potential set of individuals (Arnaud-Hanod et al. 2007; Kamvar et al., 2015).

Genotypic diversity was determined calculating the Stoddart and Taylor's index (G) (Stoddart and Taylor, 1988), Simpson index (Simpson, 1949), Shannon index (H) (Shannon, 1948), evenness (E5) (Grünwald et al., 2003) and the number of expected multilocus genotypes (eMLH), which was estimated by rarefaction of the population with sample size of 10 (Hurlbert, 1971; Heck et al., 1975; Grunwald et al., 2003). Nei's gene diversity (1978) was estimated. To evaluate if random mating is occurring in the populations, the linkage disequilibrium between the SSR loci was evaluated by standardized association index (r_d) from clone-corrected data set with 999 permutations (Brown et al., 1980; Smith et al., 1993; Agapow and Burt, 2001). The indices of genotype diversity and linkage disequilibrium were calculated with the poppr package.

Hill numbers were calculated based on the MLG frequency of each population using the iNEXT (Hsieh et al., 2016) package for R. These numbers correspond to estimates of genotype richness (0), Shannon index (1) and Simpson concentration index (2) (Chao et al., 2014). To compare the Hill numbers, integrated curves that allow rarefaction and extrapolation were used (Colwell et al., 2012). A 95% confidence interval (95% CI) was applied to each curve to compare the diversity in the different populations. The genetic differentiation coefficient (G"$_{ST}$), which indicates whether populations are segregating by different alleles (G"$_{ST}$ = 1) or if there is no

differentiation ($G''_{ST} = 0$), was calculated using the mmod package (Hedrick, 2005). The analysis of molecular variance (AMOVA) with the genetic distance of Bruvo was obtained with poppr package to estimate the distribution of variation within and between populations (Excoffier et al., 1992; Bruvo et al., 2004; Kamvar et al., 2014). In addition, discriminant analysis of principal components (DAPC) was performed using adegenet version 2.1.0 to infer the number of clusters of genetically related individuals (Jombart, 2008). To establish an appropriate number of main components (PC) for the DAPC analysis, cross validation was performed using the xvalDapc function of the adegenet package.

Results

Analysis of the genotype accumulation curve reached the plateau (Figure 1) with the 12 loci used in this study (Table 2).

A total of 54 multilocus genotypes were identified among the 61 isolates and 3 to 12 alleles per locus were detected. The P17 locus had the lowest gene diversity (0.24) and the least evenness estimate (0.46) (Table 2). The data set was divided into three subpopulations, which were defined according to the watersheds of the Amazon river: Madeira (M), Purus (P) and Juruá (J). The highest allelic diversity was obtained for the P watershed where 2 to 12 alleles per locus were detected, whereas for the J and M watersheds the number of alleles per locus was much lower and varied from 1 to 5, and 1 to 2, respectively (Table 2).

The number of multilocus genotypes (MLG) detected for the M and J watersheds was similar, 8 and 9, respectively. However, each isolate obtained from the P watershed had a unique MLG (Table 3). The genotypic diversity indices of Shannon (H), Stoddart and Taylor (G), and Simpson (λ) were highest for the P subpopulation, with intermediate values for the J subpopulation and lowest at the M subpopulation (Table 3). The subpopulations have similar evenness (E5) values, but it equals 1 in the P subpopulation because each isolate was a unique MLG (Table 3). Lowest gene diversity was observed in the M subpopulation (h = 0.17). The estimate is approximately 3.5 times lower than the average gene diversity estimated for the P and J subpopulation (Table 3).

There was strong evidence of linkage disequilibrium of alleles between loci in the total population ($r_D = 0.061$), and in two of the three subpopulations: J ($r_D = 0.34$) and P ($r_D = 0.03$). Nevertheless, there was no evidence to reject the occurrence of random mating in the M subpopulation ($r_D = 0.01$; $p = 0.367$) (Table 3).

No significant difference was observed for Hill numbers when the data were rarefied. But when the data set was extrapolated, there was greater genotypic richness and Shanon index for the P population (Figure 2).

There was evidence of genetic differentiation among populations. The estimated G''_{ST} was significant when M was compared with the other subpopulations. The estimated G''_{ST} between P and M was 0.73, while between J and M $G''_{ST} = 0.79$. No significant differentiation was observed between the P and J populations ($G''_{ST} = 0.46$).

