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Research Article

Evidence that the *Ceratobasidium*-like white-thread blight and black rot fungal pathogens from persimmon and tea crops in the Brazilian Atlantic Forest agroecosystem are two distinct phylospecies

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Abstract

The white-thread blight and black rot (WTBR) caused by basidiomycetous fungi of the genus *Ceratobasidium* is emerging as an important plant disease in Brazil, particularly for crop species in the Ericales such as persimmon (*Diospyros kaki*) and tea (*Camellia sinensis*). However, the species identity of the fungal pathogen associated with either of these hosts is still unclear. In this work, we used sequence variation in the internal transcribed spacer regions, including the 5.8S coding region of rDNA (ITS-5.8S rDNA), to determine the phylogenetic placement of the local white-thread-blight-associated populations of *Ceratobasidium* sp. from persimmon and tea, in relation to *Ceratobasidium* species already described world-wide. The two sister populations of *Ceratobasidium* sp. from persimmon and tea in the Brazilian Atlantic Forest agroecosystem most likely represent distinct species within *Ceratobasidium* and are also distinct from *C. noxium*, the etiological agent of the first description of white-thread blight disease that was reported on coffee in India. The intraspecific variation for the two *Ceratobasidium* sp. populations was also analyzed using three mitochondrial genes (*ATP6*, *nad1* and *nad2*). As reported for other fungi, variation in nuclear and mitochondrial DNA was incongruent. Despite distinct variability in the ITS-rDNA region these two populations shared similar mitochondrial DNA haplotypes.

Key words: ecological speciation, fungal plant pathogens, host specialization, population divergence, phylogenetics.

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Introduction

The white-thread blight and black rot (WTBR) associated with fungal pathogens from the *Ceratobasidium* species complex is considered an emerging plant disease for Brazilian crop species in the Ericales, especially persimmon (*Diospyros kaki* L.) and tea [*Camellia sinensis* (L.) Kuntze] (Furtado, 1997; Cavalcante and Sales, 2001). However, the species identity of the pathogen associated with either of these hosts is still unclear; it is also unknown whether the fungal pathogens associated with WTBR in persimmon and tea are the same or distinct species. WTBR is particularly important in Brazilian tropical agroecosystems, such as those neighbouring the Amazon and Atlantic Forests, because of the high temperatures and humidity that are conducive to disease development (Lourd and Alves, 1987; Gasparotto and Silva, 1999).

The first description of the WTBR pathogen was reported by Cooke in 1876, based on material collected from infected coffee plants in India (Tims *et al.*, 1954). Cooke considered the pathogen a hyphomycete and named it *Pellicularia koleroga* Cooke 1876. In 1910, Von Hoehnel re-described the pathogen naming the fungus as *Corticium koleroga* (Cooke) von Höhnel 1910 (Tims *et al.*, 1954). In addition to *Corticium koleroga*, synonymy of *P. koleroga* has included *Botryobasidium koleroga* (Cooke) Venkatarayan, *Hyphocnus koleroga* Stevens & Hall, *Koleroga noxia* Donk and *Ceratobasidium noxium* (Donk) P. Roberts (Venkatarayan, 1949; CAB-International, 2004).

Historically, initiatives for a better taxonomical positioning of the fungal species related to WTBR pathogens were begun by Rogers (1943), who positioned *P. koleroga* within the *Botryobasidium* complex. Donk (1954) pro-

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posed the monotypic genus *Koleroga* to accommodate *P. koleroga* (characterized by secondary spores and tetra-spored basidia). Talbot (1965) considered the genus *Pellicularia* as a synonym of *Ceratobasidium*; other species with secondary spores were transferred from *Botryobasidium* to the genera *Thanatephorus* and *Uthatobasidium*. While one of the most important species within *Thanatephorus* (*T. cucumeris*) is a pathogen for several plant species (Sneh *et al.*, 1991), the *Botryobasidium* complex (which includes *Botryohypochnus* and *Uthatobasidium*) is basically composed of wood decomposing fungal species. Currently, the white-thread blight-causing species of several tree plants are grouped within the genus *Ceratobasidium*, more precisely *C. anceps*, *C. cornigerum* and *C. noxium* (Roberts, 1999).

The first report of WTBR caused by *Ceratobasidium* in Brazil dates back to 1978 when the occurrence of a white-thread blight-like fungal pathogen was observed in a Pomelo [*Citrus maxima* (Merr., Burm. f.)] orchard in the Amazon region. The pathogen associated with this disease was identified as *P. koleroga* (Rosseti *et al.*, 1982). Lourd and Alves (1987) reported that 27 plant species were hosts for this pathogen in the Amazon. Another 18 species of native fruit trees from the Amazon were subsequently described as hosts for *P. koleroga* (Gasparotto and Silva, 1999). Among these host species, WTBR has been reported to affect soursop (*Annona muricata* L.), black-pepper (*Pipper nigrum* L.), cacao (*Theobroma cacao* L.), *Citrus* sp., coffee (*Coffea arabica* L), mango (*Mangifera indica* L.), rubber trees [*Hevea brasiliensis* (Willd. Ex Adr. de Juss.) Muell. & Arg.] and several other tree species (Fawcett, 1914; Rosseti *et al.*, 1982; Furtado, 1997; Pereira *et al.*, 2000; Benchimol *et al.*, 2001). WTBR has also been reported to affect several tree crops worldwide and occurs on most continents. In the Americas, WTBR has been reported in Argentina, Brazil, Columbia, Venezuela, and the Guianas, in several Central American countries, Mexico and the United States (Tims *et al.*, 1954; CAB-International, 2004). These *Ceratobasidium*-associated diseases have recently been reported to affect persimmon (white-thread blight) and tea (black-rot) in areas near the Atlantic Forest agroecosystem of São Paulo State, such as Guararema, in the Mogi das Cruzes region (Pereira *et al.*, 2003) and Registro, in the Vale do Ribeira (Ribeira River Valley) (Furtado, 1997), respectively. Signs of the pathogen and symptoms of the disease on persimmon and tea are shown in Figure S1.

In Brazil, there are several tree species for which the identity of the fungal pathogen that causes white-thread blight is unknown (Lourd and Alves, 1987). We are particularly interested in resolving the phylogenetic placement of the *Ceratobasidium* species associated with persimmon and tea from the Brazilian Atlantic Forest agroecosystem. This is the first study to establish the identity of the pathogen(s) associated with WTBR on these Brazilian crops. Similarly, there is no information on the recent levels of intraspecific genetic variation in these two populations of *Ceratobasidium* sp. or on the extent of recent or historical genetic divergence between the persimmon- and tea-derived populations of the pathogen.

DNA sequence information from ribosomal DNA (rDNA) genes and their transcribed spacer regions (ITS1 and 2) has been extensively used to establish the relationship among distinct and closely related taxa within the *Ceratobasidium* complex by phylogenetic methods (Gonzalez *et al.*, 2001). Variation in the rDNA genes has also been used for studies of fungal population divergence to determine the population-species interface (White *et al.*, 1990; James *et al.*, 2001; Ciampi *et al.*, 2005).

In contrast to nuclear genes, fungal mitochondrial DNA (mtDNA) has accumulated much fewer changes compared with mammalian mtDNA (Clark-Walker, 1991). However, no intraspecific studies have surveyed nuclear and mitochondrial variation in any *Ceratobasidium* species or even among sister species of the important plant pathogenic genus *Thanatephorus*. There is currently no information as to whether the variation in mtDNA regions is less than or equal to that for nuclear genes in these fungi.

The initial objective of this study was to use sequence variation in the ITS-5.8S region of rDNA to determine the phylogenetic placement of the local white-thread-blight-associated populations of *Ceratobasidium* sp. from persimmon and tea in relation to *Ceratobasidium* species already described around the world. We also analyzed the intraspecific sequence variation in nuclear rDNA (ITS1, ITS2 and 5.8S) to test the hypothesis that the two distinct populations of *Ceratobasidium* (one in each host) represent a single species with a very low level of recent or historical divergence between them. The intraspecific variation in the two *Ceratobasidium* sp. populations from tea (Cs) and persimmon (Dk) was analyzed using a previously sequenced coding region from the mitochondrial gene coding for ATP synthase 6 (ATP synthase subunit 6, *ATP6* (Kretzer and Bruns, 1999) and two others from two mitochondrial NADH dehydrogenase genes (NADH dehydrogenase subunit 1, *nad1*, and subunit 2, *nad2*). The NADH subunit sequences were obtained specifically for this study based on sequences of ortholog genes from other Basidiomycota. Since fungal mtDNA has a lower mutation rate than nuclear DNA (Clark-Walker, 1991) we hypothesized that the variation in mtDNA in *Ceratobasidium* sp. would be lower than in nuclear DNA.

Materials and Methods

Population samples

Two populations of the WTBR fungal pathogen *Ceratobasidium* sp. were sampled. One population was obtained from persimmon “Rama Forte” in Guararema (23° 32’ 32”S, 46° 12’ 00” W), in the Mogi das Cruzes re-

gion, São Paulo state (SP), Brazil, in February 2005. The other population was obtained from tea "IAC 259" in Registro (24° 29' 15" S, 47° 50' 37" W), in the Vale do Ribeira region (SP) (Figure 1). The samples were collected from infected actively growing vegetative material from adult plants; 10-12 disease foci were sampled from transects along the fields and 5-6 infected branches were collected from each focus. Thirty-one isolates were obtained from persimmon and another 31 from tea. The isolates were initially obtained using alkaline water agar (pH 8.5) with subsequent transfer of fungal hyphal tips to potato-dextrose agar (PDA, Difco) (Gutierrez *et al.*, 1998). For long-term storage, four-day-old fungal colonies grown on potato-dextrose agar supplemented with 50 µg of kanamycin/mL were transferred to sterilized parboiled rice (containing 50 µg of chloramphenicol/mL), grown for about five days at 25 °C, air-dried under laminar-flow for two days and finally preserved at -20 °C (Ceresini and Souza, 1996). Four extra isolates (from persimmon, tea, soursop and mango) previously obtained in our lab were included in the sample. All isolates showed typical *Ceratobasidium* growth on potato-dextrose-agar and were characterized as binucleate by classic cytomorphological methods (Herr and Roberts, 1980; Sneh *et al.*, 1991; Ceresini and Souza, 1996).

DNA extraction

Fungal mycelia for DNA extraction were produced by growing the *Ceratobasidium* isolates in 25 mL of PD broth supplemented with kanamycin (50 µg/mL) in 50 mL Falcon tubes (Greiner Bio-one, Germany). After five days of incubation at 25 °C on a rotor-shaker at 180 rpm, the mycelia were harvested, frozen and lyophilized. Total DNA was extracted from lyophilized mycelial tissue using DNeasy Plant mini extraction kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR amplification and sequencing of the ITS region of rDNA

Polymerase chain reactions (PCR) to amplify the ITS1-5.8S-ITS2 rDNA region were done on a Biometra T-Gradient thermocycler (Göttingen, Germany) in 20 µL reactions containing 20-50 ng of total DNA, 2 µL of 10X reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, 20 mM $MgSO_4$, 1% Triton X-100, pH 8.8; NEB [New England Biolabs]), 0.5 µM of each primer (ITS4/ITS5; White *et al.*, 1990), 0.1 mM of each dNTP and 0.5 units of *Taq* polymerase (NEB – New England Biolabs). The PCR conditions were 96 °C for 2 min, 35 cycles of denaturation for 1 min at 96 °C, annealing for 1 min at 55 °C and elongation for 1 min at 72 °C. The extension time of the final cycle was 5 min. The PCR products were purified using Nucleofast-96-PCR clean-up plates (Macherey-Nagel, Germany).

