



Erasing the Past: A New Identity for the Damoclean Pathogen Causing South American Leaf Blight of Rubber

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Abstract

Background: South American leaf blight (SALB) of rubber has been the main constraint to production in its neotropical centre of origin since commercial plantations were first established. The fungal causal agent was identified and described more than a century ago but its precise placement within the Ascomycota still remains uncertain. Indeed, such is the ambiguity surrounding the pathogen that each of the spore morphs would, according to their present classification, be placed in different ascomycete families: the *Microcyclus* sexual morph in the Planistromellaceae and the two purported asexual morphs - *Fusicladium* and *Aposphaeria* - in the Venturiaceae and Lophiostomataceae, respectively. Given the historical importance of the fungus and the ever-menacing threat that it poses to rubber production in the Palaeotropics - and, thus to the rubber industry and to the global economy - its phylogeny, as well as its biology, should be resolved as a matter of urgency.

Methods and Results: Here, six genomic regions (LSU rRNA, mtSSU, MCM7, EF-1 α , Act and ITS) were used for reconstructing the molecular phylogeny of the SALB fungus based on material collected throughout Brazil. The analyses support the classification of the fungus in the family Mycosphaerellaceae s. str. (Capnodiales, Dothideomycetes) and place it firmly within the clade *Pseudocercospora* s. str., now accepted as one of the distinct genera within Mycosphaerellaceae. The new combination *Pseudocercospora ulei* is proposed and the life cycle of the fungus is confirmed, based on both experimental and phylogenetic evidence, with the *Aposphaeria* morph shown to have a spermatial rather than an infective-dispersal function.

Conclusions: Because the phylogeny of the SALB fungus has now been clarified, new insights of its epidemiology and genomics can be gained following comparison with closely-related, better-researched crop pathogens.

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Introduction

South American leaf blight (SALB) of the rubber tree *Hevea brasiliensis* (Willd. ex A. L. Juss.) Muell.-Arg., caused by *Microcyclus ulei* (Henn.) Arx (Ascomycota), is recognized as the most serious threat to the natural rubber industry worldwide [1–4]. Epidemics of SALB led to the failure of rubber plantations in tropical America in the early 20th century, as epitomized by the demise of Fordlandia in the Lower Amazon region of Brazil despite enormous investment in research and development [5,6]. Currently, the world supply of natural rubber is highly dependent on the plantations established in Southeast Asia [3]. The magnitude of the threat represented by the SALB fungus is highlighted by Money [7] who described the relevance of natural rubber as an irreplaceable prime matter for the world's industry in a plethora of applications besides tires, machinery belts and

condoms and as an industry itself providing the livelihood of 30 million people, concluding that “Nothing else (but *M. ulei*) has the power to terminate the global flow of latex.” Because of the potential serious economic consequences, there are strict quarantine measures in place to prevent SALB from establishing in the rubber tree production areas in the Palaeotropics, especially in Southeast Asia, a SALB-free zone [3,8]. The fungus infects young leaves, stems and fruits of *Hevea brasiliensis*, as well as *H. benthamiana* Muell.-Arg., *H. spruceana* (Benth.) Muell.-Arg., *H. guianensis* Aublet and *H. camporum* Ducke [2], resulting in defoliation and, potentially, after repeated outbreaks in tree death.

The fungus was first observed and collected by E. Ule in 1900 in the Upper Amazon region of Peru and Brazil and was later described by Hennings [9]. Initially, two spore morphs were recognised: the sexual morph, *Dothidella ulei*; and a supposed asexual pycnidial morph, *Aposphaeria ulei*. The hyphomycete

asexual morph was described soon after by J. Kuyper in Surinam in 1911 as *Fusicladium macrosporium*. In 1917, G. Stahel observed the connection of hyphae from different fungal structures within the leaf tissue and linked the sexual and asexual morphs of the fungus and renamed the former as *Melanopsammopsis ulei* [1]. Much later, von Arx (in [10]) transferred this to the genus *Microcyclus* and suggested a close relationship with the genus *Mycosphaerella*, based on the morphology of the hyphomycete *Passalora*-type morph. Subsequently, he suggested that *Fusicladium* should only be used for asexual morphs belonging to the family Venturiaceae rather than to the Mycosphaerellaceae [11]. *Microcyclus* is characterized by erumpent ascostromata on living leaves having a foot-like hypostroma, similar to some *Mycosphaerella* pathogens of pine trees [12].

Each of the spore morphs in *M. ulei*'s life cycle would, according to the present classification for the genera where they are placed, belong to a different ascomycete family, namely: Planistromellaceae/incertae sedis (*Microcyclus* sexual morph) [13–15], Venturiaceae (*Fusicladium* asexual morph) [16] and Lophiostomataceae (*Aposphaeria* asexual morph) [17,18]. This is clearly inadequate and requires an explanation. Although there would be grounds for speculating that SALB is a disease complex involving three unrelated fungal species, perhaps with the involvement of a mycoparasite, previous authors that have dealt with the SALB disease and its etiology have not reached such conclusion. Nevertheless, Langford [19] and more recently, Guyot and Doaré [20] have inoculated conidia and ascospores on rubber plants and were able to reproduce the symptoms of SALB, demonstrating that the *Microcyclus* and *Fusicladium* morphs are part of the cycle of a single fungus. Ascospores were shown to play an essential role in the perpetuation of the disease outside the host's growth periods, in the resumption of epidemics, and in long-distance dispersal and the conidia contributed primarily to the stepwise and short-distance spread of the disease [21]. A conclusive Koch's postulates have never been performed with the pycnidial morph of the fungus. This might play a different role in the life cycle of the fungus or even be a mycoparasite of *M. ulei*. Conversely, this puzzling situation may just result from the lack of proper understanding of the life cycle and classification of the fungus behind SALB.

The general lack of DNA sequence data for all three purported morphs (*Microcyclus*, *Fusicladium* and *Aposphaeria*) contributes to the confusion surrounding the taxonomy of the causal agent of SALB. Until relatively recently, the genus *Microcyclus* was classified in the Mycosphaerellaceae (order Capnodiales), as a stromatic counterpart of the family [22,23], but has since been reclassified in the Planistromellaceae (Dothideales), initially to accommodate genera with ascostromatal locules that open schizogenously by a periphysate ostiole [13,14]. More recently, a phylogenetic analysis showed that the core Planistromellaceae belong in the Botryosphaeriales, from which *Microcyclus* – represented only by ITS sequences of *M. ulei* – was excluded based on BLAST searches of GenBank, and its familial position was considered to be uncertain [15]. After a taxonomic review of the hyphomycete conidial morph, this asexual morph was retained in *Fusicladium* s. lat. [16]; some species of which have now been assigned to the newly recognised family Sympoventuriaceae in the new order Venturiales [24]. However, in the absence of type material, the species was neotypified and the name changed to *F. heveae* since it was adjudged that the original epithet could be confused with *F. macrosporium* Bonord. 1864 [16,25]. The latter authors added the rider that: “*Fusicladium heveae* is an unusual species, since its teleomorph, *Microcyclus ulei*, is placed in the Mycosphaerellaceae and not in the Venturiaceae”. The coelomy-

cete genus *Aposphaeria* is recognized as a member of the family Lophiostomataceae (order Pleosporales), as a well-supported group [17,18]. Thus, questionable issues regarding the classification of both the purported asexual morphs, as well as the sexual morph, at the genus, family and order levels of the causal agent of SALB need to be addressed. “Clearly, a re-examination of its taxonomic position would be justified” [26] and a single unifying generic name should be adopted in accordance with the new nomenclatural rules of one fungus one name system and the promotion of progressive plant pathology [27].