According to the AMOVA (Table 4), the variation was highest within the subpopulations, accounting for 70.7% of the total variance, but there was moderate percentage of variance among subpopulations (29.3%). Considering the total population, the level of differentiation was high ($\Phi = 0.292$, $P = 0.001$), indicating that there are significant differences among the populations studied.

Another set of analysis was conducted to try to find clusters without any previous information other than the MLG data. A PCA was conducted and two clusters were identified (Figure 3). Cluster 1 was comprised of isolates from Manaus, Manicoré, Rio Preto da Eva and Beruri. While cluster 2 was composed of individuals from Bujari, Xapuri, Eirunepé, Boca do Acre, Rio Branco, Rodrigues Alves and Mâncio Lima.

DAPC was performed by grouping the populations with the first 30 major components. The first discriminant axis separated the M isolates from the other individuals. Two clusters were defined according to the analysis: one with isolates from the Madeira river and the other with isolates from the Juruá and Purus rivers (Figure 4).

Discussion

The *P. ulei* populations of the Amazon region studied in the present work are structured, which can be explained by the moderate level of percentage variance found between samples. Structured populations were also found to different populations of *P. ulei* from the Brazilian coast (Hora Júnior, 2012) and in groups from Ecuador,

Guatemala and French Guiana (Barrès et al., 2012). However, the structuring of the population in these areas were somewhat expected because isolates were sampled from commercial plantations. In the present study, samples came from reminiscent trees of old and abandoned plantations. Thus, no resistant clones were planted and isolates recovered could have originated from native trees in the forest.

The high differentiation between the individuals of Madeira in relation to those from the Purus and Juruá rivers, the latter being more closely related, may indicate a structuring according to the Amazon river watersheds, as observed for their host. The populations of rubber trees formed three clusters of populations, one within the watersheds of the Purus and Juruá rivers in the Acre-Madre de Dios cluster (Peru), the other in the Madeira river watershed for the population of the state of Rondônia (Brazil) and the last one in the watersheds of the Tapajós river for populations of the state of Mato Grosso (Brazil). The genetic differentiation found for the pathogen population can be explained by the geographic distance, an important parameter of differentiation between populations that consequently results in the isolation by distance of the populations (Le Guen et al., 2009).

Principal component analysis indicates that Purus and Juruá constitute a single cluster. The close relationship observed between these populations may be the result of the occurrence of gene flow between these subpopulation. It seems that in the populations of Juruá and Purus there is migration effect due to the mixture of genotypes found, despite geographic separation of the watersheds. These observations differ from the populations of *P. ulei* in Brazil (Bahia, Rondônia and Mato Grosso), Ecuador, Guatemala and French Guiana, in which it was demonstrated that there was no gene flow (Barrès et al., 2012). However, there is evidence for gene flow from the Acre population to the areas in the East coast in Brazil (Rio de Janeiro and Espírito Santo) (Hora Júnior, 2012). Similar process can occur between Juruá and Purus.

The lack of continuous cultivation in the Amazon region may have contributed to the structuring of the populations and probably the mixture of Purus and Juruá genotypes was due to long distance dispersion, which may result from passive transport by wind. Passive air transport has been reported for *Puccinia melanocephala* in sugarcane (Purdy et al., 1985), *Melampsora larici-populina* in poplar (Barrés et al., 2008) and *Puccinia striiformis* f. sp. *tritici* on wheat (Wang et al., 2010). However, the spread of *P. ulei* by wind is less likely considering the formation of two genetic groups

in the Amazon region, certainly influenced by the degree of isolation by distance, suggesting a gradual dispersion (Barrès et al., 2008). Based on geographical distance, the dissemination may have occurred naturally through wild trees in the Amazon rainforest or by human-mediated transport of infected leaves in stumps or sprouts (Holliday, 1970; Chee and Holliday, 1986).

The structuring of populations supports the idea that conidia play a key role in SALB epidemics in the center of origin of both the host and the pathogen. Conidia were considered responsible for the infection for many years and the participation of ascospores in the cycle, generally found in small numbers, was only observed in 1945 (Langford, 1945). These results do not corroborate with studies of populations located in different regions of Brazil, which presented predominant sexual reproduction (Hora Júnior, 2012). A more intensive sampling is required to properly address this issue.

