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Why species delimitation matters for fungal ecology: *Colletotrichum* diversity on wild and cultivated cashew in Brazil

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ABSTRACT

Anthracnose is one of the most important plant diseases globally, occurring on a wide range of cultivated and wild host species. This study aimed to identify the *Colletotrichum* species associated with cashew anthracnose in Brazil, determine their phylogenetic relationships and geographical distribution, and provide some insight into the factors that may be influencing community composition. *Colletotrichum* isolates collected from symptomatic leaves, stems, inflorescences, and fruit of cultivated and wild cashew, across four Brazilian biomes, were identified as *Colletotrichum chrysophilum*, *Colletotrichum fragariae*, *Colletotrichum fructicola*, *Colletotrichum gloeosporioides sensu stricto*, *Colletotrichum queenslandicum*, *Colletotrichum siamense* and *Colletotrichum tropicale*. *Colletotrichum siamense* was the most dominant species. The greatest species richness was associated with cultivated cashew; leaves harbored more species than the other organs; the Atlantic Forest encompassed more species than the other biomes; and Pernambuco was the most species-rich location. However, accounting for the relative abundance of *Colletotrichum* species and differences in sample size across strata, the interpretation of which community is most diverse depends on how species are delimited. The present study provides valuable information about the *Colletotrichum*/cashew pathosystem, sheds light on the causal agents identification,and highlights the impact that species delimitation can have on ecological studies of fungi.

1. Introduction

The genus *Anacardium* L. comprises about 38 species of wild and cultivated plants, each with socioeconomic and/or ecological relevance (Agostini-Costa et al., 2006; TPL, 2017). One such group of *Anacardium* species includes plants from the Amazon Forest and the Brazilian 'Cerrado' popularly known as cashew (Agostini-Costa et al., 2006; Barros et al., 2002; Cardoso and Freire, 2002; Mitchell and Mori, 1987), Among the cashew species, only *Anacardium occidentale* L. is cultivated for commercial purposes in different parts of the world. *Anacardium ottonianum* Rizzini is wild-harvested for subsistence in poor communities in the Central region of Brazil, while other wild species, such as *Anacardium humile* St. Hilaire, *Anacardium nanum* St. Hilaire and *Anacardium corymbosum* Barb. Rodr., serve as a source of food for native animals (Agostini-Costa et al., 2006).

Currently, *A. occidentale* is cultivated in at least 33 countries around the world, among which Brazil is ranked among the top (FAO, 2017). The juicy part of the cashew is a pseudo-fruit or accessory fruit, as it is not derived from the ovary but is a swollen pedicel. It can be directly consumed, or may be used in the production of juice, jam, alcoholic beverages, and soft drinks, whereas the fruit is usually eaten as roasted nuts (Agostini-Costa et al., 2006; Freire et al., 2002; Freire and Cardoso, 2003). Brazil is the leading producer of cashew pseudo-fruit and the tenth largest producer of cashew nuts (FAO, 2017). Most of the cashew nuts produced in Brazil come from the Northeast region (Brazilian Institute of Geography and Statistics – IBGE, 2017), where small farmers frequently organize themselves in cooperatives aiming to raise the quantity and quality of their products. Aside

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from stressful abiotic conditions, several diseases compromise yield and quality of cashew, among which anthracnose is reported as the most important due to yield loss that can surpass 40% (Freire and Cardoso, 2003). This is usually associated with *Colletotrichum* Corda (1831) species and occurs during both vegetative growth and fruiting in commercial (Freire et al., 2002) and wild species of cashew. In general, symptoms of anthracnose start with water-soaked lesions, which become orange to slightly reddish during sporulation (Freire et al., 2002; Freire and Cardoso, 2003; Menezes, 2005). Infection may result in premature abscission of leaves or depressed lesions on the fruit (Menezes, 2005).

Colletotrichum is a cosmopolitan genus of fungi that includes pathogenic and non-pathogenic species (Cannon et al., 2012; Hyde et al., 2009; Prihastuti et al., 2009; Vieira et al., 2014). It has been found associated with approximately 2200 plant species (Farr and Rossman, 2015), and represents one of the most important etiological agents of plant diseases worldwide (Dean et al., 2012). In addition, numerous *Colletotrichum* species have been isolated from asymptomatic organs of native and cultivated plants, especially in the tropics where it is very common (Bragança et al., 2016; Gazis et al., 2011; Lima et al., 2013; Rojas et al., 2010; Vieira et al., 2014). Surveys in tropical moist forests have revealed that individual plants and even single leaves may harbor a diverse assemblage of endophytic fungal species (Arnold et al., 2000; Arnold and Lutzoni, 2007), including a diversity of *Colletotrichum* species from individual hosts. Reliable species recognition within *Colletotrichum* eluded fungal taxonomists prior to the advent of molecular systematics due to morphological plasticity and a paucity of taxonomically-informative morphological characters (Cai et al., 2009; Cannon et al., 2012; Freeman et al., 1998; Sutton, 1992). Multilocus phylogenetic data have become the standard for the delimitation of species (Dettman et al. 2003; Doyle et al., 2013; Liu et al. 2015, 2016; Rojas et al., 2010; Taylor et al., 2000) and provide the opportunity to more reliably characterize diversity and understand the biology of important but morphologically cryptic lineages within *Colletotrichum* (Damm et al. 2012a, b; De Silva et al., 2016; Haung et al., 2013; Manamgada et al. 2013; Prihastuti et al., 2009; Sharma et al., 2015; Tao et al., 2013; Wang et al., 2016; Weir et al., 2012).

The identification of etiological agents is particularly important in agroecosystems because it is the foundation from where pest management strategies are developed. For example, it was thought that anthracnose in mango was caused only by *Colletotrichum gloeosporioides*, but multilocus phylogenetic data revealed eight distinct lineages associated with mango (Lima et al., 2013; Sharma et al. 2013, 2015; Vieira et al., 2014). *Colletotrichum gloeosporioides* is the only species thus far associated with anthracnose on *A. occidentale* (Freire et al., 2002; Lopez and Lucas, 2010; Menezes, 2005; Serra et al., 2011; Uaciquete et al., 2013). However, the studies published to date have used molecular phylogenetic data with relatively low information content, making it impossible to recognize the many cryptic species that have been described within the *C. gloeosporioides* species complex, and there is no information about the species that infect wild species of cashew. Moreover, cashew species are distributed across several biomes with remarkable biodiversity and diverse agricultural environments and the aforementioned studies tend to be geographically restricted. The purpose of this study was to investigate the diversity of *Colletotrichum* species associated with cultivated and wild species of cashew across a broad geographic distribution representing four Brazilian biomes. We employed multilocus phylogenetic data to infer evolutionary relationships among the species of *Colletotrichum*, as well as their prevalence by geography, biome, host species, and plant organ.