Additionally, knowledge about the evolutionary history of *M. ulei* and of related species is scarce and molecular studies could help to resolve the true affinity of this fungus [15,25,28]. Thus, the objectives of the present study were: i) To obtain molecular evidence of the connection of the three spore morphs of *M. ulei*; ii) to elucidate the phylogenetic relationships of *M. ulei* using molecular approaches; iii) to determine the adequate nomenclatural treatment for the fungus causing SALB; iiiii) to obtain experimental evidence on the function of the intermediate pycnidial morph; iv) to prepare an updated model of life-cycle of the SALB fungus. Conceivably, this should also lead to a better understanding of the biology and ecology of one of the most threatening plant pathogens to mankind's welfare.

Material and Methods

Ethics statement

No specific permits were required for the described field studies. No endangered or protected species were involved in the studies.

Sampling, isolation and DNA extraction

Leaves with 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 Brazilian states of Acre, Rondônia, Mato Grosso, Minas Gerais, Espírito Santo and Bahia between 2008 and 2010 (Table 1). 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 M4 culture medium [29] in the dark for 2 months at 24 ± 1 °C. Pycnidial stromata of *A. ulei* and ascostromata of *M. ulei* were excised from a single lesion of an infected leaf with a sterilized razor blade. Each lesion was examined under the microscope to check for possible contamination by mycoparasites and selected stromata (approximately 10 structures) were transferred to a microtube (1.5 mL). The procedure was repeated from another lesion on the same leaf. 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 [30].

DNA phylogeny

All phylogenetic analyses were performed using DNA sequence of six loci as the first 900 bp at the 5' end of the 28S rRNA gene (LSU), the first and second internal transcribed spacer (ITS), the mitochondrial region of the mtSSU-rDNA and partial sequences of nuclear genes such as the mini-chromosome maintenance protein (MCM7), translation elongation factor 1-alpha (EF-1 α) and actin (ACT). Specific primers utilized were LR0R [31] and LR5 [32], ITS1 and ITS4 [33], NMS1 and NMS2 [34], Mcm7-709for and Mcm7-1384rev [35], EF1-728F and EF1-986R and ACT-512F and ACT-783R [36], respectively.

Table 1. Origin of the *Microcyclus ulei* isolates used in the phylogenetic study.

Isolate	Location ¹	Coordinates in decimals (Lat/Lon)	GenBank accession number (ITS, ACT, EF-1 α , LSU, MCM7, mtSSU)
<i>Fusicladium heveae</i> UFVMu01RO	Buritis-RO	-10.211944/-63.828889	KC800717, KC800725, KC800733, KC800741, KC800755, KC800768
<i>Fusicladium heveae</i> UFVMu05MT	Itiquira-MT	-17.208889/-54.150000	KC800718, KC800726, KC800734, KC800742, KC800756, KC800769
<i>Fusicladium heveae</i> UFVMu01ES	Sooretama-ES	-19.220087/-40.121414	KC800719, KC800727, KC800735, KC800743, KC800757, KC800770
<i>Fusicladium heveae</i> UFVMu77BA	Porto Seguro-BA	-16.378001/-39.366433	KC800720, KC800728, KC800736, KC800744, KC800758, KC800771
<i>Microcyclus ulei</i> AC	Xapuri-AC	-10.651944/-68.503889	KC800721, KC800729, KC800737, KC800745, KC800759, KC800772
<i>Microcyclus ulei</i> MG	Oratórios-MG	-20.415833/-42.908889	KC800722, KC800730, KC800738, KC800746, KC800760, KC800773
<i>Aposphaeria ulei</i> RO	Ariquemes-RO	-9.913333/-63.040833	KC800723, KC800731, KC800739, KC800747, KC800761, KC800774
<i>Aposphaeria ulei</i> ES	Cachoeiro do Itapemirim-ES	-20.752609/-41.290358	KC800724, KC800732, KC800740, KC800748, KC800762, KC800775

¹Brazilian states: Acre (AC), Bahia (BA), Espírito Santo (ES), Mato Grosso (MT), Minas Gerais (MG) and Rondônia (RO).
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The polymerase chain reaction (PCR) was done with a mixture containing 20 ng of DNA, 0.2 μ M of each primer and 1 \times of DreamTaq DNA Polymerase Master mix as described by the manufacturer (Thermo Fisher Scientific). PCR cycles were carried out in a PTC100 thermal cycler (MJ Research, Incline Village, NV) and consisted of a 5 min denaturation step at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C for LSU, mtSSU, EF-1 α , ACT and ITS primers or 57 °C for MCM7 primers and 1 min at 72 °C with a final extension of 10 min at 72 °C. PCR products were visualized by ultraviolet fluorescence following 1% agarose gel electrophoresis in 1 \times 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 1000 DNA Sequencing System (GE Healthcare). A consensus sequence was generated after manually editing with The Staden Package, v. 1.6.0 [37]. Genbank accession numbers are provided in Table 1. Additional sequences used in the analyses were obtained from GenBank and the Fungal Genomics Portal of the Joint Genome Institute [38](Table S1). Sequences were aligned with the Muscle v. 3.6 software [39] implemented in the MEGA 5.0 program [40]. Statistics resulting from sequence alignment such as variable, parsimony-informative and uninformative sites were estimated in MEGA.

Bayesian analysis was conducted with MrBayes v. 3.1.2 [41] to determine generic relationships based on the LSU, mtSSU and MCM7. Aligned datasets were inspected with MrModeltest v.2.2 [42] to select the suitable nucleotide substitution model and all trees were rooted with *Aspergillus niger*. Additionally, another dataset at species level was constructed and Bayesian phylogeny was derived from the concatenated ITS, EF-1 α and ACT alignments with *Pseudocercospora* s. str. sequences. *Passalora eucalypti* was used as the outgroup. For this analysis, the alignment gaps were treated as a fifth character state and the MrModeltest v.

2.2 selected the best nucleotide substitution model for each partition. 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 1000 generations, resulting in 50,000 saved trees. Burn-in was set at 5,000,000 generations after which the likelihood values were stationary, leaving 35,000 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 v. 1.5 [43]. The resulting phylogenetic trees were prepared using FigTree v. 1.4 (<http://tree.bio.ed.ac.uk/software/figtree>). All alignments and resulting trees were deposited into TreeBASE (14357), and the nomenclatural novelty in MycoBank [44].

Taxonomy

Based on newly obtained information and information available in the literature and on the re-examination of newly collected material a model was prepared. Observations of the morphology of fungal structures belonging to each morph in the life cycle were made based on the examination of microscope slides containing sections of such structures mounted in lactophenol or lactofuchsin and observed under a light microscope (Olympus BX 51) equipped with a drawing tube. At least 30 measurements were made of each fungal structure.