A high genotypic diversity was detected in all populations. Similar results were observed for populations of the Atlantic Coast and other populations of the Amazon region sampled in commercial plantations a few years ago (Hora Júnior, 2012). The high genetic variability of the pathogen population may explain the successive and devastating infections caused by *P. ulei* when the Ford Motor Company attempted to establish rubber plantations in the state of Pará using high- performance Fx clones (Medeiros and Bahia, 1971). In addition, according to studies of physiological factors and of variability in large and small scales, the presence of the pathogen in all the rubber producing areas of the region probably favors the adaptation of the pathogen to different biophysical environments and may increase the severity of the epidemics (Junqueira et al., 1986; Mattos et al., 2003). The lack of durability of qualitative resistance of *Hevea* to *P. ulei* clones is explained by the presence of high genotypic variability of the fungi (Chee and Wastie, 1980; Ploetz, 2007; Le Guen et al., 2007). Additionally, the existence of genetically distinct populations implies that clone assessments for resistance level must consider different pathogen genotypes. Considering the results of the present study, resistant clones should be tested in areas located in the Madeira watershed and in another location between the Juruá and Purus rivers.

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Figure legends

Figure 1. Genotype accumulation curves for *Pseudocercospora ulei* populations from Amazon region, Brazil. Dashed lines indicate the number of multilocus genotypes identified in each population.

Figure 2. Diversity accumulation curves for different sample sizes with 95% confidence intervals of the Hill's numbers or effective number of genotypes of orders 0, 1 and 2 estimated for *Pseudocercospora ulei* populations from Amazon region, Brazil. The 0, 1 and 2 numbers correspond to genotype richness, the exponential of Shannon's entropy, and the inverse of the Simpson's concentration indices, respectively. Solid lines correspond to rarefaction (interpolation) and dashed lines to extrapolation curves. The 95% confidence intervals were obtained by a bootstrap method based on 200 replications.

Figure 3. Analysis of principal components (PCA) of *Pseudocercospora ulei* isolates from the Amazon region.

Figure 4. Discriminant analysis of principal components (DAPC) on *Pseudocercospora ulei* genotypes from the Amazon region.

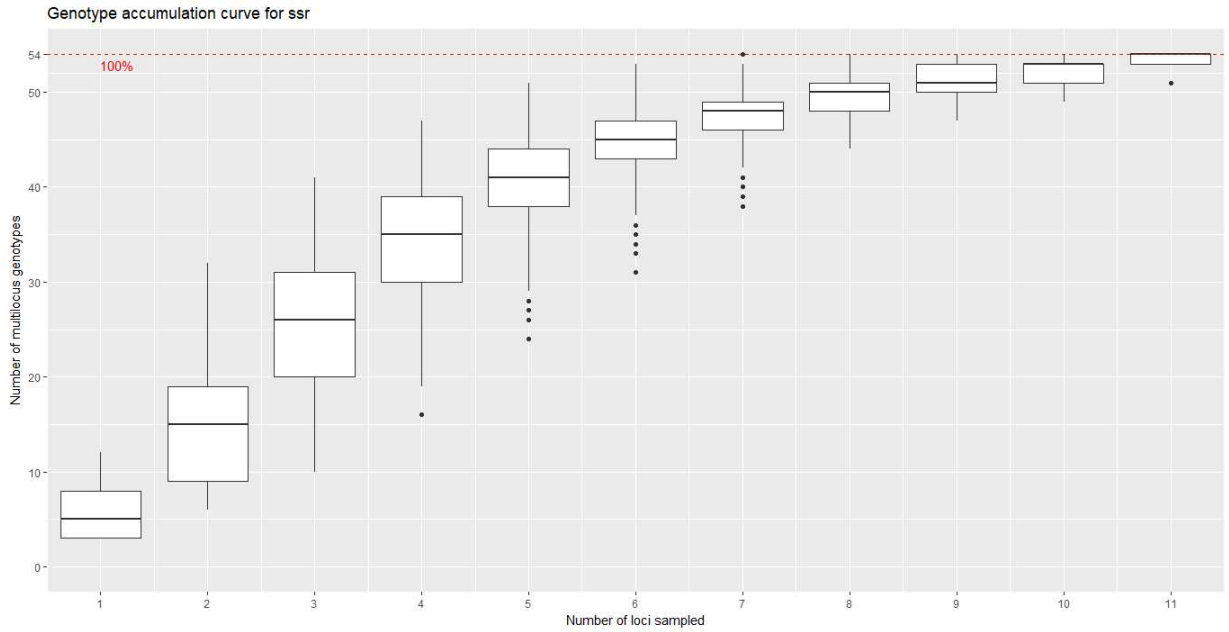

Figure 1. Santos et al.