2. Materials and methods

2.1. Sampling and fungal isolation

Cultivated and wild species of cashew were collected from nine Brazilian states, including Alagoas (AL, 13 sampled plants), Ceará (CE, 20), Minas Gerais (MG, 104), Pará (PA, 33), Pernambuco (PE, 158), Paraíba (PB, 26), Goiás (GO, 8), Santa Catarina (SC, 5), and Rio Grande do Norte (RN, 67), plus Distrito Federal (DF, 46). The collection sites are distributed across the following four Brazilian biomes that are unique with respect to climate, biodiversity, and human intervention: Atlantic Forest, Amazon Rainforest, Caatinga, and Cerrado. Multiple organs, including leaves (5 per plant), stems, inflorescences and/or fruit showing symptoms of anthracnose were collected and shipped in plastic bags to the Mycology Laboratory of the Universidade Federal Rural de Pernambuco (UFPRE) in Recife, Brazil. Fragments bordering healthy and necrotic zones in leaves, stems, inflorescences, and fruit were cut and surface disinfested by submersing in 70 % ethanol for 30 s, 1.5 % sodium hypochlorite for 1 min, rinsed three times with sterile water, and dried on sterilized filter paper. Five fragments of each plant organ were evenly spaced in glass Petri dishes containing potato-dextrose-agar (PDA: 200 g of potato, 20 g of dextrose and 20 g of glucose, in 1 L of distilled water) and amended with 0.5 g streptomycin sulfate (PDAS) to suppress bacterial growth. The Petri dishes were incubated at ambient temperature for 5 d and mycelium from the colony edges were transferred to new Petri dishes containing PDA. Single isolates were generally recovered from individual lesions, except in the case of coalesced lesions where multiple isolates were recovered if colony morphology (mycelium texture and color) suggested they were distinct.

Pure cultures were established from single spores for those isolates identified as *Colletotrichum* spp. based on morphological characteristics. Spores were suspended in 30 μL of sterile water and a 5 μL aliquot was uniformly distributed in glass Petri dishes containing PDA using a Drigalski spatula. A single conidium of each isolate was transferred to a new PDA plate for preservation and DNA extraction. Mycelium discs from these colonies were preserved by placing them in microtubes filled with 700 μL of sterile water. Representative isolates were deposited as vouchers at the "Coleção de Fungos Fitopatogênicos Professora Maria Menezes (CMM)" of the Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil.

2.2. Extraction of DNA, PCR amplification and sequencing

Single spore cultures of *Colletotrichum* isolates were grown on PDA incubated for five to seven days at ambient temperature. Mycelium was scraped from the colony surface using a sterile pipette tip and genomic DNA was extracted using the Sodium Dodecyl Sulfate (SDS) protocol with some modifications (Moller et al., 1992). Stock DNA was suspended in 1x TE buffer and stored at −20 °C. The stock DNA concentration was measured with the NanoVue Plus spectrometer (GE Healthcare, USA) and diluted to 25 ng/μL for PCR.

The intergenic spacer between the 3′ end of the DNA-lyase and the mating type locus MAT1-2 (APN2/MAT-IGS) was sequenced for all isolates in order to select representative isolates for multilocus sequencing. Distinct haplotypes were identified using DnaSP 4.0 (Rozas et al., 2003) and the selection of representative isolates was based on host species, host organ, Brazilian biome, and geographical sampling site. PCR amplification and sequencing of DNA-lyase (APN2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glyceraldehyde-3-phosphate dehydrogenase-IGS (GAP2-IGS), β-tubulin (TUB2), glutamine synthetase (GS), and calmodulin (CAL)

were done for all representative isolates. Information on primers used for PCR amplification and sequencing is shown in Supplementary Table S1.

The PCR cycling parameters for APN2/MAT-IGS and APN2 consisted of initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 62 °C for 45 s, 72 °C for 1 min and a final cycle at 72 °C for 10 min. For GAPDH and GAP2-IGS they involved an initial denaturation step at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and a final cycle at 72 °C for 10 min. The optimal annealing temperature differed for each of the other genes: TUB2 – 53 °C; GS – 55 °C; and CAL – 57 °C. When amplification resulted in multiple bands, annealing temperatures and/or time were adjusted. PCR primers used during this study are listed in Table 1. Each 12.5 μL PCR reaction volume included 4.0 μL of PCR-grade water, 0.625 μL of each primer (10 μM), 1.0 μL of DNA template and 6.25 μL of PCR Master Mix (2X) (Promega GoTaq® Master Mix, Wisconsin, USA). The PCR amplification products were visualized on an 1.5 % agarose gel stained with GelRed™ (Biotium). PCR purification and sequencing were carried out by Beckman Coulter Genomics (Danvers, Massachusetts, USA).

2.3. Sequence alignment and phylogenetic analyses

Sequence reads were edited and consensus sequences automatically assembled using Geneious 8.1 (Kearse et al., 2012). All consensus sequences were used as queries against the National Center for Biotechnology Information (NCBI) nucleotide database using the *blastn* algorithm (Johnson et al., 2008). Sequences representing ex-types and related published sequences were retrieved from GenBank (Supplementary Table S2). Multiple sequence alignments (MSA) of each locus were estimated with the online version of MAFFT version 7 (Katoh et al., 2002; Katoh and Toh, 2013) with the G-INS-i iterative refinement method and the 200PAM/$\kappa = 2$ nucleotide scoring matrix. Each MSA was manually edited in MEGA5 (Tamura et al., 2011). All sequences generated for the present study were deposited in GenBank (Supplementary Table S2). The alignment length, number of parsimony informative characters, percentage of parsimony informative characters and substitution model of each locus are given in Supplementary Table S3.