Nomenclature

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, the new combination introduced in this work has been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank

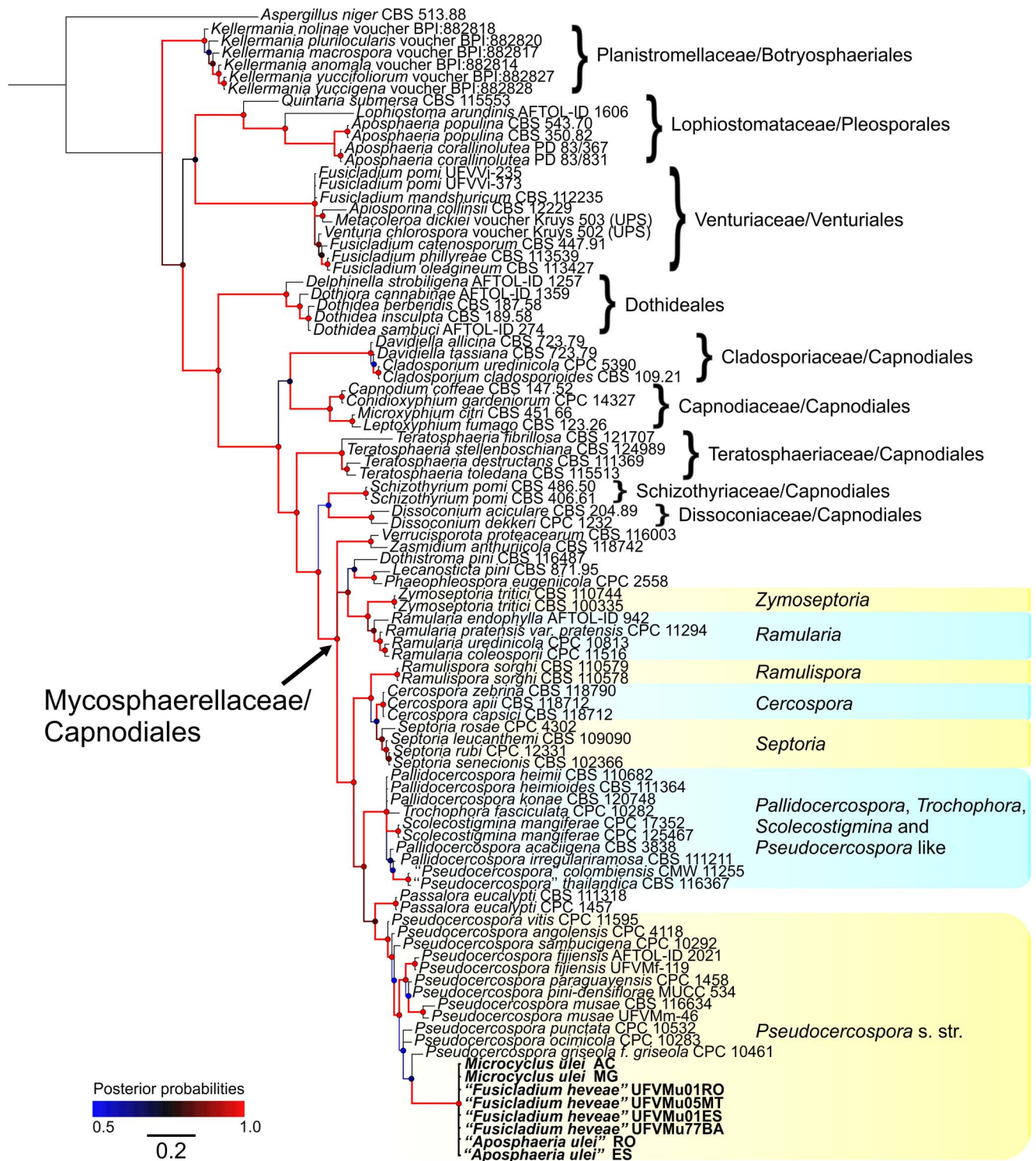


Figure 1. Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the LSU sequence alignment. Bayesian posterior probabilities are given at the nodes and coded according to the colored scale bar. The black line scale bar shows 0.2 expected changes per site. The tree was rooted with *Aspergillus niger*. doi:10.1371/journal.pone.0104750.g001

number contained in this publication to the prefix <http://www.mycobank.org/MB/>. The online version of this work is archived and available from the following digital repositories: [PubMed Central, LOCKSS].

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

The development of the pathogen in the rubber leaf was monitored under environmental conditions favorable to the

development of SALB. At the Michelin Plantation of Bahia (Brazil), 90 leaves at the B2 developmental stage [45] of eight rubber trees of the RO38 clone were tagged with a label and observations of the disease were made until maturity (stage D), from December 15, 2011 to February 24, 2012 (Experiment 1) and September 19 to December 03, 2012 (Experiment 2). All trees were pruned 45 days before each experiment started. Scoring of sporulation in lesions naturally infected was performed at every four days using a 1–6 scale for sporulation intensity of the asexual morph (conidia) adapted from Junqueira et al. [46], 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. The weighted average was computed in each observation from total of leaves in each phenological stage and score of conidial sporulation intensity and spermogonia and ascostromata density.

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

Suspension of pycniospores was obtained from pycnidia formed in near mature leaves (C/D stage) of the RO38 rubber clone. There were no conidia or ascospores. Suspension of hyphomycete asexual morph was used as positive control. Both inoculum suspensions were adjusted to 2×10^5 spores/mL in a Tween 80 at 0.05% solution. The lower surface of three young leaves from the Fx 3864 rubber tree clone were spray-inoculated until runoff with an inoculum suspension of pycniospores or conidia separately using a HS Airbrush Complete set (Paasche Airbrush company) in an inoculation chamber at 24°C, relative humidity greater than 85%, artificial daylight of 2000 lux and 12 h photoperiod. The 0.05% Tween 80 solution was used as a negative control. Sporulation was scored after 12 days on all inoculated leaves. The suspensions of pycniospores and conidia were incubated in the dark at 25 °C on both water agar and M4 culture media. Germination assessments were conducted at 6, 12, 24 and 120 h of incubation at $24 \pm 1^\circ\text{C}$. The experiment was conducted twice.

Results

Phylogeny: LSU, mtSSU and MCM7 datasets

Strongly supported clades provide molecular evidence of asexual-sexual morph connection between the three morphs of the SALB fungus and thus the holomorph belongs to the family Mycosphaerellaceae s. str., order Capnodiales (Figures 1–3). For this study, two specimens each of *M. ulei* and *A. ulei* and four of *F. heveae* collected in Brazil were analyzed. The generic relationships were determined with datasets for LSU, mtSSU and MCM7 that included 89, 55 and 36 taxa, respectively (available in TreeBASE) and the same nucleotide substitution model, GTR+I+G was used in all analyses.

The alignment of the partial sequence of the LSU region had 838 sites including alignment gaps, of which 243 sites were parsimony-informative, 57 were variable and parsimony-uninformative, and 534 were constant. The LSU phylogeny (Figure 1) resulted in *Aposphaeria populina* and *A. corallinolutea* (members of the Lophiostomataceae: Pleosporales), species of the genus *Fusicladium* (members of the Sympoventuriaceae: Venturiales), species of *Kellermania* (members of Planistromellaceae: Botry-

sphaeriales), as well as members of the Dothideales forming well-supported monophyletic groups. Representatives of the Capnodiales grouped within well-established families as Cladosporiaceae, Capnodiaceae, Teratosphaeriaceae, Schizothyriaceae, Dissoconiaceae and Mycosphaerellaceae. In the Mycosphaerellaceae, several well-supported clades were formed with *Mycosphaerella* s. str. (asexual morph *Ramularia*) and mycosphaerella-like with the asexual morphs *Cercospora*, *Pallidocercospora*, *Pseudocercospora*, pseudocercospora-like, *Ramulispora*, *Septoria*, and *Zymoseptoria*, amongst others. *Microcyclus ulei* and its morphs *A. ulei* and *F. heveae* were identical and grouped in the well-defined *Pseudocercospora* s. str. clade of the Mycosphaerellaceae, distinct from *Mycosphaerella* s. str. (*M. punctiformis*, represented by *Ramularia endophylla*), showing clearly that the holomorph of the SALB fungus is a species of *Pseudocercospora* in the Mycosphaerellaceae.