The ITS-5.8S region of rDNA was sequenced using a pre-mixed kit for cycle-sequencing based on dye-terminator chemistry (ABI-PRISM Big-Dye Terminator v. 3.0 Cycle Sequencing Ready Reaction, Applied Biosystems, USA) according to the manufacturers instructions. Around 50 ng of purified PCR amplicon DNA and 1 µM of each of the primers (ITS4 and ITS5) were used in each of two independent sequencing reactions. The sequencing products were separated by electrophoresis in the 16-capillary array

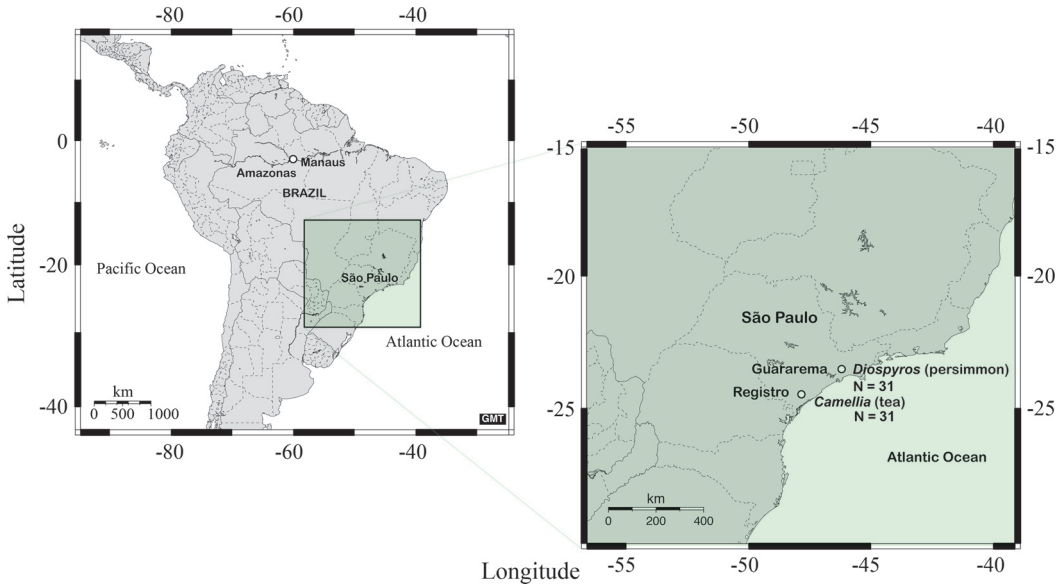

Figure 1 - Geographic origin of *Ceratobasidium* sp. isolates obtained from persimmon (*Diospyros kaki*, Ericales, Ebenaceae) and tea (*Camellia sinensis*, Ericales, Theaceae) in São Paulo state, Brazil. White-thread blight diseased persimmon plants were sampled in Guararema while black-rot infested tea plants were collected in Registro. Both sampling locations belong to the Brazilian Atlantic Forest agroecosystem. Four extra isolates obtained from persimmon and tea in São Paulo and from mango (*Mangifera indica*, Sapindales, Anacardiaceae) and soursop (*Anonna muricata*, Magnoliales, Annonaceae) in Manaus, Amazonas, Brazil, were included in the analyses.

of an automated ABI-3100 sequencer (PE Applied Biosystems) using a POP6 separation matrix (Applied Biosystems). The sequences obtained were analyzed for quality, base content and contig assembly using the software Sequencher 4.5 (Gene Code Corporation, Ann Arbor, MI, USA).

Cloning of PCR amplicons

The predominant heterokaryotic condition in the *Ceratobasidium* life cycle means that heterokaryotic in the DNA sequences from non-cloned PCR amplicons is commonly observed. To separate distinct alleles of the ITS-5.8S rDNA operon from the same heterogeneous PCR product the heterogeneous amplicons were cloned into the vector PCR2.1-TOPO (Invitrogen). Selected recombinant plasmids from *Escherichia coli* One Shot DH5a-T1R (Invitrogen) were extracted from each one of the cloned heterogeneous samples and purified using QIAprep Spin Miniprep kits (Qiagen). Primers for one of the multiple cloning sites of the vector were used for reamplification and sequencing.

Cloning of nad1 and nad2 orthologs and mitochondrial haplotyping using *nad1*, *nad2* and *ATP6* genes

Orthologous sequences from two mitochondrial genes (*nad1* and *nad2*) from the Basidiomycota fungi *Crinipellis perniciosa* (NCBI accession number AY376688), *Cryptococcus neoformans* var. *grubii* (NC_004336 and AY560608) and *Schizophylum commune* (NC_003049) available at GenBank/NCBI were aligned and used to design degenerate primers targeting conserved regions of the two loci. Fungal genomic DNA was amplified with degenerate primers designed from the *nad1* and *nad2* sequences (see Table S1). The PCR conditions were the same as described above and the products were separated by electrophoresis on a 0.8% low-melting agarose gel. Amplicons of the expected size were cut from the gel and cloned into the vector PCR2.1-TOPO and transformed in *E. coli* One Shot DH5a-T1R. Positive colonies were selected and the plasmid DNA was purified and sequenced as described above. Specific primer pairs designed from conserved regions were able to amplify sequences encoding *nad1* and *nad2* from *Ceratobasidium* and its sister genus *Thanatephorus*.

In addition to *nad1* and *nad2*, another mitochondrial gene (*ATP6*) was used for mitochondrial haplotyping of *Ceratobasidium* sp. populations from persimmon and tea. The Basidiomycota *ATP6*-specific primers used in this study were the same as those recommended by Kretzer and Bruns (1999).

Data analysis

Initially, the sequences of the ITS-5.8S rDNA region from the Brazilian isolates of *Ceratobasidium* sp. were compared with *Ceratobasidium* spp. sequences deposited in GenBank/NCBI. Similarity comparisons among sequences were done using BLASTN (nucleotide-nucleotide) version 2.2.12 (Altschul *et al.*, 1997).

Phylogenetic analyses

For the phylogenetic analyses, we included sequences of the ITS-5.8S rDNA region retrieved from GenBank/NCBI for the following species of *Ceratobasidium*: *C. albasitensis*, *C. anceps*, *C. angustisporum*, *C. bicorne*, *C. cereale (R. cerealis)*, *C. cornigerum*, *C. papillatum* and *C. ramicola*. These sequences shared 78%-86% identity with the ITS-5.8S rDNA sequences from the Brazilian *Ceratobasidium* isolates from persimmon and tea. All of the sequences were aligned using the program ClustalX (Thompson *et al.*, 1994, 1997). We also included a sequence of the ITS-5.8S region of *C. noxium* type strain (CBS154-35) introduced from CBS (Centraalbureau voor Schimmelcultures, The Netherlands).

The phylogenetic analyses were done using maximum likelihood (ML), maximum parsimony (MP) and Bayesian methods. For both ML and MP analyses, the software PAUP 4.0b10 was used (Swofford, 2002). The Bayesian analysis was done using the metropolis coupled Monte Carlo Markov Chain algorithm (MCMCMC), implemented in MrBayes v. 2.10 (Huelsenbeck and Ronquist, 2001).

For the ML and MCMCMC analyses, MODELTEST 3.7 (Posada and Crandall, 1998) was used to determine the model of nucleotide evolution that best fitted the data. A hierarchical likelihood ratio test implemented by MODELTEST selected the model GTR + G + G (proportion of invariable sites = 0.4309; base frequencies: A = 0.3097, C = 0.2189, G = 0.2226 and T = 0.2488; substitution rates R[A-C] = 0.3423, R[A-G] = 5.1103, R[A-T] = 0.4034, R[C-G] = 0.0.0381, R[C-T] = 5.1103, R[G-T] = 1, and gamma distribution shape parameter = 0.7787). Under this model for base substitution, the ML value for the tree was -ln(L) = 1835.11.

The parsimony analysis was done by weighting all bases equally and treating indels as missing data. One hundred and nine of the characters were parsimony informative. Heuristic searches were done with maxtrees set to automatically increase, random taxon addition and TBR branch swapping (839 trees with scores ≥ 295 were saved). Bootstrap analysis was used to determine the statistical support for each branch of the trees generated (for ML and MP optimality criteria) with 1,000 pseudo-replications in which all characters were re-sampled in each replication.

The phylogenetic analysis by MCMCMC was done using the *a priori* model of nucleotide evolution selected by MODELTEST. We searched for 3,000,000 generations using four chains and eliminated the first 250 trees as burning; one tree in every 500 sampled was stored. Posterior proba-

bility values (PP) were generated from a 50% majority rule consensus tree of 6,000 saved trees.

Intraspecific evolution of *Ceratobasidium* sp. populations from persimmon and tea inferred by analysis of a haplotype network

To infer intraspecific evolution, networks of haplotypes sampled from populations of *Ceratobasidium* sp. from persimmon and tea were built using the algorithm recommended by Posada and Crandall (2001) and the "shareware" TCS developed by Clement *et al.* (2000). This method starts by estimating the maximum number or differences between haplotypes as a result of single substitutions (*i.e.*, those not resulting from multiple substitutions in a single site) with a statistical significance level of 95% (Templeton *et al.*, 1992). This is called the limit of parsimony or the limit of parsimony connection. Haplotypes differing by only one change are then connected, followed by those differing by two, three and so on until all of the haplotypes are included in a single network, or until the limit of parsimony connection is reached. The estimation of phylogeny from DNA sequences was done using TCS (Clement *et al.*, 2000).

Test for population subdivision and coalescent analysis

We used the SNAP Workbench Java program package to analyze gene genealogies and population parameters (Price and Carbone, 2005). SNAP Map was used to collapse sequences into haplotypes and to remove indels and infinite sites violations among the mutations (Aylor *et al.*, 2006). Coalescent methods make strict assumptions, such as neutrality and a lack of recombination, that must be verified beforehand. To test for departures from neutrality, Tajima's D (Tajima, 1989) and Fu and Li's D and F* (Fu and Li, 1993) test statistics were calculated using the statistical tests of neutrality of mutations against an excess of recent mutations (rare alleles).