Evolutionary relationships were inferred using both Bayesian (BI) and maximum likelihood (ML) approaches for each individual locus and the concatenated matrix. BI analysis was carried out using MrBayes v. 3.2.6 program (Ronquist et al., 2012) implemented on the CIPRES cluster (https://www.phylo.org/portal2/home.action) using the best-fit model of nucleotide evolution estimated by MrModeltest 2.3 (Nylander, 2004) following the Akaike Information Criterion (AIC) (Table 3). Each analysis was run for 5×10^7 generations, sampling every 1000, with four Markov Chain Monte Carlo (MCMC) chains (3 heated, 1 cold). The first 25 % of the samples were discarded as burnin and convergence of the parameter estimates and likelihoods was visually confirmed in the program Tracer v. 1.6 (Rambaut and Drummond, 2010). Each analysis was also considered to have converged when effective sample sizes (ESS) were greater than 200. Maximum likelihood topologies were inferred in GARLI v. 2.01 (Zwickl, 2006) using the High Performance Computational Resources at Louisiana State University applying the best-fit models of nucleotide substitution selected using Akaike's Information Criterion corrected for small sample sizes (AICc) in jModelTest2 v. 2.1.6 (Darriba et al., 2012; Guindon and Gascuel, 2003). Each maximum likelihood tree represents the best tree from 20 replicate searches when the ML tree was found more than once or 100 replicate searches when the best tree was not found at least twice. Independent searches were terminated after 10,000 generations without an improvement in the likelihood score greater than 0.01 log-likelihood units. Node support was estimated in a bootstrap analysis with 1008 pseudoreplicates, with the tree with the highest likelihood after 10 replicate searches to represent each bootstrap pseudoreplicate dataset. Support values were mapped to the ML tree using the program SumTrees 3.3.1, which utilizes the DendroPy Phylogenetic Computing Library version 3.12.0 (Sukumaran and Holder, 2010). All sequence alignments and phylogenetic trees are archived in TreeBase (Study ID: S20942; treebase.org).

2.4. Species recognition

Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Dettman et al., 2003; Taylor et al., 2000) was applied to recognize *Colletotrichum* species associated with cashew. Based on GCPSR a lineage is considered as independently evolved if it is strongly supported as monophyletic in the concatenated analysis and fits at least one of the following criteria: a clade is concordant when it is present in the majority of the individual gene trees (i.e., at least in 4 of the 7); a clade is non-discordant when the clade is well supported by both BI (posterior probability ≥ 0.95) and ML (bootstrap ≥ 70 %) analysis in a single gene tree and is not in conflict with any other single genealogy at the same level of support.

Given recent discussions in the literature about the species-level status of several lineages within what is currently recognized as *Colletotrichum siamense*, strongly supported monophyletic lineages within *C. siamense* were treated under two alternate delimitation regimes. The first represents the inclusive view of Liu et al. (2016) that *C. siamense* represents a diverse and broadly distributed species composed of many monophyletic subclades. The alternate approach treats *C. siamense* as a species complex and recognizes previously described species (recently synonymized by Liu et al., 2016), strongly supported monophyletic subclades within *C. siamense*, and divergent singletons as independent lineages. The latter view is one that has been taken in previous publications (e.g. Sharma et al., 2015) and represents the other end of the spectrum when it comes to species diversity within *C. gloeosporioides s.l.* These alternate approaches were devised to explore the impact that different approaches to species recognition can have on diversity analyses.

2.5. Prevalence of Colletotrichum species

The prevalence of *Colletotrichum* species collected from different cashew species, host organ, Brazilian biome and geographical sampling site was determined by calculating the Isolation Rate (IR) as follows: IR(%) = (Cx/Ct) x 100, where Cx is the number of isolates belonging to one species and Ct is the number of isolates per host, plant organ, biome and geographical site, respectively.

2.6. Rarefaction and extrapolation curves with Hill numbers

The diversity of *Colletotrichum* species associated with cultivated and wild cashew, host organ, Brazilian biome and geographical sampling site was determined by estimating rarefaction (interpolation) and prediction (extrapolation) curves based on Hill numbers. These are known as the effective number of species, and represent a mathematically unified group of diversity indices that differ from each other only by an exponent q (Hill, 1973). As proposed by Chao et al. (2014), we applied a unified approach for individual-based data to estimate rarefaction and extrapolation curves for the first three Hill numbers: species richness ($q = 0$), Shannon diversity ($q = 1$, the exponential of Shannon entropy), and Simpson diversity ($q = 2$, the inverse of Simpson concentration). The exponent q determines the sensitivity of the measure to the relative abundance, where $q = 0$ counts species equally, regardless of their relative abundances; $q = 1$ counts individuals equally, thus

Table 1
Strains of *Colletotrichum* included in this study with information about culture collection number, *Anacardium* host, plant organ, Brazilian biomes and geographical sampling sites.