The phylogeny reconstructed with the partial sequence of the mtSSU sequences (Figure 2) had 724 characters (248 parsimony-informative and 99 singletons), while the dataset of the partial sequence of the MCM7 region (Figure 3) was based on a dataset with 466 characters (254 variable sites of which 221 were parsimony-informative). The OTUs from the Venturiales, Pleosporales, Dothideales and Capnodiales (Capnodiaceae, Cladosporiaceae, Dissoconiaceae and Mycosphaerellaceae) for mtSSU region, and those from the Pleosporales (Lophiostomataceae and Pleosporaceae), Venturiales and Capnodiales (Teratosphaeriaceae and Mycosphaerellaceae) for MCM7 formed well-supported clades. In both analyses, OTUs of the genus *Pseudocercospora* in Mycosphaerellaceae were the nearest relatives of the holomorph of the SALB pathogen.

Phylogeny: Concatenated ITS, EF-1 α and ACT datasets

After the analyses at the genus level, phylogeny at species level was conducted with some OTUs of *Pseudocercospora* s. str. using sequences of ITS, EF-1 α and ACT regions combined (Figure 4). The nucleotide substitution models, GTR+I+G, GTR+G and SYM+I+G, were used for each partition, respectively. For this dataset, 1126 characters were used, 517 were constant, 367 were parsimony-informative and 144 were singletons. Two well-defined clades were observed, both with posterior probability of 0.96, and the holomorph was closely related to *Pseudocercospora angolensis*.

Pleomorphic development and function of intermediate pycnidial morph in the life cycle of *Microcyclus ulei*

The SALB symptoms were assessed in two consecutive experiments from trees after pruning. In the first period, December 15, 2011 to February 24, 2012, conidial lesions started in leaves in the B2 stage on December 19 and were observed up to the D stage leaves, which corresponded to 26 days of monitoring (Figure 5A). *A. ulei* first emerged from the upper side of infected leaves in the C/D stage on December 29. Ascstromata arose after 32 days (January 17) and were found only in the upper side of D stage leaves. In the second period, September 19 to December 3, 2012, conidial lesions were found on September, 28 in B2 stage leaves and in D leaves within a 28 day-period (October 17) (Figure 5B). *A. ulei* appeared in the C/D stage on October 9 (20 days) and ascstromata arose after 36 days of monitoring and were found only in stage D leaves. Both stages occurred in the adaxial side of leaves.

The main weather descriptors during the course of the experiment 1 (72 days) and experiment 2 (75 days) were, respectively: average maximum temperature 29 and 27.7 °C; average minimum temperature 22 and 20.2 °C; average relative humidity 83% and 83.9%. Total (cumulative) rainfall was 267 and 267.8 mm.

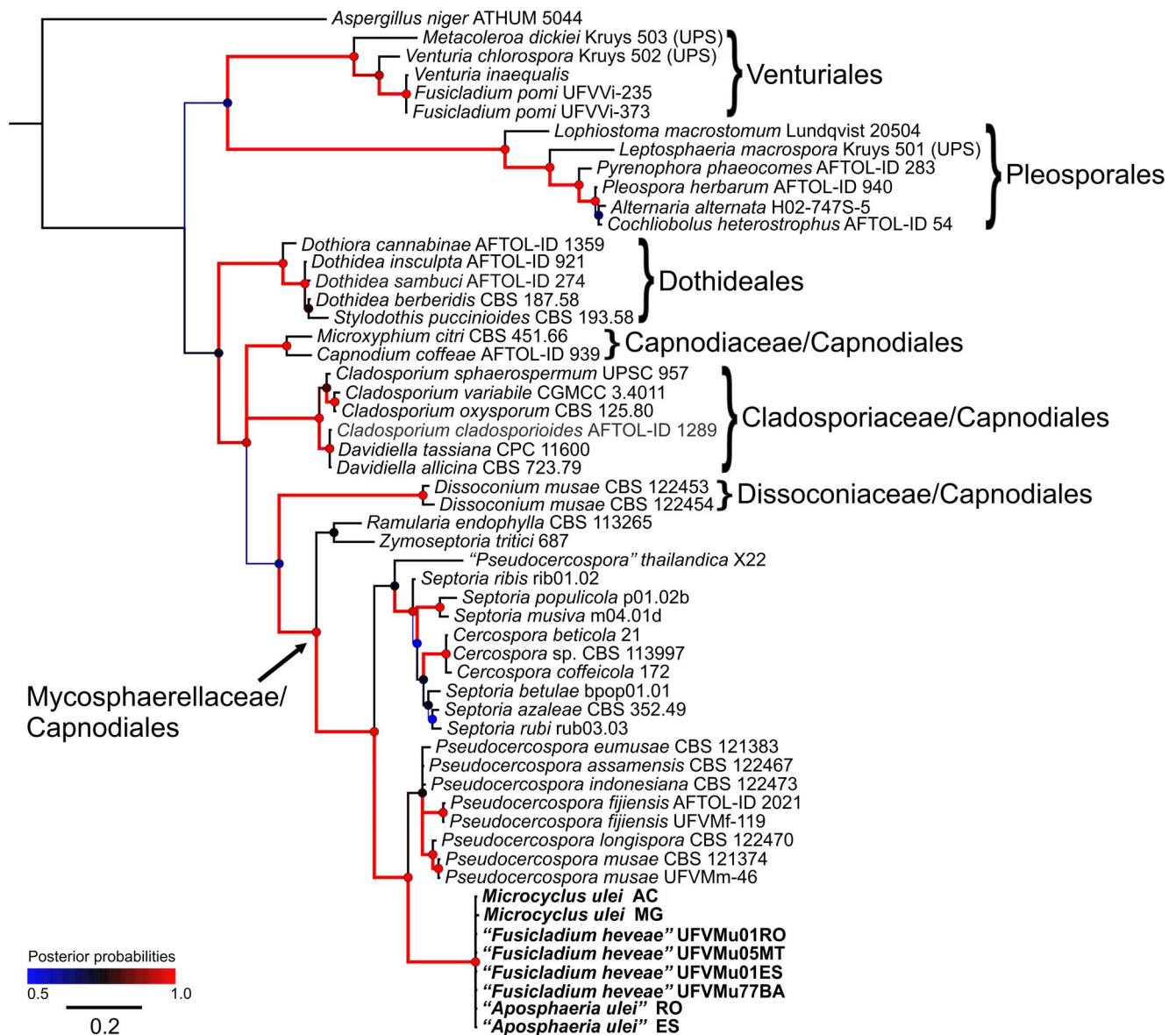


Figure 2. Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the mtSSU sequence alignment. Bayesian posterior probabilities are given at the nodes and coded according to the colored scale bar. The black line scale bar shows 0.2 expected changes per site. The tree was rooted with *Aspergillus niger*.
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The possible contribution of the so-called pycniospores for disease initiation was investigated by inoculating a concentrated suspension of "pycniospores" onto young leaflets under controlled conditions and also by assessing spore germination. Inoculation of pycniospores did not cause lesions, but signs of the disease were visible in leaflets inoculated with conidia after 12 days. The pycniospores did not germinate *in vitro*, on both culture media, while conidia germination started at 6 h of incubation.