SNAP Clade and SNAP Matrix were used to screen the variable sites and identify those showing homoplasy (Figure 2). To generate compatible sequence alignments for coalescent analysis, Carbone and Kohn (2001) suggested the removal of haplotypes whereas Stukenbrock *et al.* (2007) suggested the manual removal of conflicting sites in each gene alignment ; we used the latter approach. 17 polymorphic sites were removed from the dataset [13 incompatible sites (see Figure 2), one that violated the infinite sites model and three that were invariable after removal of the Amazonian haplotypes (HCs05 and HCs11); see Table S2 for details], resulting in 32 compatible polymorphic sites after manipulation of the alignments. To determine the order of coalescent events for haplotypes backward in time it is necessary to determine the amount of migration that occurred between populations. Haplotypes from populations linked by migration are postulated to coalesce before haplotypes from unlinked populations (Carbone and Kohn, 2001). The program MIGRATE 2.1.3 (Beerli and Felsenstein, 2001) was used to construct a migration matrix that indicated the number of migrants exchanged between populations. This backward migration matrix was used for ancestral inference in Genetree version 9.0 (Griffiths and Tavaré, 1994, 1996) and was also incorporated in the SNAP Workbench. Subsequently, we reconstructed the genealogy with the highest root probability, the ages of mutation and the T_{MRCA} (time to the most recent common ancestor) of the samples using 100,000 coalescent simulations with five runs that had distinct starting random number seeds; this analysis took into consideration population subdivision and assumed populations of the same size. The program allows estimation of the ancestral history of each haplotype and shows the distribution of mutations on a coalescent scale, thus allowing for comparison of the divergence of haplotypes within and between each population.

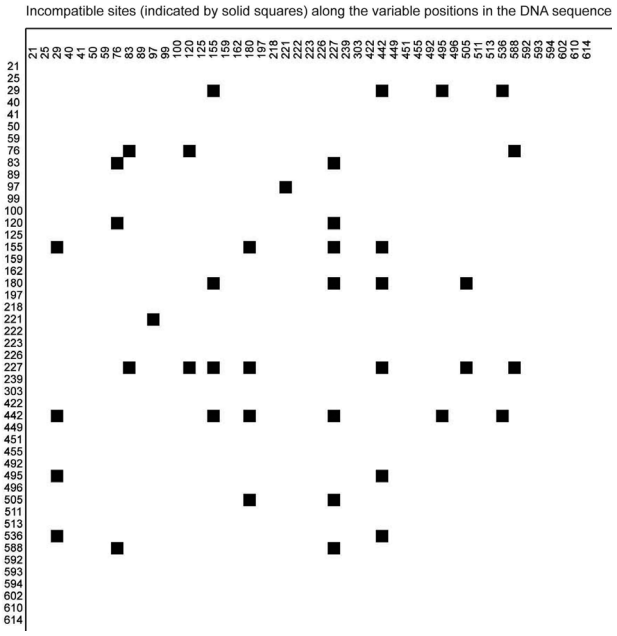

Figure 2 - Site compatibility matrices for *Ceratobasidium* sp. homoplasious ITS-rDNA haplotypes from persimmon and tea. The numbers along the top and the left of the matrix indicate variable positions in the DNA sequence alignments and the left of the matrix of the data. Incompatible sites are indicated by solid squares; all other sites in the matrix are compatible.

Population size fluctuation (θ_t and θ_{now}), demographic parameters (N_e) and the migration rates for each population ($M_{Lamarc} = m/\mu$) were tested using an MCMC method implemented by the computer program LAMARC 2.1 (Kuhner, 2006; Kuhner and Smith, 2007). We used the initial parameters of gene flow among populations with the migration rates obtained with MIGRATE 2.1.3 (Beerli and Felsenstein, 2001). Our search strategy included 10 replicates of 10 initial chains and five long final chains. The initial chains were constructed with 500 samples and a

sampling interval of 20 (10,000 steps), using a burn-in of 1000 samples for each chain. The five final chains were constructed with 5,000 samples and a sampling interval of 200 (1,000,000 steps) and a burn-in of 10,000. The confidence interval for theta (θ) and growth rate (*g*) was calculated using the percentile approach. The ancestral N_e at '*t*' time ago was calculated using the following equation for population growth: $\theta_t = \theta_{now}\ e^{-gt}$, where 'θ' represents a measure of effective population size (for diploids, theta = $4N_e\mu$, μ is the mutation rate inferred for the locus), '*t*' is the time in mutational units and '*g*' is the exponential growth parameter.

We also used the Bayesian skyline plot method implemented in the program BEAST 1.2 (Drummond *et al.*, 2005; Drummond and Rambaut, 2006) to estimate the time dynamics of population size fluctuation. This Bayesian method uses an MCMC integration under a coalescent model to incorporate uncertainty in the genealogy, where the timing of divergence dates provides information about the effective population sizes through time. The T_{MRCA} for all sequences and for the two major clades (*Camellia* and *Diospyrus*) were also estimated using two clock rate values: $8.806e^{-4}$ and $16.983e^{-4}$ substitutions/gene*10^3 years which are, respectively, the average and the highest substitution rates proposed for the ITS-rDNA region in fungi (Kasuga *et al.*, 2002).

Analysis of mitochondrial haplotype diversity

We estimated the level of mtDNA polymorphism at three loci (*ATP6, nad1* and *nad2*) by determining the number of polymorphic sites, the average number of nucleotide differences (k) between two haplotypes and the sharing of haplotypes between the two populations of *Ceratobasidium* sp. (Cs and Dk). The divergence between the Cs and Dk populations was contrasted with that between two sister phylospecies within the *Thanatephorus* complex (which has *R. solani* anamorphs): *R. solani* anatomosis group AG-1 IA (*Rs* AG-1 IA) and IB (*Rs* AG-1 IB). The measurement and DNA sequence variation within and between populations of *Ceratobasidium* sp. and the comparison between *Ceratobasidium* and *Thanatephorus* were done using DnaSP v. 4.0 (Rozas *et al.*, 2003).

Table S1 shows the primers used to amplify the three mtDNA loci and Table S2 provides more information on the haplotype polymorphisms along the ITS-rDNA region of *Ceratobasidium* sp from persimmon and tea. Sequences from the *ATP6*, *nad1* and *nad2* haplotypes detected in this study were deposited in GenBank/NCBI under the following accession numbers: EU810057 to EU810092 (*ATP6*), EU810093 to EU810128 (*nad1*) and EU810129 to EU810156 (*nad2*).

Results

All 62 isolates obtained from persimmon and tea and also the isolates from mango and soursop had ITS-5.8S rDNA sequences similar to the *Ceratobasidium* spp. deposited in GenBank/NCBI. Thirty-nine distinct haplotypes were identified among the global database of ITS-5.8S rDNA sequences from *Ceratobasidium* spp., which included those originating from persimmon, tea, mango, soursop, the CBS type strain of *C. noxium* (CBS 154-35) from coffee in India and others derived from several *Ceratobasidium* species and retrieved from GenBank, such as: *C. albasitensis* (H15), *C. raminicola* (H17), *C. cereale* (H04, H06 to H12), *C. cornigerum* (H03, H20 to H25), *C. bicorne* (H13), *C. angustisporum* (H16), *C. anceps* (H05) and *C. papilatum* (H14). The *Ceratobasidium* sp. isolates from persimmon, tea, mango and soursop were grouped into 17 distinct haplotypes: H01 and H02, H18 and H19, and H26 to H38 (Table 1, phylogenetics item). The ITS-5.8S sequence from *C. noxium* (CBS 154-35) was characterized as haplotype H39, distinct from all the other *Ceratobasidium* sequences isolated from persimmon and tea.

A comparison of the ITS-5.8S rDNA sequences from Brazilian *Ceratobasidium* identified 28 haplotypes (Table 1, reticulate phylogeny item). This increase in haplotype numbers from 17 to 28 was attributable to several polymorphic sites present only in the Brazilian sample. Haplotype 14 was excluded from the subsequent phylogenetic analyses because of its incomplete ITS-5.8S rDNA sequence. In a separate analysis of the Brazilian sample of *Ceratobasidium*, 20 haplotypes were found to originate mainly from tea (but also included isolates from mango and soursop) and eight were found in the persimmon sample (Table 1).

Of the 31 *Ceratobasidium* sp. isolates from tea, 29 (93.5%) were heterokaryons, with two distinct alleles detected for the ITS-5.8S in each isolate. The isolate from mango was also a heterokaryon, while that from soursop had only a single allele for this rDNA region. In contrast, 71% of the *Ceratobasidium* isolates from persimmon (a total of 22) were heterokaryons. A single isolate, obtained in an earlier sample, was a homokaryon. No ITS-5.8S rDNA haplotypes were shared by isolates from the persimmon and tea populations.

Phylogenetic analyses

The ML tree indicated that the ITS-5.8S rDNA haplotypes of *Ceratobasidium* sp. from persimmon and tea constitute an independent clade relative to all the other species of *Ceratobasidium* analyzed, including *C. cornigerum*, *C. cereale*, *C. anceps*, *C. bircorne* and *C. noxium*. The species *C. albasitensis* and *C. raminicola* were used as outgroups for rooting the trees (Figure 3). The topologies of the ML, MP and Bayesian trees were congruent.

In addition to assessing the global phylogenetic position of our samples relative to the other species of *Ceratobasidium* described worldwide, we examined whether the population of white-thread-blight *Ceratobasidium* sp. from persimmon was similar to the

Table 1 - World and Brazilian haplotypes of *Ceratobasidium* spp. determined based on polymorphisms detected in sequences of the ITS1-5.8S-ITS2 rDNA region[a].

World haplotypes (phylogeny)[a]	Number	Brazilian haplotypes (coalescent)[a]	Number	Brazilian haplotypes (reticulate phylogeny)[a]	Number	Accession code GenBank® (NCBI)	Species	Isolates	Hosts and places of origin
H01	41	F	40	HDk01	40	EU810046, EU810055	*Ceratobasidium* sp.	DK11b1a, DK11c1a, DK11e1a, DK12a1, DK12b1a, DK12d1a, DK12e1a, DK1a1a, DK1c1a, DK1e1a, DK2a1a, DK2c1a, DK2d1a, DK2e1a, DK3a1a, DK3b1a, DK3c1a, DK3d1a, DK4d, DK4da, DK5b, DK5ba, DK5c, DK5ca, DK5e1a, DK6a, DK6aa, DK6d, DK6da, DK7a, DK7aa, DK7d, DK7da, DK7e, DK7ea, DK8e, DK8ea, DK9e1, DKCAF, DKCAFa	*Diospyros kaki* (Ericales, Ebenaceae), Guararema county, Mogi das Cruzes region, SP, Brazil
H01		G	1	HDk28	1	EU810051	*Ceratobasidium* sp.	DK9e2	*D. kaki*, Guararema, SP, Brazil
H02	1	B	1	HCs02	1	EU810036	*Ceratobasidium* sp.	Cs631	*Camellia sinensis* (Ericales, Theaceae), Registro, SP, Brazil
H03	1	-		-		AJ301903.1	*C. cornigerum*	CBS 132.82	*Festuca* sp. (Poales, Poaceae), Pennsylvania, USA
H04	5	-		-		AX195385.1	*C. cereale*	Sequence 17 from the patent WO0151653	Switzerland
H04						AX195386.1	*C. cereale*	Sequence 18 from the patent WO0151653	Switzerland
H04						AX195388.1	*C. cereale*	Sequence 20 from the patent WO0151653	Switzerland
H04						AX195393.1	*C. cereale*	Sequence 25 from the patent WO0151653	Switzerland
H04						AF063019.1	*Rhizoctonia cerealis* (*C. cereale*)	-	*Poa annua* (Poales, Poaceae), Wisconsin, USA
H05	1	-		-		AJ427402.1	*C. anceps*	CBS 152.32	*Pteridium aquilinum* (Tracheophyta, Filicales, Dennstaedtiaceae), UK
H06	1	-		-		AX195391.1	*C. cereale*	Sequence 23 from the patent WO0151653	Switzerland
H07	1	-		-		AX195392.1	*C. cereale*	Sequence 24 from the patent WO0151653	Switzerland
H08	1	-		-		AF222793.1	*R. cerealis (C. cereale)*	99125	*Agrostis palustris* (Poales; Poaceae), Ontario, Canada
H09	1	-		-		AJ302008.1	*C. cereale*	CBS 558.77	*Secale cereale* (Poales, Poaceae), Germany
H10	1	-		-		AX195390.1	*C. cereale*	Sequence 22 from the patent WO0151653	Switzerland