Colletotrichum species	Strain	*Anacardium* species	Host organ	Brazilian biomes	Geographical sampling sites
C. chysophilum	CMM3007	*A. occidentale*	Stem	Caatinga	CE
	CMM3204	*A. humile*	Leaf	Cerrado	MG
	CMM3217	*A. occidentale*	Leaf	Caatinga	PB
	CMM3218	*A. occidentale*	Leaf	Caatinga	PB
	CMM3231	*A. occidentale*	Stem	Atlantic Forest	PE
	CMM3239	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3268	*A. occidentale*	Stem	Atlantic Forest	RN
C. fragariae	CMM3214	*A. occidentale*	Leaf	Caatinga	PB
	CMM3220	*A. occidentale*	Leaf	Caatinga	PB
	CMM3221	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3224	*A. occidentale*	Stem	Atlantic Forest	PE
	CMM3245	*A. occidentale*	Leaf	Caatinga	RN
C. fructicola	CMM3013	*A.humille*	Leaf	Cerrado	DF
	CMM3102	*A.othonianum*	Leaf	Cerrado	DF
	CMM3207	*A.othonianum*	Leaf	Cerrado	MG
	CMM3208	*A.humille*	Leaf	Cerrado	MG
	CMM3209	*A. occidentale*	Leaf	Cerrado	MG
	CMM3238	*A. occidentale*	Leaf	Atlantic Forest	PE
C. gloeosporioides	CMM3272	*A. occidentale*	Inflorescence	Atlantic Forest	PE
	CMM3279	*A. occidentale*	Inflorescence	Atlantic Forest	PE
C. queenslandicum	CMM3233	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3236	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3237	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3240	*A. occidentale*	Inflorescence	Atlantic Forest	PE
	CMM3241	*A. occidentale*	Inflorescence	Atlantic Forest	PE
	CMM3242	*A. occidentale*	Inflorescence	Atlantic Forest	PE
C. siamense	CMM2990	*A. occidentale*	Leaf	Atlantic Forest	AL
	CMM2994	*A. occidentale*	Leaf	Atlantic Forest	AL
	CMM2998	*A. occidentale*	Stem	Atlantic Forest	AL
	CMM2974	*A. occidentale*	Stem	Atlantic Forest	AL
	CMM3001	*A. occidentale*	Leaf	Caatinga	CE
	CMM3011	*A. occidentale*	Leaf	Cerrado	DF
	CMM3014	*A. humile*	Leaf	Cerrado	DF
	CMM3015	*A.othonianum*	Leaf	Cerrado	DF
	CMM3020	*A.othonianum*	Leaf	Cerrado	DF
	CMM3101	*A.othonianum*	Leaf	Cerrado	DF
	CMM3103	*A.othonianum*	Leaf	Cerrado	DF
	CMM3202	*A.othonianum*	Leaf	Cerrado	DF
	CMM3203	*A.othonianum*	Leaf	Cerrado	MG
	CMM3205	*A.othonianum*	Leaf	Cerrado	MG
	CMM3206	*A.othonianum*	Leaf	Cerrado	MG
	CMM3210	*A. occidentale*	Leaf	Cerrado	MG
	CMM3211	*A. occidentale*	Leaf	Cerrado	MG
	CMM3212	*A. occidentale*	Stem	Cerrado	MG
	CMM3215	*A. occidentale*	Leaf	Caatinga	PB
	CMM3216	*A. occidentale*	Leaf	Caatinga	PB
	CMM3219	*A. occidentale*	Leaf	Caatinga	PB
	CMM3222	*A. occidentale*	Leaf	Caatinga	PB
	CMM3223	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3225	*A. occidentale*	Stem	Atlantic Forest	PE
	CMM3226	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3227	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3229	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3232	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3234	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3235	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3243	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3251	*A. occidentale*	Leaf	Caatinga	RN
	CMM3253	*A. occidentale*	Leaf	Caatinga	RN
	CMM3260	*A. occidentale*	Inflorescence	Caatinga	RN
	CMM3261	*A. occidentale*	Stem	Caatinga	RN
	CMM3264	*A. occidentale*	Stem	Caatinga	RN
	CMM3284	*A. occidentale*	Leaf	Atlantic Forest	SC
	CMM3286	*A. humile*	Leaf	Cerrado	GO
	CMM3298	*A. occidentale*	Leaf	Amazon Rainforest	PA
	CMM3301	*A. occidentale*	Leaf	Amazon Rainforest	PA
	CMM3304	*A. occidentale*	Leaf	Amazon Rainforest	PA
	CMM3308	*A. occidentale*	Inflorescence	Amazon Rainforest	PA
	CMM3313	*A. occidentale*	Inflorescence	Amazon Rainforest	PA
	CMM3320	*A. occidentale*	Leaf	Amazon Rainforest	PA
	CMM3322	*A. occidentale*	Leaf	Amazon Rainforest	PA

Table 1 (*continued*)

Colletotrichum species	Strain	*Anacardium* species	Host organ	Brazilian biomes	Geographical sampling sites
C. tropicale	CMM2999	*A. occidentale*	Leaf	Caatinga	CE
	CMM3213	*A. occidentale*	Leaf	Caatinga	PB
	CMM3228	*A. occidentale*	Leaf	Atlantic Forest	PE
	CMM3303	*A. occidentale*	Leaf	Amazon Rainforest	PA

CMM: Culture Collection of Phythopathogenic Fungi "Prof. Maria Menezes", Recife, Brazil; AL = Alagoas, CE = Ceará, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, PA = Pará, PB = Paraíba, PE = Pernambuco, RN = Rio Grande do Norte, and SC = Santa Catarina.

Table 2
Diversity measures represented by Hill numbers based on abundance data of *Colletotrichum* species (considering *C. siamense* as a single species) associated with different organs of cultivated and wild cashew in four Brazilian biomes and various geographical sampling sites.

Source of the *Colletotrichum* isolates	Reference sample size[a]	Hill numbers (95 % confidence interval)		
		Richness ($q = 0$)[b]	Shannon ($q = 1$)[c]	Simpson ($q = 2$)[d]
Anacardium species				
A. humile	26	4.0 (2.46–5.54)[b]	2.3 (1.44–3.19)[a,b]	1.8 (1.14–2.40)[a]
A. occidentale	215	7.0 (6.27–7.73)[a]	3.2 (2.75–3.73)[a]	2.1 (1.79–2.48)[a]
A. othonianum	29	2.0 (2.00–2.00)[c]	1.8 (1.62–2.09)[b]	1.7 (1.42–2.06)[a]
Host organ				
Fruit	1	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[b]	NaN[e]
Inflorescences	19	4.0 (2.33–5.66)[b]	2.9 (2.03–3.74)[a]	2.4 (1.40–3.38)[a]
Leaves	219	7.0 (6.27–7.73)[a]	3.4 (2.89–4.00)[a]	2.4 (2.04–2.76)[a]
Stems	41	5.0 (4.02–5.98)[b]	2.8 (1.98–3.60)[a]	2.0 (1.35–2.67)[a]
Brazilian biome				
Amazon Rainforest	10	2.0 (1.72–2.28)[b]	1.6 (1.06–2.24)[b]	1.5 (0.89–2.05)[a]
Atlantic Forest	126	6.0 (5.27–6.73)[a]	3.5 (2.95–3.98)[a]	2.5 (2.05–2.89)[a]
Caatinga	71	5.0 (4.36–5.64)[a]	2.5 (1.85–3.19)[a,b]	1.8 (1.37–2.21)[a]
Cerrado	73	4.0 (2.55–5.45)[a,b]	2.4 (1.96–2.76)[b]	2.2 (1.97–2.34)[a]
Geographical sampling sites				
Alagoas	9	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[b]
Ceará	7	3.0 (2.15–3.85)[b]	2.9 (2.28–3.61)[a,b]	2.9 (2.00–3.76)[a]
Distrito Federal	36	3.0 (2.02–3.98)[b]	2.0 (1.60–2.46)[b]	1.8 (1.36–2.21)[a,b]
Goiás	2	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[b]
Minas Gerais	35	4.0 (3.13–4.87)[b]	2.4 (1.94–2.78)[b]	2.0 (1.51–2.53)[a,b]
Pará	10	2.0 (1.27–2.73)[b]	1.6 (1.08–2.22)[b]	1.5 (0.94–2.00)[a,b]
Paraíba	28	4.0 (3.21–4.79)[b]	2.8 (1.99–3.59)[a,b]	2.3 (1.53–2.99)[a,b]
Pernambuco	116	6.0 (5.21–6.79)[a]	3.7 (3.07–4.30)[a]	2.7 (2.10–3.27)[a]
Rio Grande do Norte	36	4.0 (2.22–5.78)[a,b]	1.6 (0.89–2.29)[b,c]	1.3 (0.95–1.57)[b]
Santa Catarina	1	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[c]	NaN[e]