Taxonomy

Based on the multi-gene phylogeny analyses, the pleomorphic fungus *M. ulei* was shown to cluster unmistakably within the *Pseudocercospora* s. str. clade and, in accordance with Art. 59 of the ICN (International Code of Nomenclature for Algae, Fungi and Plants), a new combination is hereby introduced.

***Pseudocercospora ulei* (Henn.) Hora Junior & Mizubuti, comb. nov.** MB 804653 (Fig. 4)

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

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

≡ *Aposphaeria ulei* Henn. Notizbl. Bot. Gart. Berlin-Dahlem 4: 135 (1904)

≡ *Apiosphaeria ulei* Henn., Hedwigia 43: 245 (1904)

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

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

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

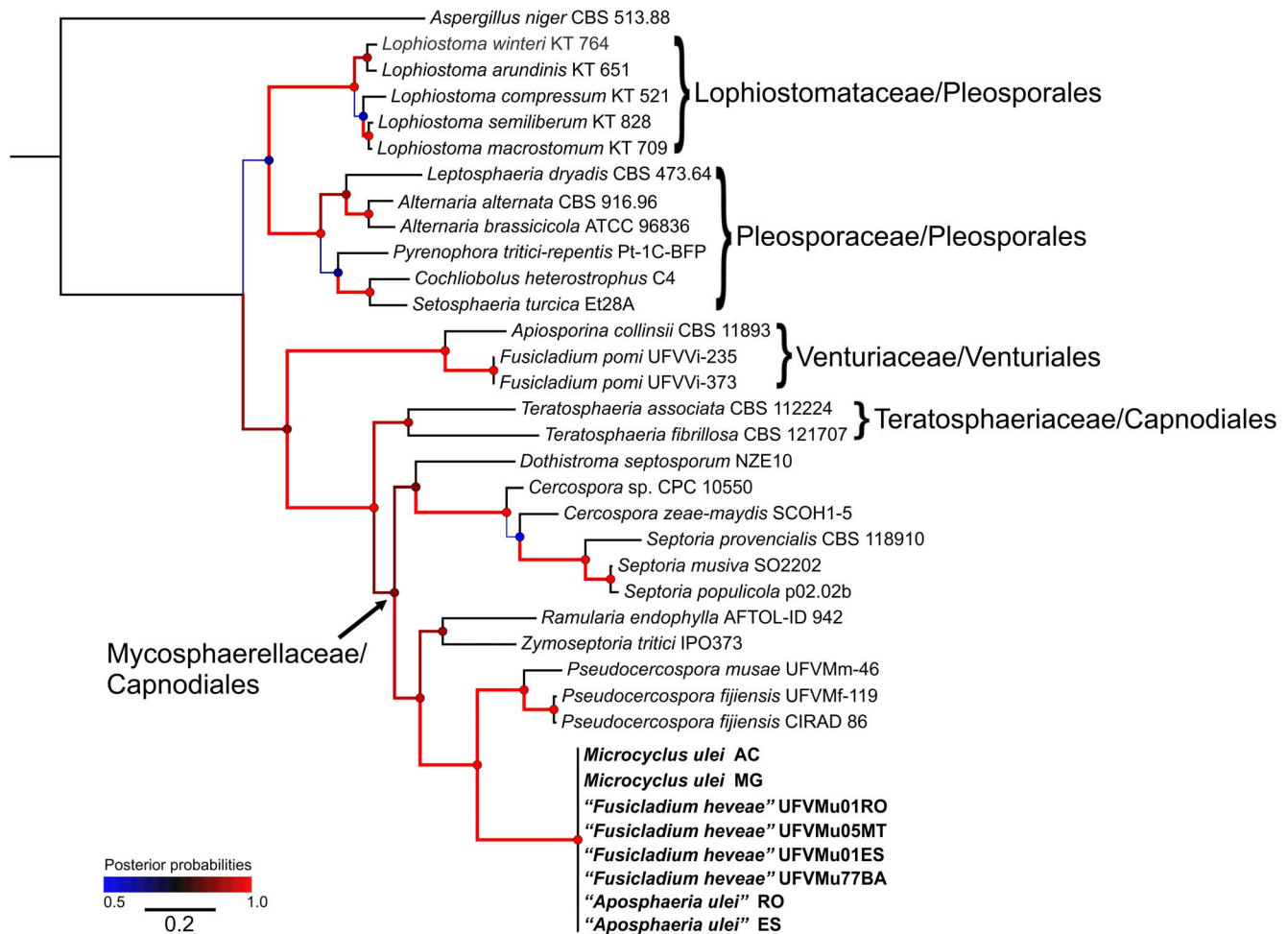


Figure 3. Bayesian analysis showing the phylogenetic relationships of *Microcyclus ulei* based on the MCM7 sequence alignment. Bayesian posterior probabilities are given at the nodes and coded according to the colored scale bar. The black line scale bar shows 0.2 expected changes per site. The tree was rooted with *Aspergillus niger*. doi:10.1371/journal.pone.0104750.g003

≡ *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)

Lesions on young stems, petioles, inflorescences, fruits and (mainly) on leaves. **Sexual morph lesions**, initially punctiform, becoming circular to subcircular, necrotic, pale brown centrally surrounded with a ring of prominent black stromata, growing with age and leading to loss of subcircular fragments (shot-holes), 1–3 × 1–6 mm diam. leading to foliage distortions and, when abundant to leaf drop. **Spermogonial morph lesions** as for sexual morph. **Asexual morph lesions**, leaf spots of variable shape, subcircular to elongating along leaf veins to angular or irregular 1–7 × 1–2.5 mm, greyish brown to black, scattered over lamina, sometimes somewhat raised, coalescing with age and leading to premature leaf drop. **Internal mycelium**, 3–6 µm diam, branched, septate, hyaline to pale brown, smooth.

Ascomata pseudothecial, superficial, epiphyllous, in large erumpent ascostromata having a foot-like hypostroma, spherical, 128–165 × 90–192 µm, walls of brown *textura angularis*, 6–9 cells, 42–57.5 µm thick, smooth. **Dehiscence** ostiolate, 2–10 µm in diam; **Asci** bitunicate, clavate, 66.5–90 × 13–16.5 µm, 8-spored. **Ascospores** ellipsoidal, 15–20 × 4–5 µm, 1-septate, constricted at septum, hyaline, smooth. **Conidiophores** amphigenous, sometimes

emerging from a thin layer of brown cells or ill-developed stroma, mostly reduced to conidiogenous cells, sparse or subfasciculate to forming dense parallel groups, cylindrical, bulbous at the base, erect, straight or slightly flexuous to geniculate toward the apices, unbranched, 31–56 × 4–6 µm, 0–1 septate, pale brown, smooth. **Conidiogenous cells** holoblastic, integrated, cylindrical to subcylindrical, terminal, proliferating sympodially with 1–3 loci, 2 µm diam, flat, unthickened, not darkened. **Conidia** solitary, obclavate, straight to usually curved or twisted into a somewhat sigmoid shape, 27.5–62 × 6–11 µm, apex rounded, base attenuated to a truncate hilum, 0–1-septate, somewhat constricted at the septum, subhyaline to pale brown, smooth to somewhat roughened, thin-walled, hilum 2 µm wide, unthickened, not darkened. **Spermogonia** adaxial superficial, in stromatic groups, globose, 112.5–138 × 87.5–150 µm, walls of pale to dark brown *textura angularis*, 4–8 cells-thick, 37.5–92.5 µm, ostiolate, smooth. **Spermatophores** phialidic, lageniform, integrated, 10–16 × 1–2 µm, hyaline, smooth. **Spermatia** dumb-bell-shaped, 7–4 × 1 µm, aseptate, hyaline, smooth.