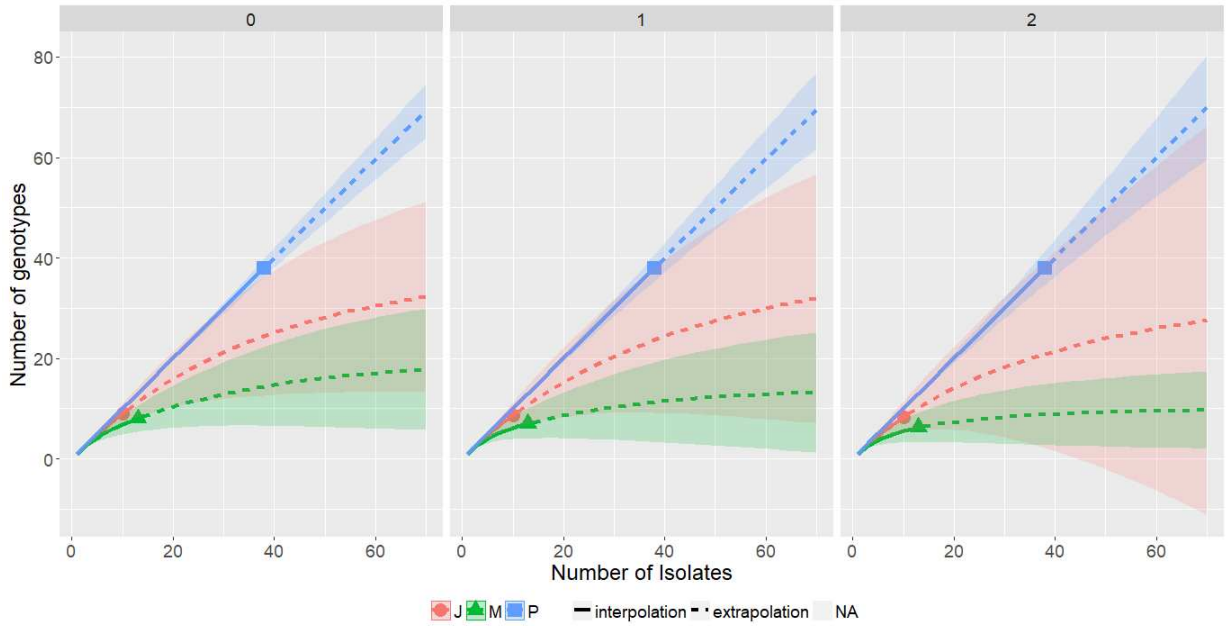

Figure 2. Santos et al.