Table 1 (cont)

World haplotypes (phylogeny)[a]	Number	Brazilian haplotypes (coalescent)[a]	Number	Brazilian haplotypes (reticulate phylogeny)[a]	Number	Accession code GenBank® (NCBI)	Species	Isolates	Hosts and places of origin
H11	1	-		-		AX195387.1	*C. cereale*	Sequence 19 from the patent WO0151653	Switzerland
H12	1	-		-		AJ302009.1	*C. cereale*	CBS 559.77	*Triticum aestivum* (Poales, Poaceae), Germany
H13	1	-		-		AF200514.1	*C. bicorne*	1231	*Polytrichastrum formosum* (Bryophyta, Polytrichales), Finland
H14	1	-		-		AJ427401.1	*C. papillatum*	CBS 570.83	*Sarcochilus dilatatus* (Tracheophyta, Asparagales, Orchidaceae), Queensland, Australia
H15	2	-		-		AJ427399.1	*C. albasitensis*	Eab-T2	*Crocus sativus*, (Embryophyta, Asparagales, Iridaceae), Spain
H15						AJ427398.1		Eab-T2	*Crocus sativus*, Spain
H16	1	-		-		AJ427403.1	*C. angustisporum*	CBS 568.83	*Pterostylis mutica* (Tracheophyta, Asparagales, Orchidaceae), South Australia
H17	1	-		-		AJ427404.1	*C. ramicola*	CBS 758.79	*Pittosporum* sp. (Tracheophyta, Apiales, Pittosporaceae), Florida, USA
H18	1	D	1	HDk18	1	EU810050	*Ceratobasidium* sp.	DK5e2a	*D. kaki*, Guararema, SP, Brazil
H19	1	Not included	-	HCs05	1	EU810054	*Ceratobasidium* sp.	Mango2a	*Mangifera indica* (Sapindales, Anacardiaceae), Manaus, AM, Brazil
H20	1	-		-		AJ301899.1	*C. cornigerum*	CBS 133.82	*Pittosporum* sp., Florida, USA
H21	1	-		-		AJ301902.1	*C. cornigerum*	CBS 137.82	*Erigeron canadensis* (Asterales, Asteraceae), USA
H22	1	-		-		AJ302010.1	*C. cornigerum*	Eab-aB	*Alfala, Medicago sativa* (Fabales, Fabaceae), Spain
H23	1	-		-		AJ301901.1	*C. cornigerum*	CBS 136.82	*Taxus* sp. (Coniferales, Taxaceae), Rhode Island, USA
H24	1	-		-		AJ301900.1	*C. cornigerum*	CBS 135.82	*Juniperus* sp. (Coniferales, Cupressaceae), North Carolina, USA
H25	1	-		-		AJ302006.1	*C. cornigerum*	CBS 139.82	*Pittosporum* sp., Florida, USA
H26	14	K	3	HCs04	3	EU810028	*Ceratobasidium* sp.	Cs1011, Cs1021, Cs331	*C. sinensis*, Registro, SP, Brazil
H26		K	1	HCs15	1	EU810041		Cs911	
H26		K	1	HCs16	1	EU810042		Cs921	
H26		K	5	HCs19	5	EU810026		CHA1, Cs131, Cs221, Cs321, Cs341	
H26		K	3	HCs21	3	EU810035		Cs621, Cs641, Cs651	

Table 1 (cont)

World haplotypes (phylogeny)[a]	Number	Brazilian haplotypes (coalescent)[a]	Number	Brazilian haplotypes (reticulate phylogeny)[a]	Number	Accession code GenBank® (NCBI)	Species	Isolates	Hosts and places of origin
H26		N	1	HCs17	1	EU810030		Cs1031	
H27	3	M	3	HCs06	3	EU810037	*Ceratobasidium* sp.	Cs721, Cs731, Cs741	*C. sinensis*, Registro, SP, Brazil
H28	2	K	2	HCs08	2	EU810031	*Ceratobasidium* sp.	Cs1081, Cs512	*C. sinensis*, Registro, SP, Brazil
H29	1	J	1	HCs07	1	EU810027	*Ceratobasidium* sp.	CHA11Fa	*C. sinensis*, Registro, SP, Brazil
H30	24	A	24	HCs09	24	EU810029, EU810052	*Ceratobasidium* sp.	Cs1012, Cs1022, Cs1032, Cs1082, Cs132, Cs21, Cs21a, Cs222, Cs242 , Cs322, Cs332, Cs342, Cs362, Cs5 11, Cs622, Cs632, Cs642, Cs652, Cs812, Cs831, Cs842, Cs851, Cs86 2, Cs932	*C. sinensis*, Registro, SP, Brazil
H31	17	C	2	HCs03	2	EU810043	*Ceratobasidium* sp.	Cs94, Cs94a	*C. sinensis*, Registro, SP, Brazil
H31		K	3	HCs10	3	EU810038		Cs722, Cs732, Cs742	
H31		K	3	HCs12	3	EU810040		Cs811, Cs852, Cs861	
H31		K	3	HCs13	3	EU810033		Cs162, Cs912, Cs922	
H31		K	6	HCs20	6	EU810034		Cs241, Cs361, Cs762, Cs832, Cs84 1, Cs931	*C. sinensis*, Registro, SP, Brazil
H32	2	Not in-cluded	-	HCs11	2	EU810053	*Ceratobasidium* sp.	Graviola, Mango1a	*Anonna muricata* (Magnoliales, Annonaceae), and *M. indica,* Manaus, AM, Brazil
H33	1	L	1	HCs14	1	EU810032	*Ceratobasidium* sp.	Cs161	*C. sinensis*, Registro, SP, Brazil
H34	1	K	1	HCs22	1	EU810039	*Ceratobasidium* sp.	Cs761	*C. sinensis*, Registro, SP, Brazil
H35	2	H	2	HDk23	2	EU810045	*Ceratobasidium* sp.	DK10a2a, DK10c2a	*D. kaki*, Guararema, SP, Brazil
H36	14	I	1	HDk24	1	EU810049	*Ceratobasidium* sp.	DK2c2a	*D. kaki*, Guararema, SP, Brazil
H36		I	13	HDk26	13	EU810047		DK11b2a, DK11c2a, DK11e2a, D K1a2a, DK1c2a, DK1e2a, DK2a2a, DK2d2a, DK2e2a, DK3a2a, DK3b 2a, DK3c2a, DK3d2a	
H37	4	E	4	HDk25	4	EU810048	*Ceratobasidium* sp.	DK12a2, DK12b2a, DK12d2a, DK 12e2a	*D. kaki*, Guararema, SP, Brazil
H38	2	F	2	HDk27	2	EU810044	*Ceratobasidium* sp.	DK10a1a, DK10c1a	*D. kaki*, Guararema, SP, Brazil
H39	1	-	-	-	-	EU810056	*Ceratobasidium noxium*	CBS 154-35	*Coffea arabica* (Rubiales, Rubiaceae), India

[a]The ITS-5.8S rDNA sequences for the majority of the World haplotypes of *Ceratobasidium* spp. used in the phylogenetic analyses were from GenBank (NCBI). The following example explains the coding used to designate Brazilian isolates of *Ceratobasidium* ssp.: isolate Dk10a, from persimmon, for which two distinct copies of the ITS-5.8S were identified: Dk10a1a [corresponding to the World haplotype H38 in the phylogenetic analysis and the Brazilian haplotypes F (in the coalescent analysis) and HDk27 (in the reticulate phylogeny)] and Dk10a2a [World haplotype H35 in the phylogenetic analysis and Brazilian haplotypes H (in the coalescent analysis) and HDk23 (in the reticulate phylogeny)].

species causing black rot on tea in the Vale do Ribeira. In this context, the ML tree (Figure 3) revealed two distinct main clades that grouped the ITS-5.8S haplotypes of *Ceratobasidium* sp. essentially according to their host of origin. While the persimmon-associated clade consisted exclusively of haplotypes from persimmon isolates collected in São Paulo, the tea-associated clade contained haplotypes identified in isolates of *Ceratobasidium* from tea, mango and soursop, indicating that this last clade may be more widespread in Brazil, particularly in native plant populations.

Intraspecific evolution of *Ceratobasidium* sp. from persimmon and tea inferred by analysis of reticulate haplotype networks

The statistical parsimony analyses of ITS-5.8S rDNA haplotypes from *Ceratobasidium* sp. from persimmon and from tea, mango and soursop isolates indicated two distinct, unconnected networks (Figure 4). The *Ceratobasidium* sp. population from the *Camellia*-associated clade was more diverse as judged by the number of ITS-5.8S haplotypes that formed the statistical parsimony network (20 haplotypes). In contrast, the *Diospyros*-associated clade consisted of eight haplotypes. The most frequent haplotype in the network from the *Camellia*-associated clade was HCs9 (n = 24), while for the *Diospyros* clade the most frequent haplotype (from which all the ramifications of the network were derived) was HDk1 (n = 40).