Hosts and Distribution: on *Hevea* spp. (Euphorbiaceae), *H. benthamiana* (Brazil); *H. brasiliensis* (Bolivia, Brazil, Colombia, Costa Rica, French Guyana, Guatemala, Guyana, Honduras, México, Nicaragua, Panamá, Peru, South America, Suriname,

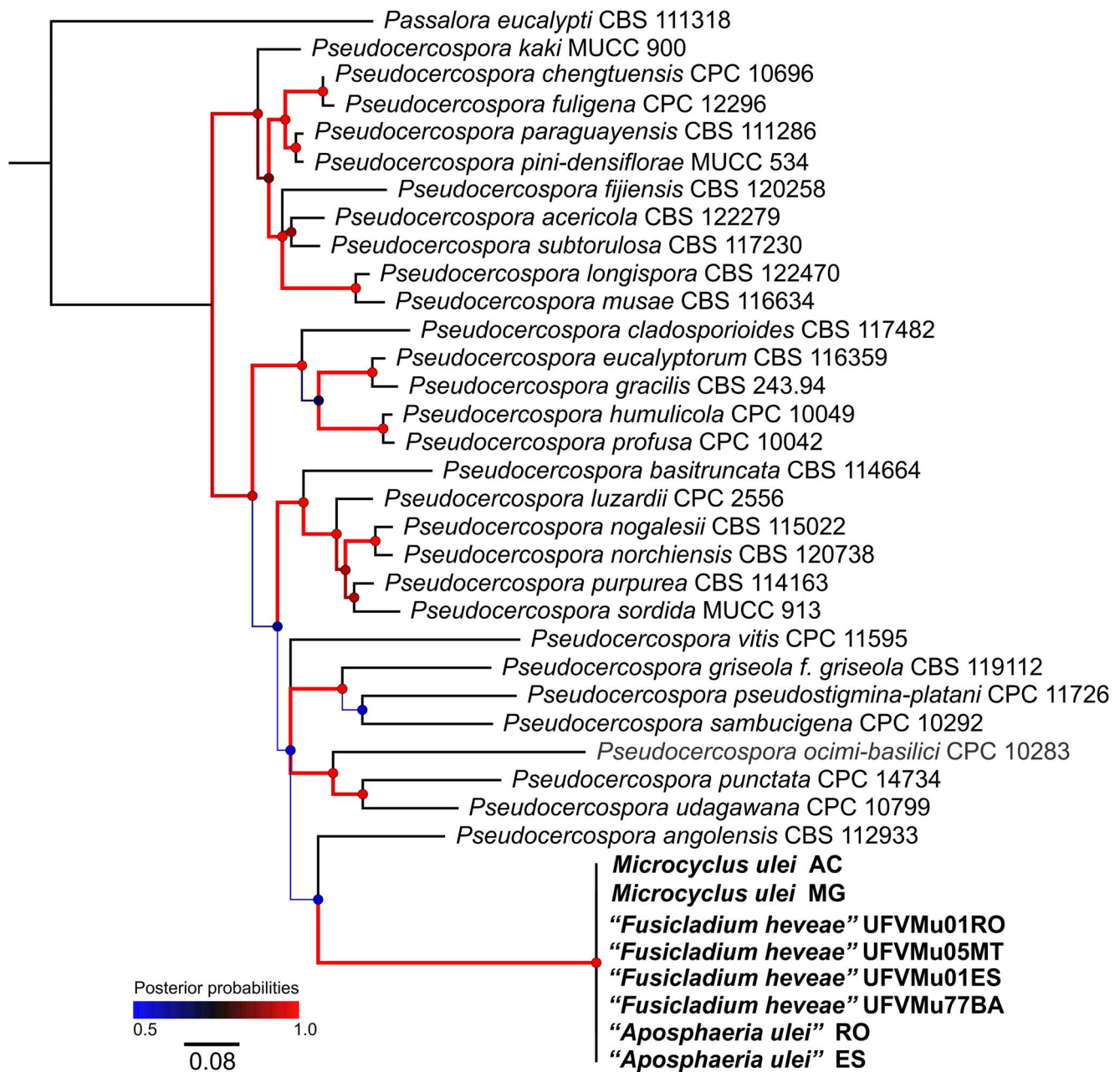


Figure 4. Phylogenetic relationships of *Microcyclus ulei* based on the combined ITS, EF-1 α and ACT sequences alignment. Bayesian posterior probabilities are given at the nodes and coded according to the colored scale bar. The black line scale bar shows 0.08 expected changes per site. The tree was rooted with *Passalora eucalypti*. doi:10.1371/journal.pone.0104750.g004

Trinidad & Tobago, Venezuela); *H.colina* (Brazil); *H. confusa* (Guyana); *H. guyanensis* (Brazil, Guyana, South America, Suriname); *H. guianensis* (South America); *H. guianensis var. lutea* (Peru); *H. lutea* (Peru); *H. paludosa* (Brazil); *H. randiana* (Brazil); *H. spruceana* (Brazil, Costa Rica, Guyana, Panama, South America, Suriname, Trinidad & Tobago); *Hevea* spp. (Brazil, Trinidad & Tobago) [47,48].

Material examined: sexual morph and spermatogonial morph - BRAZIL, Pará, Belém, on living leaves of *Hevea brasiliensis*, 14 March 2007, H. C. Evans (VIC 30547), **asexual morph** - Bahia, Porto Seguro, on living leaves of *Hevea*

brasiliensis, September 2008, B. T. Hora Junior (VIC 39722 – COAD 1339)

Additional material examined: sexual morph - BRAZIL, Pará, Belém, 14 March 2007, H. C. Evans (VIC 30549), Acre, Xapuri, January 2010, B. T. Hora Junior (VIC 39728); Minas Gerais, Oratórios, Fazenda Experimental EPAMIG, September 2010, B. T. Hora Junior (VIC 39729); **asexual morph** - BRAZIL, Rondônia, Buritis, July 2010, J. Honorato Junior (VIC 39723 – COAD 1340); Mato Grosso, Itiquira, February 2009, B. T. Hora Junior (VIC 39724 – COAD 1341); Espírito Santo, Sooretama, December 2010, B. T. Hora Junior (VIC 39725 – COAD 1342); **spermatogonial morph** - BRAZIL, Rondônia,