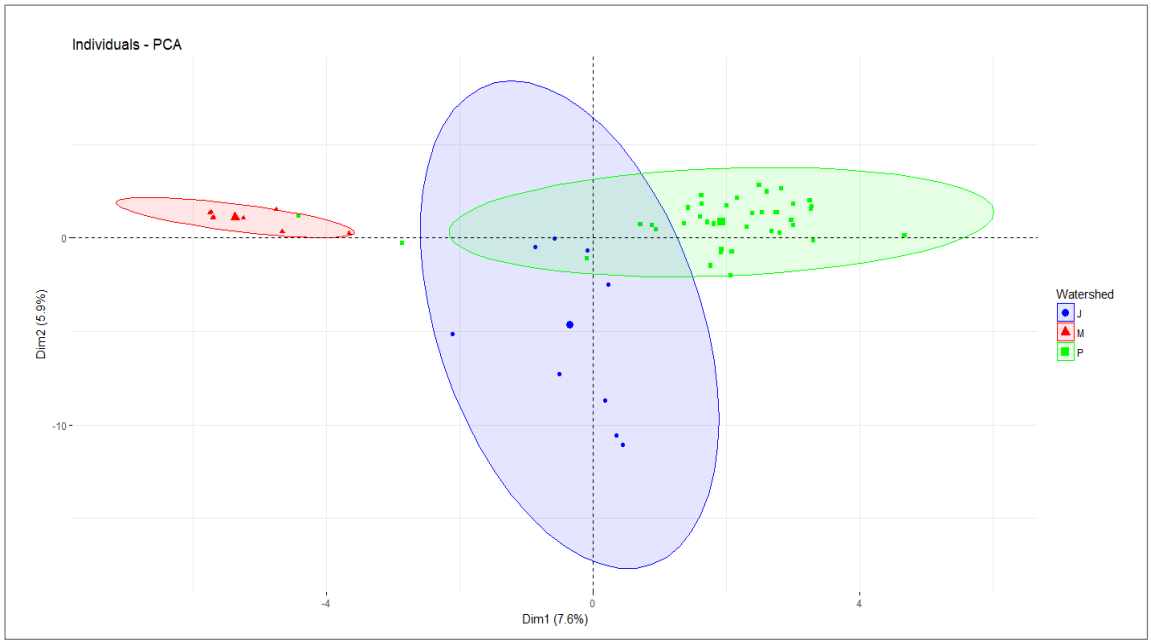

Figure 3. Santos et al.

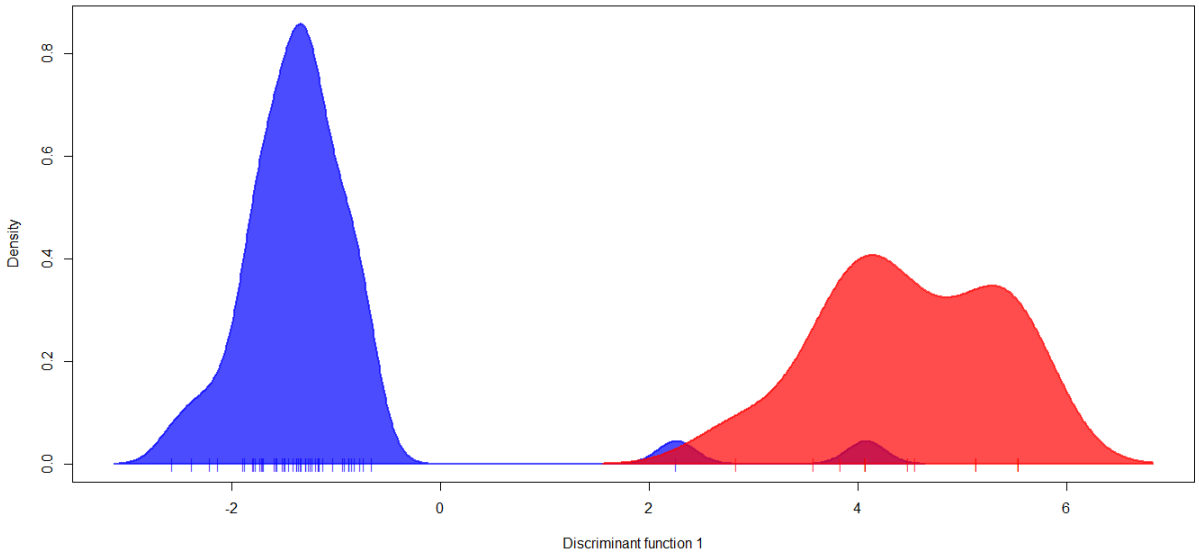

Figure 4. Santos et al

Table 1. Location and sampling data of the 61 *Pseudocercospora ulei* isolates collected in the southwest of the Amazon.

Watershed of the Amazon river	State	Location	No. of individuals	Sampling year	Geographical coordinates
Juruá	Acre	Eirunepé	5	2016	6º40'18.379"S 69º52'19.754"W
Juruá	Acre	Mâncio Lima	4	2017	7º27'6.408"S 73º23'14.957"W
Juruá	Acre	Rodrigues Alves	1	2017	7º44'16.764"S 72º39'44.474"W
Madeira	Amazonas	Beruri	2	2016	4º26'39.167"S 61º52'48.497"W
Madeira	Amazonas	Manaus	3	2016	3º8'33.371"S 60º3'27.464"W
Madeira	Amazonas	Manicoré	7	2016	5º44'2.587"S 61º37'58.818"W
Madeira	Amazonas	Rio Preto da Eva	3	2016	2º30'32"S 59º36'4.374"W
Purus	Acre	Bujari	5	2010	9º46'48.396"S 67º56'2.213"W
Purus	Amazonas	Boca do Acre	7	2014	8º45'25.189"S 67º24'42.775"W
Purus	Acre	Rio Branco	8	2010	9º58'56.392"S 67º49'17.922"W
Purus	Acre	Xapuri	16	2010	10º36'43.704"S 68º41'15.709"W

Table 2. Average allelic diversity and evenness per locus in three strata of the *Pseudocercospora ulei* populations from the Amazon region, Brazil.