Hill numbers for the same *Colletotrichum* source within the same exponent q (0, 1 or 2) followed by different letters were significantly different based on their 95 % confidence intervals.
[a] Number of *Colletotrichum* isolates.
[b] Species richness.
[c] Exponential of the Shannon entropy.
[d] Inverse Simpson concentration.
[e] Not a number.

considering species proportionally to their abundances; and $q = 2$ discounts all but the dominant species, and can be interpreted as the effective number of dominant species in the community (Chao et al., 2014; Hsieh et al., 2016). Species diversity was estimated as the mean of 200 bootstrap replications with 95 % confidence intervals. All analyses were performed using the iNEXT library (Hsieh et al., 2016) in R v. 3.1.3 (R Core Team, 2013).

3. Results

3.1. Isolation of Colletotrichum

A total of 280 *Colletotrichum* isolates were collected from both commercial and wild cashew species presenting typical symptomatology of anthracnose, and all of them produced conidia similar to *C. gloeosporioides* on PDA. Most isolates were collected from *A. occidentale* (215), intermediate numbers were obtained from *A. othonianum* (39), and the smallest number of isolates were isolated from *A. humile* (26). Regardless of the host species, the largest number of isolates were found on leaves (219), while intermediate numbers were collected from stems (41) and inflorescences (19), and a single isolate was obtained from fruit. With respect to biome, most *Colletotrichum* isolates were taken from the Atlantic Forest (126), followed by Cerrado (73), Caatinga (71) and the Amazon Rainforest (10). The majority of them were obtained in Pernambuco (116), followed by Rio Grande do Norte (36), Distrito Federal (36), Minas Gerais (35), Paraíba (28), Pará (10), Alagoas (9), Ceará (7), Goiás (2), and Santa Catarina (1).

3.2. Phylogenetic analysis and species recognition

There were 30 Apn2/MAT-IGS haplotypes among the 280 isolates, all of which we assigned to the *C. gloeosporioides* complex based on similarity to representative sequences in the NCBI database. The seven loci described by Vieira et al. (2018) as the most informative for species recognition were sequenced for seventy-five isolates representing the breadth of collection localities across Brazil, host species, host organ, and genetic diversity at APN2/MAT-IGS. These data were included in a multilocus phylogenetic analysis to assign representative haplotypes to species.

Table 3
Diversity measures represented by Hill numbers based on abundance data of *Colletotrichum* species (considering *C. siamense* as a species complex) associated with different organs of cultivated and wild cashew in four Brazilian biomes and various geographical sampling sites.

Source of the *Colletotrichum* isolates	Reference sample size[a]	Hill numbers (95 % confidence interval)		
		Richness ($q = 0$)[b]	Shannon ($q = 1$)[c]	Simpson ($q = 2$)[d]
Anacardium species				
A. humile	26	5.0 (3.26–6.74)[b]	2.6 (1.46–3.69)[b]	1.8 (1.13–2.48)[b]
A. occidentale	215	21.0 (16.76–25.23)[a]	7.8 (6.24–9.39)[a]	4.6 (3.51–5.63)[a]
A. othonianum	29	5.0 (3.75–6.25)[b]	2.9 (2.24–3.60)[b]	2.4 (1.82–2.94)[b]
Host organ				
Fruit	1	1.0 (1.00–1.00)[c]	1.0 (1.00–1.00)[c]	NaN[e]
Inflorescences	19	6.0 (3.97–8.03)[b]	4.9 (3.52–6.39)[b]	4.4 (3.13–5.78)[a]
Leaves	219	19.0 (14.96–23.04)[a]	9.1 (7.81–10.46)[a]	6.3 (4.97–7.73)[a]
Stems	41	10.0 (7.28–12.72)[b]	5.9 (4.07–7.65)[b]	3.9 (2.18–5.55)[a]
Brazilian biome				
Amazon Rainforest	10	7.0 (5.32–8.68)[b]	6.6 (4.64–8.55)[a,b]	6.2 (4.08–8.42)[a]
Atlantic Forest	126	12.0 (10.26–13.74)[a]	6.7 (5.46–7.87)[a]	4.5 (3.25–5.76)[a,b]
Caatinga	71	7.0 (5.18–8.82)[b]	4.2 (3.29–5.15)[b]	3.2 (2.28–4.06)[b]
Cerrado	73	10.0 (6.81–13.19)[a,b]	4.2 (3.30–5.18)[b]	3.1 (2.45–3.77)[b]
Geographical sampling sites				
Alagoas	9	3.0 (1.71–4.29)[c]	2.0 (1.00–2.97)[c,d]	1.6 (0.88–2.30)[c]
Ceará	7	3.0 (2.13–3.87)[c]	2.9 (2.04–3.84)[b,c]	2.9 (1.99–3.78)[b,c]
Distrito Federal	36	5.0 (3.84–6.16)[b,c]	3.2 (2.54–3.88)[b,c]	2.7 (1.96–3.35)[b,c]
Goiás	2	1.0 (1.00–1.00)[d]	1.0 (1.00–1.00)[d]	1.0 (1.00–1.00)[c]
Minas Gerais	35	8.0 (4.71–11.29)[a,b]	3.5 (2.03–5.06)[b,c]	2.3 (1.13–3.50)[b,c]
Pará	10	7.0 (4.99–9.01)[b]	6.6 (4.45–8.75)[a,b]	6.2 (4.33–8.17)[a]
Paraíba	28	5.0 (3.97–6.03)[b]	4.1 (3.39–4.89)[b]	3.7 (2.56–4.77)[a,b]
Pernambuco	116	10.0 (9.11–10.89)[a]	6.6 (5.47–7.57)[a]	4.7 (3.83–5.65)[a]
Rio Grande do Norte	36	6.0 (3.83–8.17)[b]	3.1 (1.98–4.20)[b,c]	2.4 (1.71–3.02)[b,c]
Santa Catarina	1	1.0 (1.00–1.00)[d]	1.0 (1.00–1.00)[d]	NaN[e]

Hill numbers for the same *Colletotrichum* source within the same exponent q (0, 1 or 2) followed by different letters were significantly different based on their 95 % confidence intervals.
[a] Number of *Colletotrichum* isolates.
[b] Species richness.
[c] Exponential of the Shannon entropy.
[d] Inverse Simpson concentration.
[e] Not a number.