Test for population subdivision and coalescent analysis

Table 2 provides evidence for the non-neutral evolution of ITS-rDNA in the population of *Ceratobasidium* from tea. The significant values of Fu and Li's F* and D* were negative. A significant negative test result is consistent with either population growth or shrinkage or background selection (Fu, 1997). Since the ITS and IGS are the regions of rDNA with the lowest selective constraint [fully

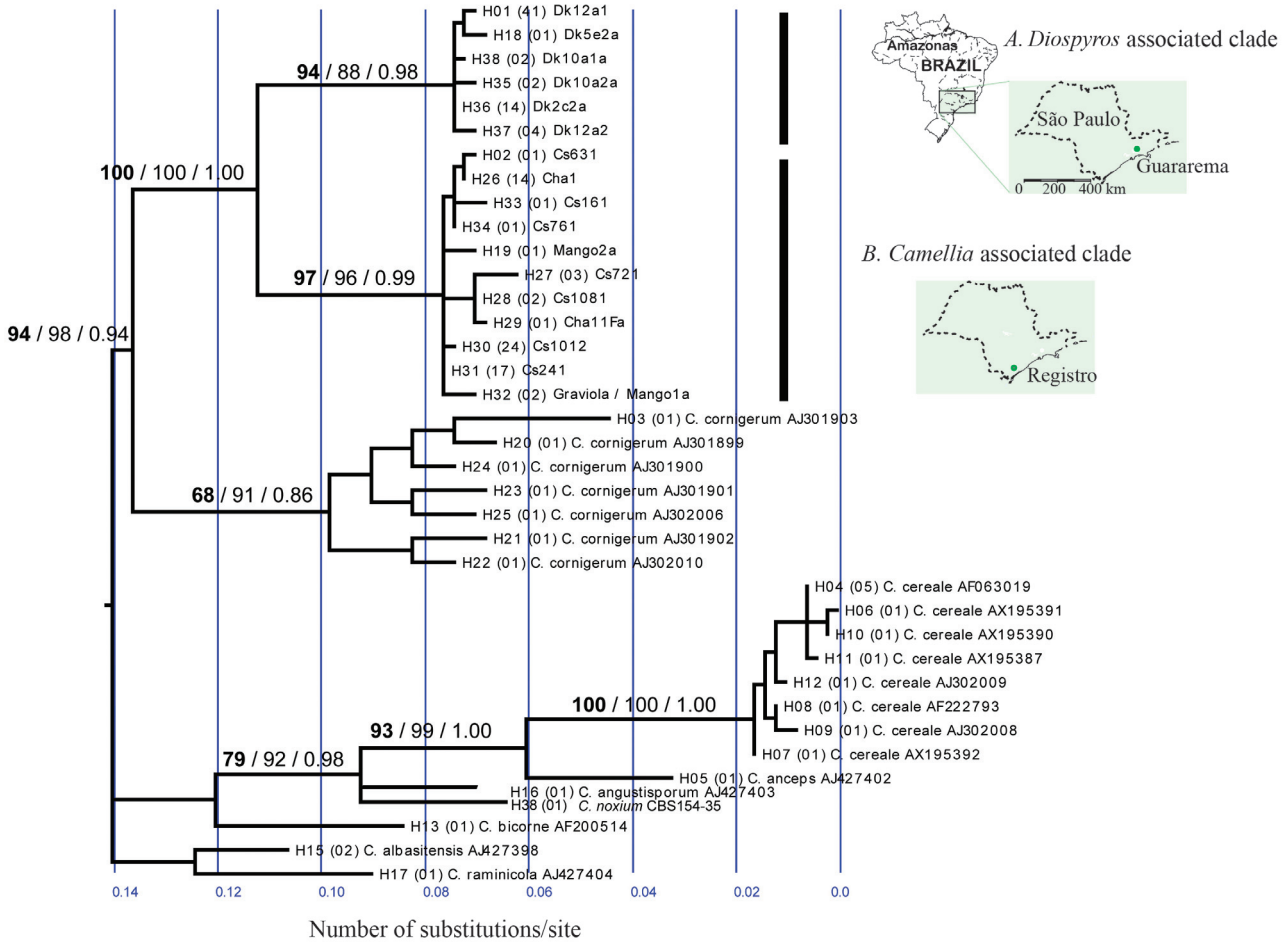

Figure 3 - Maximum likelihood (ML) tree showing the phylogenetic relationship among *Ceratobasidium* spp. based on the GTR + G evolutionary model. ML (bold), maximum parsimony (regular type) 1,000 replicate bootstrap values and Bayesian MCMCMC posterior probabilities (closer to 1.0) are given for the major clades. The taxon unit identifications consist of the ITS-5.8S rDNA haplotype designation followed by the number of haplotypes sampled, the species of *Ceratobasidium*, the code of representative isolates belonging to a particular haplotype and/or the code number for sequences obtained from GenBank/NCBI. The haplotype designations for *Ceratobasidium* spp. are given in Table 1. The tree was rooted using the ITS-5.8S-rDNA sequences from C. *albasitensis* and *C. raminicola*.

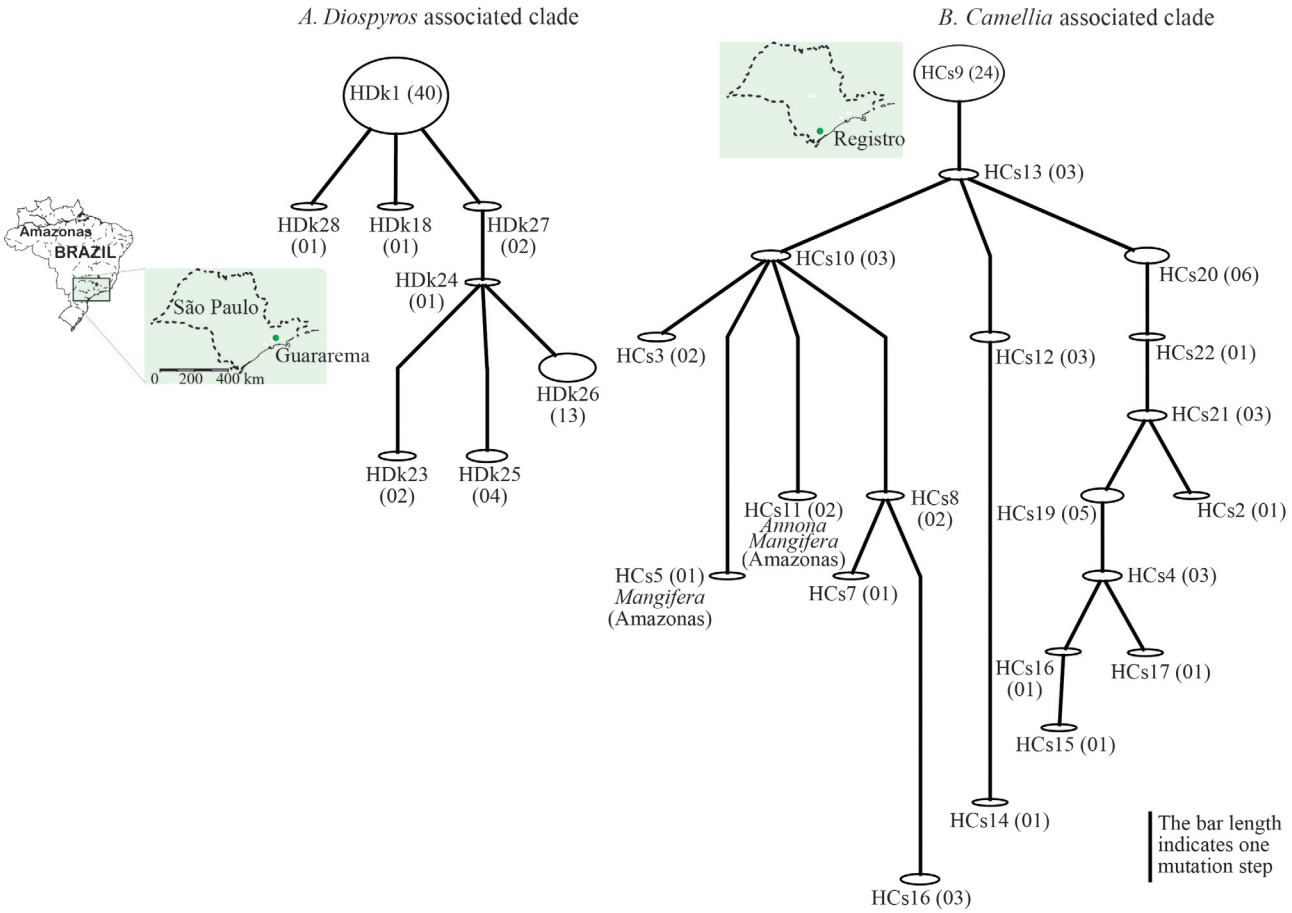

Figure 4 - The ITS-5.8S haplotype network of *Ceratobasidium* sp. from persimmon (*Diospyros kaki*) and tea (*Camellia sinensis*) from São Paulo state, Brazil, showing the most parsimonious plausible (~95%) set of connections. The haplotype network was constructed using the statistical parsimony algorithm implemented by TCA (Templeton *et al.*, 1992). The haplotype designations are shown in Table 1. Circle size is proportional to sample size (numbers in parentheses). Two unconnected major clades (the *Camellia* and *Diospyros* associated clades) were identified.

Table 2 - Estimates of gene diversity and tests of neutrality for the ITS-rDNA region.

Populations of *Ceratobasidium* sp.	Sequence length	Total no. of polymorphic sites	Polymorphic sites per population	Haplotypes[a]	Haplotype diversity (± SD)	Fu and Li's *D**	Fu and Li's *F**	Tajima's *D*
Locus: ITS-5.8S rDNA	645 (628)[b]	49 (32)[b]						
Tea (*Camellia sinensis*), Registro, SP			10	8 (64)	0.630 ± 0.039	-2.4941*	-2.5778*	-1.5649ns
Persimmon (*Diospyros kaki*), Guararema, SP			7	6 (64)	0.524 ± 0.061	-0.4041ns	-0.7275ns	-1.0584ns

[a]The numbers in parentheses indicate the total number of sequences sampled from each population.
[b]The numbers in parentheses indicate the length of the compatible sequence alignments and the number of polymorphic sites in the compatible alignments (see Table S2 for further details).
*$p < 0.05$; ns = not significant.

consistent with them being neutral variants that are being spread by homogenization (Ganley and Kobayashi, 2007)], we hypothesized that the deviation from neutrality was due to a change in effective population size. The *g* values were positive for the *Camellia*- and *Diospyrus*-associated clades, indicating population growth (Table 3). However, the growth rate for the *Camellia*-associated clade was 4.1 times higher than *g* for the *Diospyrus*-associated clade. The *g* values were not symmetrical in magnitude because of an exponential effect on population growth. Thus, a *g* value that is four times higher would indicate faster growth. This faster population growth was probably the cause of the non-neutral evolution of the ITS rDNA region detected in the *Camellia*-associated clade.

Table 3 - Estimates of coalescent parameters from the divergence between tea and persimmon populations of the white-thread-blight and black rot fungus *Ceratobasidium* sp. based on the ITS-5.8S rDNA region.

Populations	Haplotypes sampled and effective sample size	Ancestral theta (4Neμ)[a]	Population growth rate (g)[b]	Migration rate[c] 4Nm (+ = receiving population)	
				1, +	2, +
1. Tea (*Camellia*)	8 (64)	0.0111	248.17	-	1.43e-07
2. Persimmon (*Diospyrus*)	6 (64)	0.0074	60.16	0.3794	-

[a]Theta values represent a measure of effective population size (for diploids, theta = 4*N*eμ where *N*e = effective population size and μ = mutation rate inferred for the locus).
[b]Most probable estimate of population growth rate calculated by Bayesian analyses using the program Lamarc 2.0 (Kuhner, 2006; Kuhner and Smith, 2007).
[c]Migration between geographical or host populations was estimated using an isolation with migration model. The theta values and migration rates were estimated using the program MIGRATE 2.1.3 (Beerli and Felsenstein, 2001). Sink populations are shown on the left side and source populations are indicated along the top. Effective sample size for each population is given in parentheses. The estimates of population growth, theta and migration rates are at the 95% confidence interval.

A value of $p < 0.0001$ for the Hudson test estimates ($K_{ST} = 0.89$, $K_S = 0.93$, $K_T = 8.30$) indicated that the *Ceratobasidium* populations in tea and persimmon were genetically differentiated (Hudson *et al.*, 1992). However, there was evidence for asymmetric historical migration of *Ceratobasidium* sp. from the tea population into the persimmon population ($4N_{m1,+2} = 0.38$) (Table 3).