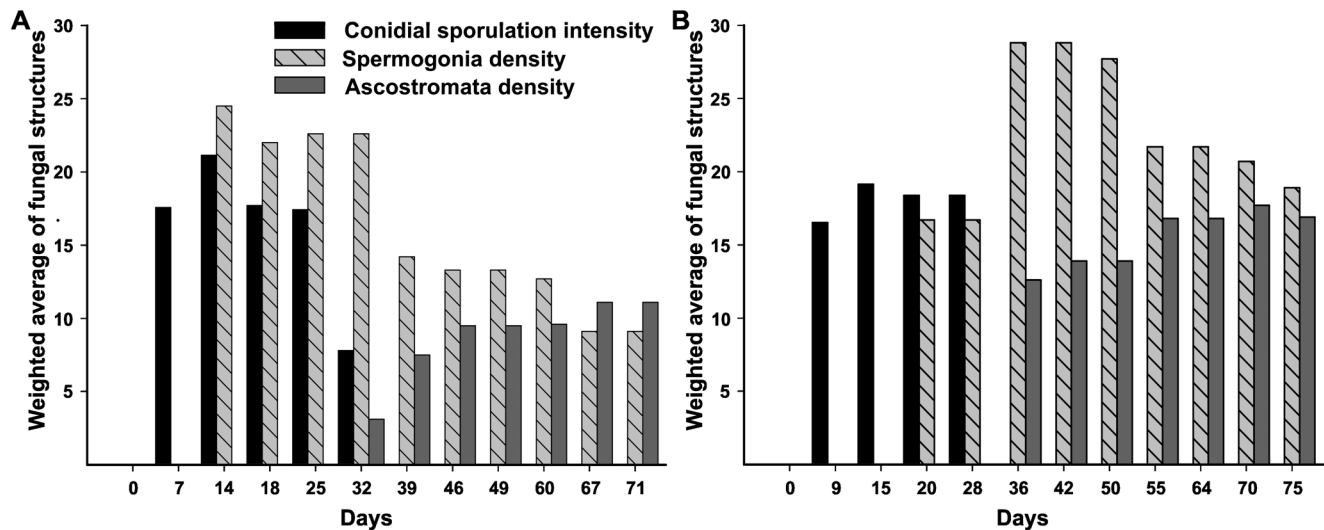


Figure 5. Pleomorphic development of the life cycle of *Pseudocercospora ulei*. Weighted average based on the score of conidial sporulation intensity and spermogonia and ascostromata density during the leaf development of RO38 rubber clone grown under field conditions. Assessments were made from December 15, 2011 to February 24, 2012 (A); and from September 19 to December 3, 2012 (B). doi:10.1371/journal.pone.0104750.g005

Ariquem, on living leaves of *Hevea brasiliensis*, July 2010, J. Honorato Junior (VIC 39726); Espirito Santo, Cachoeiro do Itapemirim, December 2010, B. T. Hora Junior (VIC 39727).

Discussion

DNA sequences of the three morphs in the life cycle of *M. ulei* collected over a wide geographic area in Brazil confirmed the asexual:sexual morph connection of this fungal species, but the current classification of the pathogen in the Planistromellaceae [13,14] was not supported by any of the phylogenies. The analysis conducted in the present study was based on nuclear and mitochondrial ribosomal rDNA, as well as on protein-coding genes, and supports the classification of *M. ulei* in the family Mycosphaerellaceae s. str., in the order Capnodiales, class Dothideomycetes of the phylum Ascomycota. Mycosphaerellaceae is a well-supported family within the Capnodiales with *Mycosphaerella punctiformis* (*Ramularia endophylla* as anamorph) as the type species [49,50]. DNA sequence data of *R. endophylla* were included in all analyses of generic relationships, corroborating the classification of the pathogen at the family level and revealing a close relationship of the SALB fungus with *Mycosphaerella*. Currently, robust multi-gene phylogenetic analysis concluded that *Mycosphaerella* is a polyphyletic group [51–53], suggesting that *Mycosphaerella* s. lat. should be subdivided to reflect natural groups (genera) as defined by their asexual morphs, since *Mycosphaerella* s. str. is restricted to species with *Ramularia* morphs [52].

The phylogeny of species representing the core genera of the Planistromellaceae formed a clade within the order Botryosphaeriales and revealed that previous morphology-based definitions of genera have resulted in an artificial classification system and thus, the genera *Planistromella* and *Planistroma* have been considered to constitute one genus, namely *Kellermania* [15]. In addition to *M. ulei*, other species previously classified in Planistromellaceae have been transferred to the Mycosphaerellaceae, as in the case of *Eruptio acicula* [54] and to the Phaeosphaeriaceae as for *Loratospora aestuarii* [53], after phylogenetic re-evaluation. The classification of *Microcyclus* as a

mycosphaerella-like organism has been discussed previously [55]. The *Microcyclus* genus has ellipsoidal, hyaline, 1-septate ascospores in clavate, bitunicate asci, typical of the genus *Mycosphaerella* Johanson [26]. The development of stromatic tissue in *Microcyclus* appears to be the only character that contributes to its separation from the genus *Mycosphaerella* [55]; although Barr [13] further characterized the genus by the presence of periphysate ostioles in the type species *Microcyclus angolensis*. Nevertheless, some mycosphaerella-like species have similar strongly erumpent ascostromata [12,26]. As already demonstrated for *Mycosphaerella*, the genus *Microcyclus*, as currently circumscribed, also appears to be polyphyletic, given the variety of asexual morphs associated with the assigned species [55].

The conidial morph of *M. ulei*, *F. heveae*, was “tentatively retained in *Fusicladium* since it is morphologically indistinguishable from other species of this genus” [25]. Bonorden [56] characterized the genus *Fusicladium* as having denticulate conodiogenous cells. In Saccardo [57], Lindau [58] and Ferraris [59] this genus was described as having sympodial conidiogenous cells (denticulate) or percurrent (annelidic). In the 1950s, Hugues [60] limited *Fusicladium* spp. to species having conidiogenous cells with sympodial proliferation. Sympodial proliferation on its own is a character that is far from adequate for grouping species within the cercosporoid complex as this is a feature widespread among genera of cercosporoids. Furthermore, our observations have consistently shown that *P. ulei* does not have denticulate conidiogenous loci but instead it has the typical locus structure of fungi in *Pseudocercospora* - truncate without thickening and pigmentation of conidiogenous scars contrarily to “slightly denticulate” as in the description included in Schubert et al. [25]. Another feature consistently observed that was in disagreement with Schubert et al. [25] as the absence of “well formed stromata from which the conidiophores emerge”. Instead, only a 1–2 cell layer of pseudoparenchymatous cells from which a dense palisade of somewhat parallel to subfasciculate conidiophores was observed. Species of *Fusicladium* s. lat. form a monophyletic group in the Venturiaceae [24,61], whilst other fusicladium-like species have been assigned to the Sympoventuriaceae, both in the Venturiales [24]. Although *F. heveae* has already been treated as a