The *Colletotrichum* isolates associated with cultivated and non-cultivated cashew were assigned to seven species previously described within the *C. gloeosporioides* complex. Most of them were strongly supported by both ML and BI analysis in the concatenated tree, and satisfied the GCPSR criteria across the individual gene trees (Fig. 1). Two isolates were assigned to *C. gloeosporioides sensu stricto* with strong support in all individual gene trees. Seven isolates were identified as *Colletotrichum chrysophilum*, recently described by Vieira et al. (2018), which formed a clade with strong support in the majority of single gene trees, except CAL (high support only in the BI) and GAPDH (not supported by ML or BI). Six isolates were assigned to *Colletotrichum fructicola*, a clade resolved with strong support in the majority of single gene trees from either ML or BI, with the exception of CAL. Six isolates were assigned to *Colletotrichum queenslandicum* with significant support in the multilocus analysis and the single gene trees of Apn2/MAT-IGS, GAPDH and TUB2. Five isolates were recognized to be conspecific with *Colletotrichum fragariae* with strong support in both the Apn2/MAT-IGS and GAP2-IGS gene trees and not contradicted in any other single gene tree at the same level of support. Four isolates were nested with *Colletotrichum tropicale*, which was supported by the majority of the independent gene trees and the concatenated analysis. Forty five isolates were assigned to *C. siamense* in a strongly supported clade in the multilocus analysis and the majority of individual gene trees (Fig. 1).

3.3. *Communities of* Colletotrichum *species across sampling strata*

The composition of *Colletotrichum* species differed among host species, host organ, biome, and geographical sampling site (Table 1, Fig. 2). *Colletotrichum siamense* and *C. fructicola* were common to all three sampled *Anacardium* species (*A. occidentale*, *A. othonianum* and *A. humile*), while *C. gloeosporioides* and *C. chrysophilum* were isolated from *A. occidentale* and *A. humile*; and *C. fragariae*, *C. queenslandicum* and *C. tropicale* were restricted to *A. occidentale*. With respect to the host organ, *C. siamense* was isolated from leaves, stems, inflorescences and fruit, while *C. chrysophilum* was found on leaves, stems and inflorescences; *C. fragariae*, *C. tropicale* and *C. fructicola* on leaves and stems; and *C. queenslandicum* and *C. gloeosporioides* on leaves and inflorescences (Table 1, Fig. 2).

With respect to geographical distribution, most of the *Colletotrichum* species occurred in two or three of the sampled Brazilian biomes (Atlantic Forest, Amazon Rainforest, Caatinga, and/or Cerrado), with *C. siamense* common to all of them and *C. queenslandicum* restricted to the Atlantic Forest (Table 1, Fig. 2). In contrast, *C. tropicale* was found in the Atlantic Forest, the Amazon Rainforest, and Caatinga; *C. chrysophilum* occurred in the Atlantic Forest, Caatinga, and Cerrado; *C. fragariae* was found in the Atlantic Forest and Caatinga; *C. gloeosporioides* was restricted to the Caatinga and Cerrado; and *C. fructicola* was collected in the Atlantic Forest and Cerrado (Table 1, Fig. 2).

While *C. siamense* was collected from all sampled federative units of Brazil (AL, CE, DF, GO, MG, PA, PB, PE, RN and SC), *C. chrysophilum* was common to five locations; *C. tropicale* was distributed across four sites; *C. fragariae*, *C. fructicola* and *C. gloeosporioides* occurred in three; and *C. queenslandicum* was restricted to Pernambuco (PE) (Table 1, Fig. 2).

3.4. *Prevalence of* Colletotrichum *species across sampling strata*

Overall, *C. siamense* was the most prevalent species among 280 *Colletotrichum* isolates collected from cultivated and wild cashew