The coalescent gene genealogy (Figure 5) was used to infer the mutational history of the ITS-5.8S rDNA and to describe the history of the variation observed between the *Diospyros*- and *Camellia*-associated clades of *Ceratobasidium* sp. All of the mutations along the branches were population-specific. Based on coalescent simulations of the estimated mutation age (measured as coalescent units of effective population size 4Neμ and expressed as the time to the most recent common ancestor or T_{MRCA}), the oldest mutations were in the *Diospyros* clade of *Ceratobasidium* sp. Mutation number 11 was the oldest in the genealogy, with a mean age of 0.94 ± 0.12 (SD) coalescent units. This clade also had six of the oldest mutations observed in the genealogy. The haplotypes from the *Diospyros* clade accumulated an average of 11-13 mutations. In addition, the persimmon-derived population of *Ceratobasidium* sp. showed a more recent expansion that occurred at ~0.23 units of coalescent time. The oldest mutation in the *Camellia*-associated clade was number 9 (Figure 5; mean age: 0.54 ± 0.05 coalescent units), which was more recent than the first six mutations in the *Diospyros*-associated clade. The ITS-5.8S haplotypes of the *Camellia*-associated clade accumulated a lower number of mutations (4-6) along the genealogy. The time for population expansion (around 0.3 units) was similar to that for the *Diospyros* clade.

The initial diversification of the white-thread blight-associated *Ceratobasidium* sp. into two major clades dates to approximately 7.6-14.5 thousand years ago, according to the coalescent approach implemented in the program BEAST 1.2 (Figure 6). The diversification within clades began more recently and, interestingly, the two major clades *Camellia* and *Diospyrus* appear to have diversified almost simultaneously between 1.7 and 3.2 thousand years ago (Figure 6). The scenario for the species complex presented by the Bayesian skyline plot showed a dual pattern: a long history of constant population size followed by a very recent expansion (Figure 6) estimated to have occurred about 1-2 thousand years ago. Since then, the populations have expanded in size by approximately four-fold.

Shared mitochondrial haplotypes

The estimates of mtDNA polymorphism at three distinct loci (*ATP6, nad1* and *nad2*) indicated very little or no variation within and between the two populations of *Ceratobasidium* sp. (Cs and Dk). A single, shared haplotype was detected for *nad1* and *nad2* genes in both populations, while for *ATP6* two out of three haplotypes were shared and only three polymorphic sites [average number of nucleotide differences (k) = 1.06] were detected among 660 bp (Table 4). We contrasted the divergence observed between the Cs and Dk populations with the divergence between two sister species within the *Thanatephorus* complex (which has *R. solani* anamorphs): *R. solani* anatomosis group AG-1 IA (*Rs* AG-1 IA) and IB (*Rs* AG-1 IB). No shared haplotypes were observed between *Rs* AG-1 IA and IB for any of the three mtDNA markers, and k varied from 0.57 (for *nad2*) to 6.4 (for *ATP6*). Much higher nucleotide diversity was observed when comparing *Ceratobasidium* with *Thanatephorus*.

Discussion

Persimmon and tea crops from the Brazilian Atlantic Forest agroecosystem harbor populations of the *Ceratobasidium* spp. complex that are genetically distinct from the originally described white-thread blight coffee pathogen *C. noxium*. The phylogenetic analysis described here indicates that the *Ceratobasidium* species infecting persimmon and tea in Brazil are distinct from each other: they most likely represent two distinct species within *Ceratobasidium* and are distinguishable from all the other

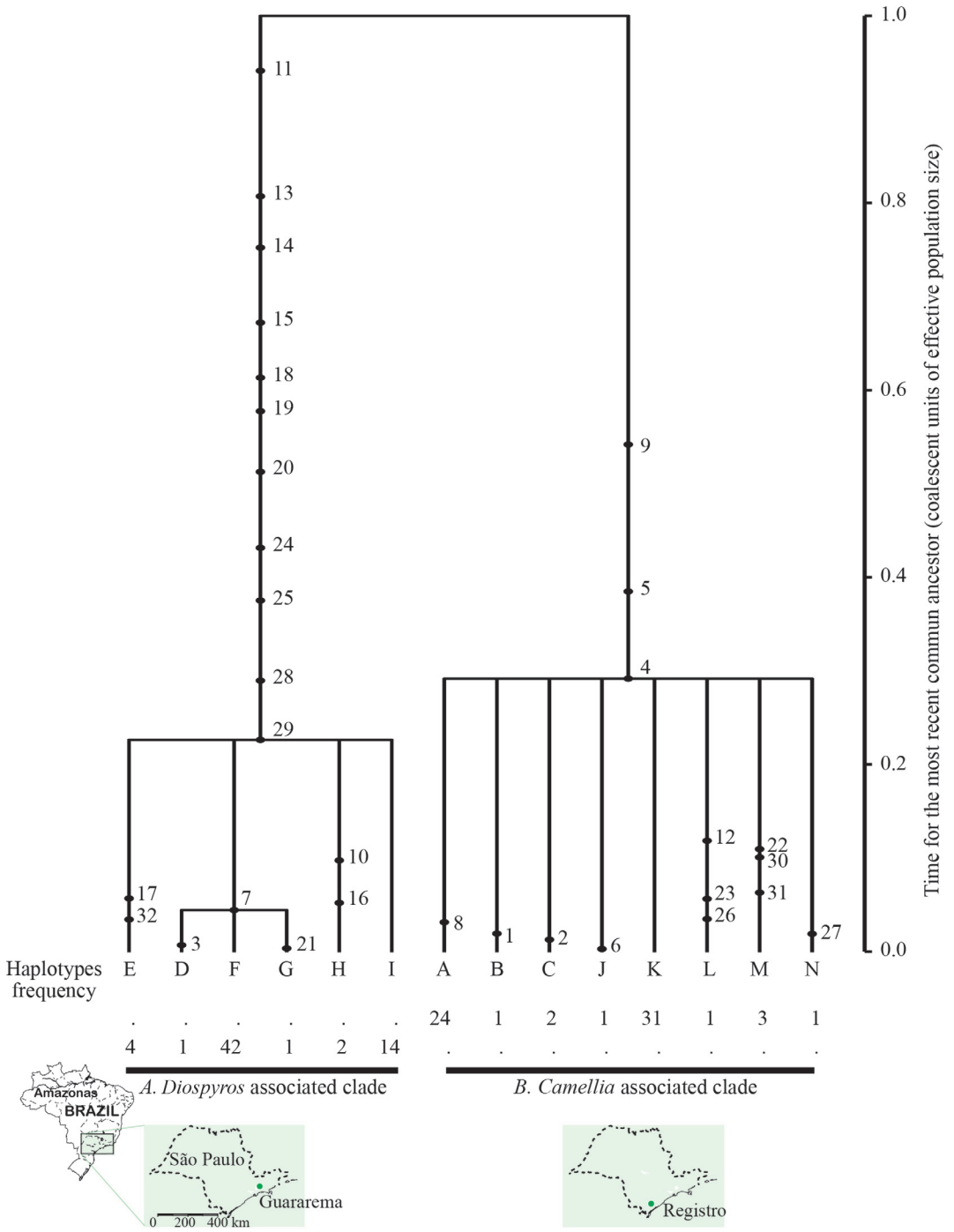

Figure 5 - Coalescent-based gene genealogy with the highest root probability (likelihood = 2.4362 e-49, SD = 1.0704 e-49) showing the distribution of ITS-5.8S rDNA mutations in populations of *Ceratobasidium* sp. from persimmon (*Diospyros*-associated clade) and tea (*Camellia*-associated clade). The inferred genealogy was based on Watersons estimate of Θ = 1.905 and constant population size and growth rates. The time scale is represented in coalescent units of effective population size. All of the mutations and bifurcations in this rooted genealogy are time ordered from top (past) to bottom (present) and follow the direction of divergence from the oldest to the youngest. The coalescent process is from the bottom to the top of the Figure. The numbers below the tree indicate each distinct haplotype and its frequency, *i.e.*, the number of occurrences of the haplotype in the samples from *Diospyros* and *Camellia*. The haplotype designations are given in Table 1.

Ceratobasidium species that cause thread blight disease worldwide.

In addition to the phylogenetic evidence (based on ML, MP and Bayesian MCMCMC analyses) for rejecting the null hypothesis that the two host-specific populations of *Ceratobasidium* sp. belong to a single species, there were other consistent indications that supported the subdivision between populations of *Ceratobasidium* from persimmon

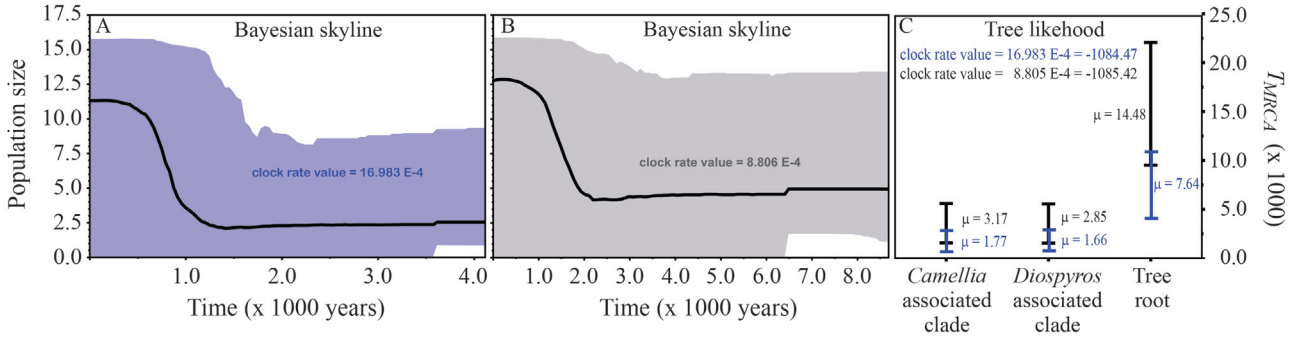

Figure 6 - Bayesian skyline plot (Drummond *et al.*, 2005; Drummond and Rambaut, 2006) showing the effective population size fluctuation over time for the combined *Ceratobasidium* sp. populations from persimmon (*Diospyros*-associated clade) and tea (*Camellia*-associated clade). (a) clock rate = $16.983e^{-4}$ substitutions/gene*10^3 years, (b) clock rate = $8.806e^{-4}$ substitutions/gene*10^3 years, (c) tree likelihood values and the T_{MRCA} under the two distinct clock rates suggested by Kasuga *et al.* (2002) for the ITS rDNA region of fungi; μ is the mean T_{MRCA}.

and tea, including the lack of shared ITS-5.8S haplotypes (Figure 3 and 4), mutations restricted to one or the other population and no recent migration between *Ceratobasidium* sp. haplotypes from the *Diospyros*- and *Camellia*-associated clades. We detected only historical asymmetric migration of *Ceratobasidium* from the tea to the persimmon population. This data on asymmetric historical migration was interpreted as evidence for ancestral polymorphisms shared by the *Ceratobasidium* populations from persimmon and tea, but with little or no recent genetic exchange.