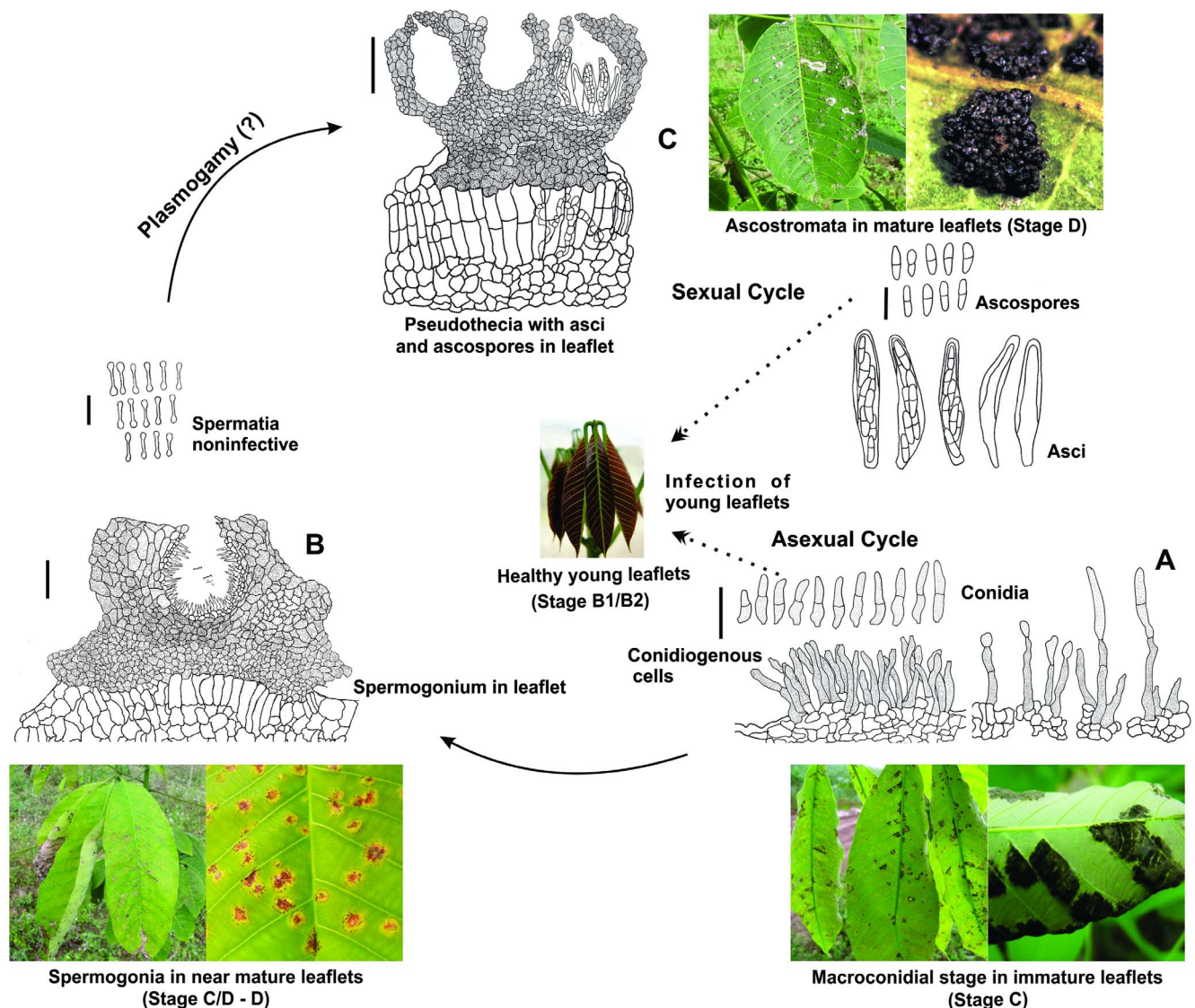


Figure 6. Hypothetical life cycle of *Pseudocercospora ulei*. **A.** Asexual morph with conidiophores and conidia (Bar = 35 μ m) and conidiogenous cells with conidia at different stages of conidial formation. Pictures: Lesions to which the asexual morph is associated (left) and close-up of leaf bearing typical lesions (right). **B.** Spermatogonial morph with stroma, spermatogonia (Bar = 30 μ m) and spermatia (Bar = 7 μ m). Pictures: Lesions to which the spermatogonial morph is associated (left), and close-up of the same lesions (right). **C.** Sexual morph with stroma, pseudothecia, asci and ascospores (Bar = 60 μ m). Pictures: Lesions to which the sexual morph is associated (left), and close-up of stomata (right). Dotted arrows indicate that both ascospores and conidia can infect young leaves.
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species of the *Passalora*-type and, therefore, a cercosporoid fungus [16], our molecular data demonstrate that the SALB pathogen is better accommodated in the clade *Pseudocercospora* s. str., as defined by Crous et al. [52,62] in which the type species, *Pseudocercospora vitis*, resides (Figures 1 and 4). Whilst in the Mycosphaerellaceae, many asexual morphs evolved in more than one clade and thus represent different genera [51], [52], the morphological convergence of '*F. heveae*' is at the order level, as is also evident for '*A. ulei*' (Figure 1).

Pseudocercospora s. str. is a well-defined genus in the Mycosphaerellaceae, based on both DNA sequence and morphological data [52,62], which is now utilized as a holomorph name with species having mycosphaerella-like sexual morphs. As observed for '*F. heveae*' in the present study, when the phylogenetic species concept is applied to other species of the genera *Cercostigmia*,

Phaeoisariopsis and *Stigmia*, they are reduced to synonymy with the genus *Pseudocercospora* [62–65].

Pseudocercospora s. str. includes several well-known and highly destructive plant pathogens affecting important crops worldwide [62]. Recognizing the SALB fungus as belonging to such genus allows for the adoption of comparative epidemiology and genomics approaches, using better studied pathogenic species such as *P. fijiensis*, the causal agent of the black leaf streak (black Sigatoka) disease of banana [66].

Based on the molecular evidence connecting the three spore morphs in the life cycle of the SALB fungus and comparative biology from phylogenetic relationships, the life cycle of *M. ulei* was then re-assessed with special attention to its intermediate 'pycnidial' morph. Physiological data indicate that leaves at the B and C stages act as sinks with high respiration rates and are almost

lignin-free [3]. Previous studies report that conidial lesions are the first stage of the disease and fertile pycnidia occur three to five weeks later on mature or near-mature diseased leaves [2,19]. Ascstromata become mature at about four to six weeks and the formation of ascospores is correlated with effete pycnidia [1]. In our study, mature 'pycnidia' were seen after two to three weeks on the upper surface of leaves in the C/D and D stages in the area previously occupied by abaxial conidial (*Pseudocercospora*) lesions. After four weeks, the ascstromata became more visible and increased in number and size.

In contrast to a previous study [1], but in accordance with an earlier study [19], our results confirmed that the pycniospores do not germinate *in vitro* and fail to infect rubber leaves. These observations corroborate the hypothesis that the supposedly erumpent pycnidial structures are in fact spermogonia and are likely to be involved in the initial stages of the sexual cycle, as suggested previously [2,19,26]. Commonly, fungi in the Mycosphaerellaceae produce spermogonia and the spermatia are thought to act as male sexual elements because of their small size and inability to germinate and to infect the host plant. Pseudothecial development begins from protoascomata, usually concurrently with the spermogonia, and the two structures are similar in size and shape [67–70]. The production of spermatia is also part of the life cycle of *Pseudocercospora fijiensis* [66,71], and such spermatia 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 elucidated [66]. Similar fertilization events can also take place in *P. ulei*.

A revised version of the life cycle of this pleomorphic fungus is presented (Figure 6). Only one asexual morph, which belongs to *Pseudocercospora* s. str., is present and conidia infect young leaves being responsible for the destructive secondary disease cycles 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 ascstromata of the *Mycosphaerella*-type.

The persistence of significant gaps in the knowledge on the biology of a fungus of the importance of *P. ulei* has puzzled

Money [7] who stated "I am astonished by the apparent ostrich-like behavior of the rubber-manufacturing companies towards the disease... the scientists endeavor to understand the fungus appears frozen... the lack of recent publications in the public domain is remarkable." His anxiety was clearly justifiable considering the lack of a proper taxonomic treatment for the fungus and of adequate understanding of its life cycle as indicated by the present findings. Much more needs to be investigated about the SALB fungus if we are willing to properly understand the biology of the fungus and prepare to deflect the threats represented by SALB.

Supporting Information

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

Newly deposited sequences are shown in bold.

(DOCX)

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Author Contributions

Conceived and designed the experiments: BTHJ CRRM LAM ESGM. Performed the experiments: BTHJ DMM. Analyzed the data: BTHJ DMM RWB HCE ESGM. Contributed reagents/materials/analysis tools: CRRM RWB ESGM. Contributed to the writing of the manuscript: BTHJ RWB HCE ESGM.

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