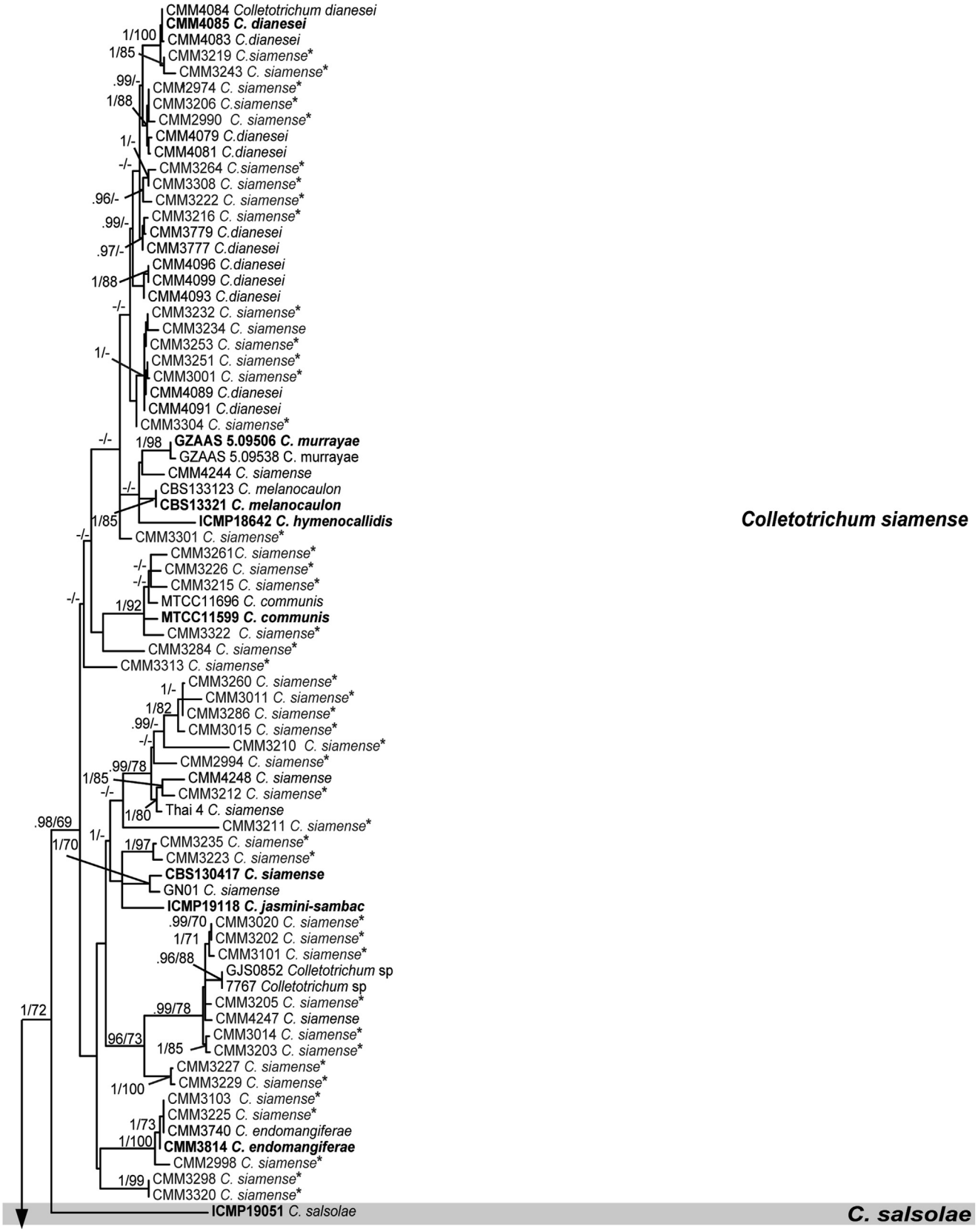

Fig. 1. Maximum likelihood tree of the *C. gloeosporioides* species complex inferred from a concatenated alignment of APN2, APN2/MAT-IGS, CAL, GAPDH,GAP2-IGS, GS and TUB2. Bootstrap support values (ML $\geq$ 70) and Bayesian posterior probability values (PP $\geq$ 0.95) are shown above the branches. "-" indicates no-significant support or absence of the branch. Ex-types are emphasized in bold and include the taxonomic name as originally described. "*" indicates isolates from cashew. *Colletotrichum fragariae, C. theobromicola* and *C. grevilleae* were used as outgroups. The scale bar indicates the estimated number of substitutions per site.

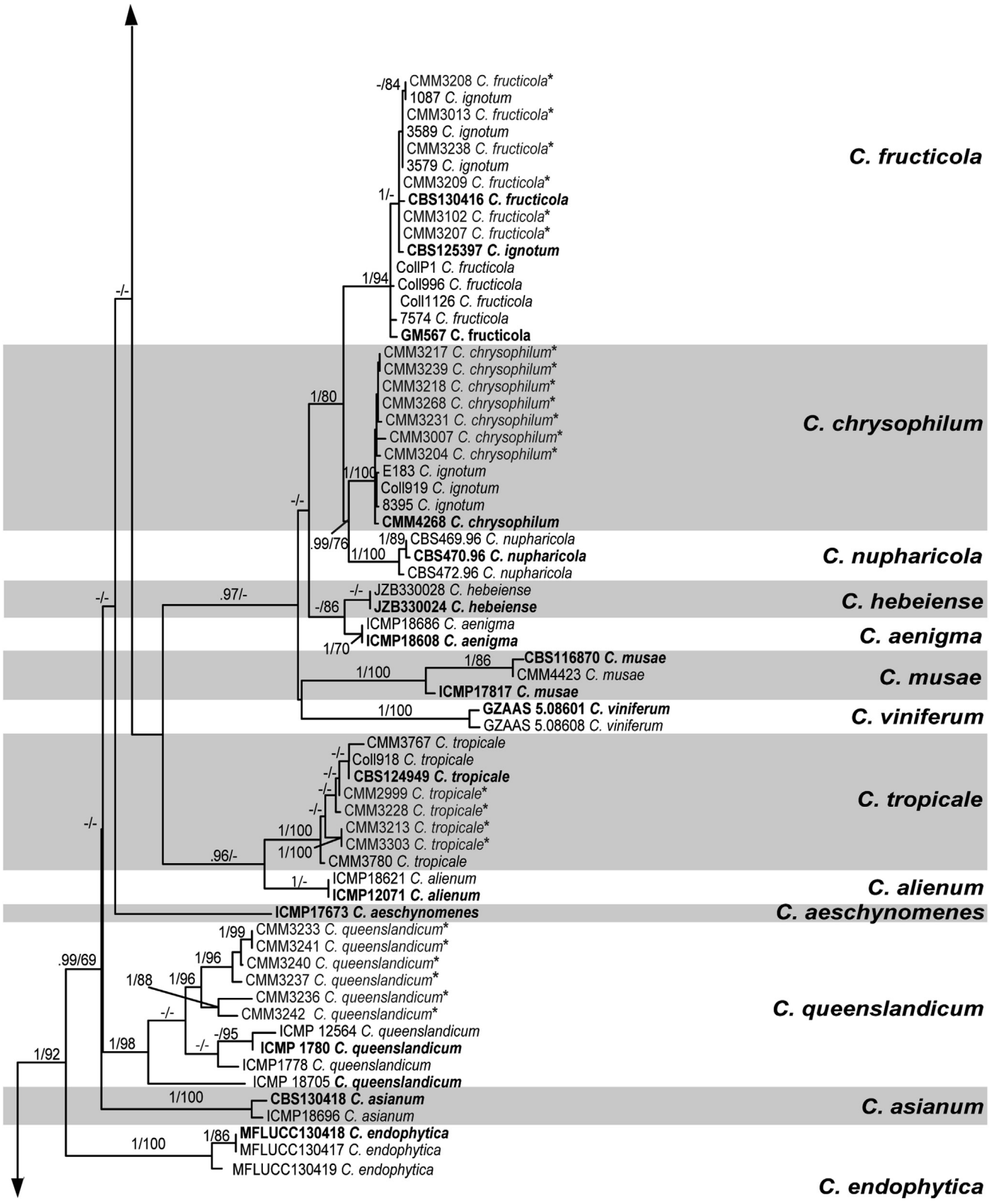

Fig. 1. (*continued*).