Divergent lineages of parasites can arise through ecological adaptations (Couch *et al.*, 2005; Huyse *et al.*, 2005). For fungal plant parasites, these ecological adaptations include host shifts or changes in pathogenicity (Couch *et al.*, 2005). Host specialization is common in plant pathogenic fungi, such as *Magnaporthe grisea* (Couch and Kohn, 2002; Couch *et al.*, 2005), *Mycosphaerella graminicola* (Stukenbrock *et al.*, 2007), *Rhynchosporium secalis* (Zaffarano *et al.*, 2008), *Ceratocystis fimbriata* (Baker *et al.*, 2003), *Microbotryum violaceum* (Bucheli *et al.*, 2000) and *Rhizoctonia solani* (Ceresini *et al.*, 2007; Bernardes-de-Assis *et al.*, 2008). There was no cross pathogenicity between *Ceratobasidium* isolates from the persimmon and tea groups or from citrus and coffee (two other host plants) (Costa-Souza *et al.*, 2009). However, although in the latter two host plants the fungus did not cause the extensive foliar lesions typically observed in the original host plants, about 30 days after inoculation the fungus showed mycelial growth on the branches of all the other host plants tested. This may indicate that ecological speciation via host specialization has occurred, but with the fungus retaining a broader host range that likely includes reservoir hosts.

There was a high level of genetic diversity in the two local Brazilian populations of *Ceratobasidium* sp., which in theory would contradict the level of genetic diversity expected for an introduced pathogen. Introduced plant pathogens usually experience a decrease in the genetic diversity of their populations when compared with the variation observed at the centre of origin (McDonald and Linde, 2002a,b). There are three plausible explanations for the high level of genetic diversity observed in the persimmon and tea populations of *Ceratobasidium* sp.: (a) the two populations of *Ceratobasidium* sp. may have experienced recent diversification following the normal phenomenon of a reduction in genetic diversity associated with introduced pathogens, (b) the establishment of these two crops in Brazil resulted in the introduction of multiple pathogen genotypes from their original centre of diversity in Asia, and (c) the WTBR pathogen originated locally from native hosts, in which a high degree of genetic diversity already existed.

There is little information concerning the pattern of genetic variation in the mtDNA of *Ceratobasidium* and *Thanatephorus* species (with *Rhizoctonia*-like anamorphs). The findings described here are the first for these Basidiomycota and show variation in three mtDNA regions, two of which were novel loci (*nad1* and *nad2*). There was little intraspecific variation in mtDNA within and between the two populations of white-thread-blight-fungus *Ceratobasidium* sp. when compared with nuclear region ITS-rDNA. Lower genetic variation in mtDNA has been reported for several other fungal species, including a few Basidiomycota (Xu *et al.*, 1998; Moncalvo *et al.*, 2000; Carlisle *et al.*, 2001; Ghimire *et al.*, 2003; Zhan *et al.*, 2003; Láday *et al.*, 2004), but there are few exceptions (Sommerhalder *et al.*, 2007).

The low levels of intraspecific variation in mtDNA observed in *Ceratobasidium* sp. from persimmon and tea may reflect the slow rates of nucleotide substitution in these regions, as reported for fungi in general (Clark-Walker, 1991; Moncalvo *et al.*, 2000). This low variation means that the mtDNA sequences used here cannot be relied on to reveal differences between closely related species, although their potential use in phylogenetic analyses of more divergent species cannot be excluded. Much higher variation was observed by contrasting two divergent phylospecies within *Thanatephorus* and between *Thanatephorus* and *Ceratobasidium* (Table 4). Other mitochondrial genes

Table 4 - Estimates of within population mtDNA polymorphism and divergence between the two populations of *Ceratobasidium* sp. from tea [*Camellia sinensis* (Cs)] and persimmon [*Diospyrus kaki* (Dk)] compared with three groups within the sister species complex *Thanatephorus cucumeris* [anamorphs *R. solani* AG-1 IA (*Rs* AG-1 IA) and IB (*Rs* AG-1 IB).

Locus	Populations of *Ceratobasidium* sp. and *T. cucumeris*				
	Cs	Dk	Cs *vs.* Dk	*Rs* AG-1 IA *vs.* *Rs* AG-1 IB	*Ceratobasidium* (Cs + Dk) *vs. Thanatephorus* (*Rs* AG-1 IA + *Rs* AG-1 IB)
ATP6					
Number of sequences	17	7	24	6	30
Sequence length analyzed (bp)	660	660	660	654	654
Polymorphic sites	3	2	3	12	72
Average number of nucleotide differences (k)	0	0	1.06	6.4	22.4
Haplotypes	3	2	3	2	5
Shared haplotypes between groups contrasted	-	-	2	0	0
nad1					
Number of sequences	16	10	26	8	34
Sequence length analyzed (bp)	333	333	333	333	333
Polymorphic sites	0	0	0	1	4
Average number of nucleotide differences (k)	0	0	0	0.57	1.33
Haplotypes	1	1	1	2	3
Shared haplotypes between groups contrasted	-	-	1	0	0
nad2					
Number of sequences	11	9	20	6	26
Sequence length analyzed (bp)	327	327	327	309	309
Polymorphic sites	0	0	0	11	31
Average number of nucleotide differences (k)	0	0	0	5.27	9.72
Haplotypes	1	1	1	2	3
Shared haplotypes between groups contrasted	-	-	1	0	0

[such as mitochondrial small subunit (mitSSU) rDNA and genes coding for subunits 1 and 3 of the cytochrome oxidase (cox1 and cox3, respectively)] have been used to resolve deeply branching metazoan/fungal relationships (Paquin *et al.*, 1995, 1997; Forget *et al.*, 2002) and could be exploited as markers for resolving phylogenetic relationships among *Ceratobasidium* species and/or among sister species of the genus *Thanatephorus.*

Another important observation from this study was the pattern of growth associated with these populations of *Ceratobasidium* sp., *i.e.*, a long history of constant population size followed by very recent expansion estimated to have occurred about 1-2 thousand years ago (Figure 6). This recent population expansion may be explained by one of two alternative scenarios: (1) historically, the pathogen may have co-evolved with its hosts in their centre of origin in China (Wight, 1959, 1962; Barua, 1965) and (2) the pathogen may have originated locally on native hosts in South America and only became a persimmon or tea pathogen after the two crop species were introduced.

The first scenario has to be considered cautiously because we have not characterized Asian populations of the pathogen and there is currently no information to support the occurrence of the WTBR pathogen associated with persimmon and tea in China or other Asian countries. The only report of a similar disease in Asia was on coffee in India (Venkatarayan, 1949). Based on our study, however, the coffee-infecting pathogen is a distinct species from those associated with WTBR on persimmon and tea.

The second scenario predicts that the establishment and development of persimmon and tea crops in Brazil led to the emergence of a new pathogen through local adaptation in already existing highly variable *Ceratobasidium* sp. populations. The changes in population size would have occurred in local pathogen populations infecting native plant species. The incidence of WTBR in several native plant species from the Amazon (Lourd and Alves, 1987; Gasparotto and Silva, 1999; Benchimol *et al.*, 2001) supports the hypothesis of a local origin for the populations of *Ceratobasidium* from persimmon and/or tea. Indeed, we have detected two ITS-5.8S rDNA haplotypes in the *Camellia*-associated clade of *Ceratobasidium* that were similar to haplotypes detected in soursop and mango isolates from the Amazon. However, based on the data described here, we cannot confidently postulate that the changes de-

tected in demographic parameters were necessarily determined by the local origin of the pathogen.

To examine the influence of the local origin for this pathogen and to assess the changes in demographic parameters it will be necessary to sample other Brazilian populations of the pathogen. These samples should include populations of *Ceratobasidium* sp. from other crops (such as coffee, citrus and rubber trees), from crops adjacent to persimmon and tea plantations and, most importantly, from native plants from other locations such as the Atlantic and the Amazonian forests adjacent to agroecosystems. Such a framework is critical to understanding the origins and evolution of WTBR-associated *Ceratobasidium* spp. as pathogens of many important tropical tree species.

Based on the results of this work, we conclude that the two sister populations of the basidiomycetous fungus *Ceratobasidium* sp. from persimmon and tea in the Brazilian Atlantic Forest agroecosystem represent two distinct, historically divergent lineages that have experienced historical diversification. These two Brazilian populations of the WTBR fungus most likely represent distinct species within *Ceratobasidium* and they are also distinct from *C. noxium* (the etiological agent of the white-thread blight in coffee firstly described in India), and *C. cornigerum* (the most closely related species).

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Supplementary Material

The following online material is available for this article:

Figure S1 – Symptoms of white-thread blight and black rot and signs of *Ceratobasidium* sp. on persimmon and tea plants.

Table S1– Degenerate and specific primers designed for PCR amplification and sequencing reactions.

Table S2 – Characterization of haplotypes of *Ceratobasidium* sp. from persimmon and tea.

This material is available as part of the online article at: http://www.scielo.br/gmb.

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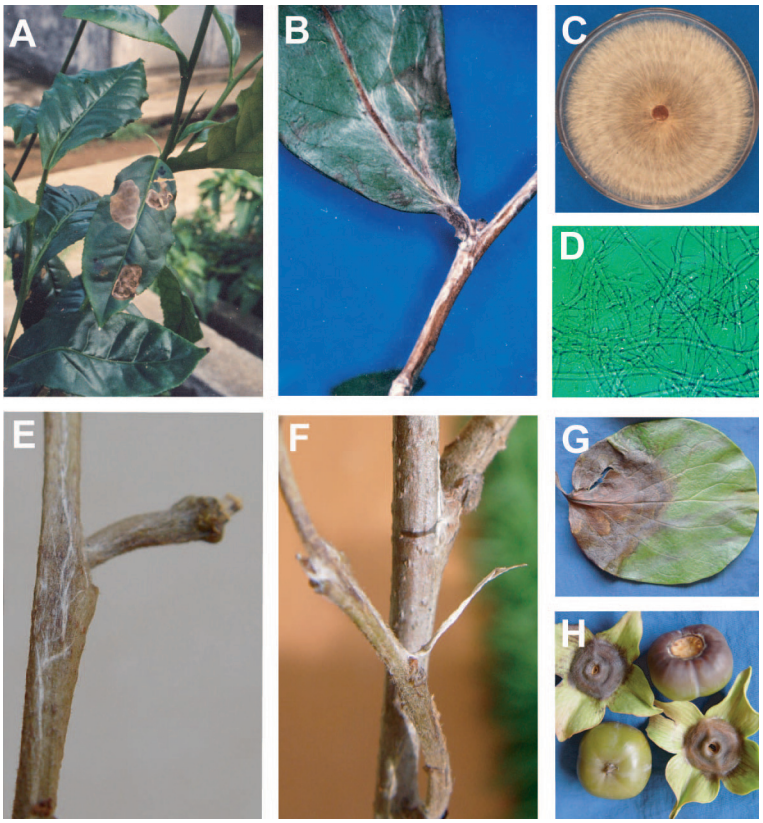

Figure S1 - Symptoms of white-thread blight and black rot and signs of *Ceratobasidium* sp. on persimmon and tea plants, respectively.