	Locus	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
Pop	Range	112-122	240-250	140-146	206-220	133-137	326-336	254-270	106-124	164-192	136-152	89-105	99-107
	NA	8.00	9.00	3.00	8.00	4.00	8.00	3.00	3.00	12.00	3.00	5.00	4.00
T	*h*	0.74	0.80	0.51	0.79	0.61	0.71	0.65	0.53	0.82	0.67	0.66	0.24
	E_5	0.69	0.74	0.92	0.73	0.79	0.68	0.93	0.84	0.69	0.98	0.71	0.46
	NA	4.00	3.00	1.00	2.00	2.00	4.00	3.00	2.00	4.00	3.00	3.00	5.00
J	*h*	0.73	0.64	0.00	0.56	0.56	0.73	0.62	0.47	0.78	0.64	0.64	0.67
	E_5	0.81	0.88	0.00	1.00	1.00	0.84	0.80	0.86	0.90	0.88	0.88	0.62
	NA	6.00	9.00	3.00	8.00	3.00	6.00	3.00	2.00	12.00	3.00	4.00	2.00
P	*h*	0.68	0.88	0.53	0.86	0.57	0.73	0.56	0.51	0.90	0.57	0.53	0.23
	E_5	0.66	0.91	0.89	0.89	0.85	0.81	0.80	0.99	0.81	0.83	0.65	0.62
	NA	2.00	2.00	2.00	1.00	2.00	1.00	2.00	1.00	2.00	1.00	2.00	1.00
M	*h*	0.15	0.15	0.15	0.00	0.54	0.00	0.38	0.00	0.54	0.00	0.15	0.00
	E_5	0.53	0.53	0.53	0.00	0.99	0.00	0.77	0.00	0.99	0.00	0.53	0.00

[a]Population strata: T = Total population, J = Juruá watershed subpopulation, P = Purús watershed subpopulation, and M = Madeira watershed subpopulation; Locus denomination according to Le Guen et al. 2004 (6) and Barrès et al., 2012 (6) and allele range size registered in the present study; NA = Number of observed alleles; h = Nei's (1978) gene diversity; E5 = Evenness index (Grunwald et al., 2003).

Table 3. Genetic diversity indices and linkage disequilibrium estimated for microsatellites data sets of tree groups of isolates of *Pseudocercospora ulei*.

Pop	N	MLG	eMLG	H	G	λ	E_s	h	r_d
M	13	8	6.80	1.95	6.26	0.840	0.871	0.173	0.017
P	38	38	10.00	3.64	38.00	0.974	1.000	0.630	0.035*
J	10	9	9.00	2.16	8.33	0.880	0.952	0.587	0.344*
Total	61	54	9.75	3.92	44.83	0.978	0.887	0.649	0.061*

Pop, Population; N, number of individuals; MLG, number of multilocus genotypes; eMLG, expected number of MLGs; H, Shannon–Weiner index; G, Stoddart and Taylor's index; λ, Simpson's Index; h, Nei's (1978) gene diversity; E5, Evenness (Grunwald et al., 2003); r_d, standardized index of association. An asterisk indicates a significant value of r_d after 999 permutations, $P \leq 0.001$.

Table 4: Hierarchical analysis of molecular variance (AMOVA) of isolates of *Pseudocercospora ulei* collected in rubber plantations of the Amazon region.

Hierarchy	d.f.	Sum of squares	% Variation	ΦStatistic	*P*
Between populations	2	49.53	29.25	-	-
Within populations	58	183.97	70.75	-	-
Total	60	233.50	100	0.29	0.001

d.f., degrees of freedom

Conclusão geral

1. O protocolo original de Doyle e Doyle (1990) foi o mais adequado para a extração de DNA de *Pseudocercospora ulei*.
2. O patógeno possui alta diversidade genética e associação aleatória de alelos.
3. Foram formados dois grupos, um isolado pelo rio Madeira e o outro composto por isolados dos rios Purus e Juruá.
4. A estruturação da população de *P. ulei* se assemelha à de *Hevea* spp.