- Symptoms of black rot on tea (A), white-thread blight on persimmon leaves (G) and fruits (H).
- Mycelial growth (B, E) and mycelial cord (B) on tea and persimmon (F) branches.
- Hyphae and mycelial growth of *Ceratobasidium* sp. on potato-dextrose-agar medium (C, D).

Table S1 – Degenerate and specific primers designed for PCR amplification and sequencing reactions of three mtDNA fragments from the Basidiomycota fungus *Ceratobasidium*.

NCBI accession number	Organism	Mitochondrial gene and genomic region	Degenerate primers for Basidiomycetes		Gene-specific primers for *Ceratobasidium* and *Thanatephorus*			
					Code	Length (bp)	Oligo	Length of PCR amplicon (bp)
AY376688	*Crinipellis perniciosa* mitochondrion, complete genome	NADH dehydrogenase subunit 1 (*nad1*) 70615 - 72832	nad1_F	5'-TAGTATTTAGT**Y**TA**Y**TAGG-3'	nad1N_F	20	5'-AGGGTGGGCTGTAATTCCTT-3'	360
NC_004336	*Cryptococcus neoformans* var. *grubii* mitochondrion, complete genome	18444 - 18609, 19731 - 20575	nad1_R	5'-C**H**TTTGA**Y**CTAC**M**AGA**R**GC-3'	nad1N_R	22	5'-TCTGGTAGGTCAAATGGTGTTC-3'	
AY560608	*C. neoformans* var. *grubii* strain IFO 410	221 - 1066						
NC_003049	*Schizophyllum commune* mitochondrion, complete genome	28257 - 29270						
		NADH dehydrogenase subunit 2 (*nad2*)						
AY376688	*C. perniciosa* mitochondrion, complete genome	65399 - 67192	nad2A_F	5'-TAAGTATTGAA**Y**TACAATC-3'	nad2N_F	22	5'-AGCCCAACAACTGTACCTATGA-3'	488
NC_004336	*C. neoformans* var. *grubii* mitochondrion, complete genome	37790 - 39589	nad2A_R	5'-TAGC**Y**TATAGTAC**M**AT**W**TC-3'	nad2N_R	20	5'-TCAGCTACTTCAGCCGGATT-3'	
NC_003049	*S. commune* mitochondrion, complete genome	15468 - 16967						
		ATP synthase subunit 6 (*ATP6*)						
			atp6_2	5'-TAATTCTANWGCATCTTTAATRTA-3' *				727
			atp6_3	5'-TCTCCTTTAGAACAATTTGA-3' *				

*The *ATP6* primers used in this study were described by Kretzer and Bruns (1999) for phylogenetic analyses of Basidiomycota fungi. Primer bases presented in blue indicate degenerate bases.

Table S2 – Characterization of haplotypes of *Ceratobasidium* sp. from persimmon and tea based on polymorphic sites detected in ITS-rDNA sequences.

Sequence code at GenBank - NCBI	Haplotypes designation for reticulate phylogeny	Number	Haplotype designation for coalescent analysis	Number	Polymorphic sites detected in ITS-rDNA sequences [a]																									
					Position	21	25	29	40	41	50	59	76	83	89	97	99	100	120	125	155	159	162	180	197	218	221	222	223	226	227
					Site number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					Site type	A	T	T	A	T	G	T	T	C	C	T	A	A	C	T	A	C	A	A	G	A	A	A	A	A	A
					Character type	t	v	t	t	v	t	t	t	t	v	t	t	t	t	t	t	t	t	t	t	t	t	t	v	t	t
						-	-	i	-	-	i	i	i	i	-	i	-	i	i	-	i	i	-	i	i	i	i	-	i	-	i
EU810054	HCs05	1	-			.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
EU810053	HCs11	2	-			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	G	.
EU810029	HCs09	24	A	24		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810036	HCs02	1	B	1		G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810043	HCs03	2	C	2		.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810050	**HDk18**	**1**	**D**	**1**		.	.	C	G	.	A	C	.	.	.	C	.	G	.	.	.	T	.	.	A	G	.	.	T	.	.
EU810048	**HDk25**	**4**	**E**	**4**		.	.	C	.	.	A	C	.	.	.	.	.	G	.	.	.	T	.	.	A	G	G	.	T	.	.
EU810046	**HDk01**	**40**	**F**	**42**		.	.	C	.	.	A	C	.	.	.	C	.	G	.	.	.	T	.	.	A	G	.	.	T	.	.
EU810044	**HDk27**	**2**	**F**			.	.	C	.	.	A	C	.	.	.	C	.	G	.	.	.	T	.	.	A	G	G	.	T	.	.
EU810051	**HDk28**	**1**	**G**	**1**		.	.	C	.	.	A	C	.	.	.	C	.	G	.	.	.	T	.	.	A	G	.	.	T	.	.
EU810045	**HDk23**	**2**	**H**	**2**		.	.	C	.	.	A	C	.	.	.	.	.	G	.	C	.	T	.	.	A	G	G	.	T	.	.
EU810049	**HDk24**	**1**	**I**	**14**		.	.	C	.	.	A	C	.	.	.	.	.	G	.	.	.	T	.	.	A	G	G	.	T	.	.
EU810047	**HDk26**	**13**	**I**			.	.	C	.	.	A	C	.	.	.	.	.	G	.	.	.	T	.	.	A	G	G	.	T	.	.
EU810027	HCs07	1	J	1		.	.	.	.	.	.	.	.	T	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
EU810028	HCs04	3	K	31		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810031	HCs08	2	K			.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
EU810038	HCs10	3	K			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810040	HCs12	3	K			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810033	HCs13	3	K			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810041	HCs15	1	K			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810042	HCs16	1	K			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810026	HCs19	5	K			.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810034	HCs20	6	K			.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810035	HCs21	3	K			.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810039	HCs22	1	K			.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
EU810032	HCs14	1	L	1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	G	.	.	.	.	.	.	G
EU810037	HCs06	3	M	3		.	.	.	.	.	.	.	C	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G
EU810030	HCs17	1	N	1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.

Sequence code at GenBank - NCBI	Polymorphic sites detected in ITS-rDNA sequences [a] (continued)																							
	Position	239	303	422	442	449	451	455	490	492	495	496	505	511	513	517	536	588	592	593	594	602	610	614
	Site number	27	28	29	30	31	32	33		34	35	36	37	38	39		40	41	42	43	44	45	46	47
	Site type	T	T	C	A	G	C	T	C	A	C	C	C	G	G	A	A	A	A	G	A	T	A	T
	Character type	t	v	t	t	t	t	t	-	t	v	v	v	v	t	-	t	t	t	t	t	t	t	t
		-	-	i	i	i	i	-	-	-	i	i	i	i	-	-	i	i	-	i	i	-	-	-
EU810054		.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
EU810053		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
EU810029		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810036		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
EU810043		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
EU810050		.	.	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810048		.	G	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	C
EU810046		.	.	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810044		.	.	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810051		.	.	T	G	A	T	C	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810045		C	.	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810049		.	.	T	G	A	T	.	.	.	.	A	.	C	.	.	.	.	.	A	G	.	.	.
EU810047		.	.	T	G	A	T	.	.	.	.	A	.	C	.	G	.	.	.	A	G	.	.	.
EU810027		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.
EU810028		.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.
EU810031		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.
EU810038		.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
EU810040		.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
EU810033		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810041		.	.	.	G	.	.	.	.	.	G	.	.	.	.	T	G	.	.	.	.	.	.	.
EU810042		.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	G	.	.	.	.	.	.	.
EU810026		.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.
EU810034		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810035		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
EU810039		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU810032		.	.	.	G	.	.	.	.	G	.	.	.	.	A	T	.	.	.	.	.	.	.	.
EU810037		.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	G	.	.	.	C	G	.
EU810030		.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	G	.	.	.	.	.

Table S2 (cont)

Haplotype designation for coalescent analysis	Number	Position	21 25	40	50 59	89 97 98 99 100	125	159 162	197 218	223	239 303 422	449 451 455 490 492	496	511 513	592 593 594 602 610 614
		Site number	1 2	3	4 5	6 7 8 9 10	10	11 12	13 14	15	16 17 18	19 20 21 22 23	24	25 26	27 28 29 30 31 32
		Site type	A T	A	G T	C T A A	T	C A	G A	A	T T C	G C T C A	C	G G	A G A T A T
		Character type	t v	t	t t	v t t t t	t	t t	t t	v	t v t	t t t v t	v	v t	t t t t t t
			- -	-	i i	- i - - i	-	i -	i i	i	- - i	i i - - -	i	i -	- i i - - -
A	24		. .	.	. .	. . G .	.	. .	. .	.	. . .	. . . . .	.	. .	. . . . . .
B	1		G .	.	. .	. . . .	.	. .	. .	.	. . .	. . . . .	.	. .	. . . . . .
C	2		. G	.	. .	. . . .	.	. .	. .	.	. . .	. . . . .	.	. .	. . . . . .
D	1		. .	G	A C	. C . G	.	T .	A G	T	. . T	A T . . .	A	C .	. A G . . .
E	4		. .	.	A C	. . . G	.	T .	A G	T	. G T	A T . . .	A	C .	. A G . . C
F	42		. .	.	A C	. C . G	.	T .	A G	T	. . T	A T . . .	A	C .	. A G . . .
G	1		. .	.	A C	. C . G	.	T .	A G	T	. . T	A T C . .	A	C .	. A G . . .
H	2		. .	.	A C	. . . G	C	T .	A G	T	C . T	A T . . .	A	C .	. A G . . .
I	14		. .	.	A C	. . . G	.	T .	A G	T	. . T	A T . . .	A	C .	. A G . . .
J	1		. .	.	. .	G . . .	.	. .	. .	.	. . .	. . . . .	.	. .	. . . . . .
K	31		. .	.	. .	. . . .	.	. .	. .	.	. . .	. . . . .	.	. .	. . . . . .
L	1		. .	.	. .	. . . .	.	. G	. .	.	. . .	. . . . G	.	. A	. . . . . .
M	3		. .	.	. .	. . . .	.	. .	. .	.	. . .	. . . G .	.	. .	. . . C G .
N	1		. .	.	. .	. . . .	.	. .	. .	.	. . .	. . . . .	.	. .	G . . . . .

[a] Conflicting sites showing homoplasy are presented in red; these incompatible sites (present among the polymorphic sites) were identified using the compatibility methods SNAP Clade and SNAP Matrix (Figure 2).

Light shading columns indicate conflicting sites removed for the coalescent analyses.

Site type: t = transition; v = transversion. Character type: i = phylogenetically informative; - = uninformative.

Sequences from the ITS-rDNA haplotypes detected in this study were deposited in GenBank/NCBI under accession numbers EU810026 to EU810056. Haplotypes in bold are associated with *Diospyrus*.

Bases in blue indicate infinite sites violations that were removed from all the analyses and the ones in blue green indicate polymorphisms detected in the haplotypes HCs5 and HCs11 (from *Anona muricata* and *Mangifera indica*), which were not included in the coalescent analyses.