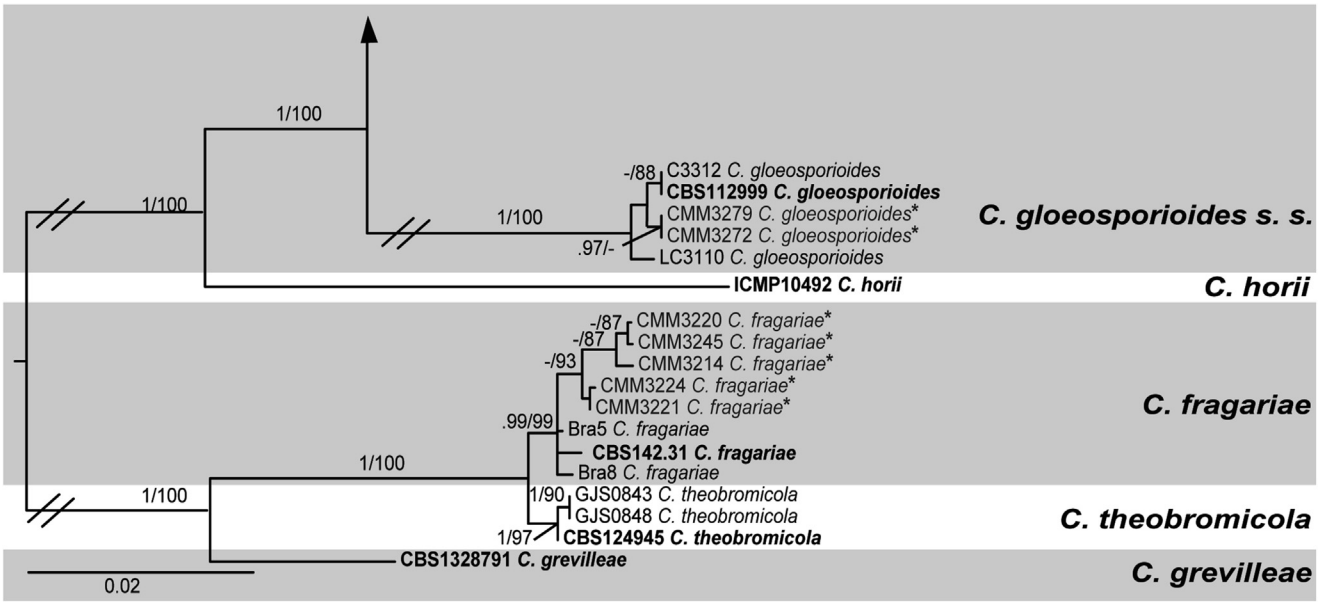

Fig. 1. (*continued*).

plants across Brazil. It represented approximately 62 % of the isolates, with other species representing 1.4–14.3 %. Nearly 66.5 % and 69.2 % of the isolates collected from *A. occidentale* and *A. othonianum*, respectively (Fig. 2A), were assigned to *C. siamense*, while *C. fructicola* was the most frequent (73.1 %) in *A. humile*. Regardless of the host, *C. siamense* was the most common species on leaves, stems, inflorescences and fruit (Fig. 2B).

With respect to geographical distribution, *C. siamense* was the most common species across the four sampled biomes and all geographical sampling sites, except in Minas Gerais, where *C. fructicola* was the most abundant species (Fig. 2C and D).

3.5. *Comparison of* Colletotrichum *species diversity based on abundance data*

To compare the diversity of *Colletotrichum* species associated with anthracnose on different organs of cultivated and wild cashew in Brazil, individual-based rarefaction and extrapolation curves were computed for the first three Hill numbers: species richness ($q = 0$), Shannon diversity ($q = 1$), and Simpson diversity ($q = 2$). Given recent discussion about recognizing *C. siamense* as a single species or a complex (Liu et al., 2016; Sharma et al., 2015), we computed rarefaction and extrapolation curves considering both situations (Figs. 3 and 4, respectively). In the first case, maximum species richness comprised seven *Colletotrichum* species, namely *C. chrysophilum*, *C. fragariae*, *C. fructicola*, *C. gloeosporioides s.s.*, *C. queenslandicum*, *C. siamense* and *C. tropicale*. On the other hand, the second scenario includes 21 independent lineages (the former seven species but *C. siamense s.s.*, plus *C. communis*, *C. endomangiferae*, *C. dianesei* and 12 lineages not currently recognized as species – see Supplementary Figure S1 for species assignments). While *C. dianesei* was supported by neither ML nor BI, other lineages within *C. siamense* s.l. formed sub clades or singletons identified as independent in the concatenated analysis and concordant across APN2, APN2/MAT-IGS, and GS individual gene trees. However, conflicts were observed when these trees were compared to GAPDH and GAP2-IGS trees. Overall, the number of significant differences in *Colletotrichum* diversity among sampling strata decreased as the exponent q increased, i.e. the richness index detected more significant differences among sources of *Colletotrichum* isolates than did Shannon, which depicted more differences than Simpson diversity (Figs. 3 and 4, Tables 2 and 3).

Considering *C. siamense* as a single species, there was virtually no expected increase in species diversity measures due to extrapolation (double the reference sample size) curves (Fig. 3A–D) across sampling strata and diversity metrics with the exception of *A. humile* ($q = 0$; Fig. 3A), inflorescence ($q = 0$; Fig. 3B), Cerrado ($q = 0$, Fig. 3C), Rio Grande do Norte and Minas Gerais ($q = 0$; Fig. 3D) and Ceará ($q = 2$; Fig. 3D). *Anacardium occidentale* exhibited greater diversity than *A. humile* and *A. othonianum* on the basis of species richness, but was not significantly greater on the basis of Shannon or Simpson diversity measures. Similarly, on the basis of species richness, cashew leaves harbored more *Colletotrichum* species than stems, inflorescences and fruit (Table 2), but not when accounting for relative abundance and species dominance ($q = 1, 2$). Species richness for the Atlantic Forest and Caatinga did not differ and were significantly greater than that observed in the Amazon Rainforest, with the Cerrado intermediate (Table 2). The Atlantic Forest also exhibited the greatest diversity based on Shannon index, and was significantly different from Cerrado and the Amazon Rainforest, with Caatinga intermediate. In contrast, no significant difference was detected among biomes based on Simpson diversity. With respect to geographical sampling site, Pernambuco was the most diverse location (Table 2), but overlapping with Rio Grande do Norte in species richness. It was also the most diverse location based on Shannon diversity, but was overlapping with Paraíba and Ceará. While Ceará and Pernambuco were the most diverse on the basis of Simpson diversity, they were only significantly greater than Alagoas, Goiás, and Rio Grande do Norte.

When *C. siamense* was treated as a species complex, conclusions about the most diverse sampling strata (Table 3, Fig. 4) differed from treating *C. siamense* as a single species. In this case, extrapolation (double the reference sample size) based on $q = 0$ predicted an increase in diversity by host species (Fig. 4A), host organ (Fig. 4B), biome, and geographical sampling site, while $q = 1$ and $q = 2$ predicted an increase only for the Amazon Rainforest and Pará (Fig. 4C and D). The trends in community diversity with respect to host were similar to that previously described when treating *C. siamense* as a single species, with *A. occidentale* hosting

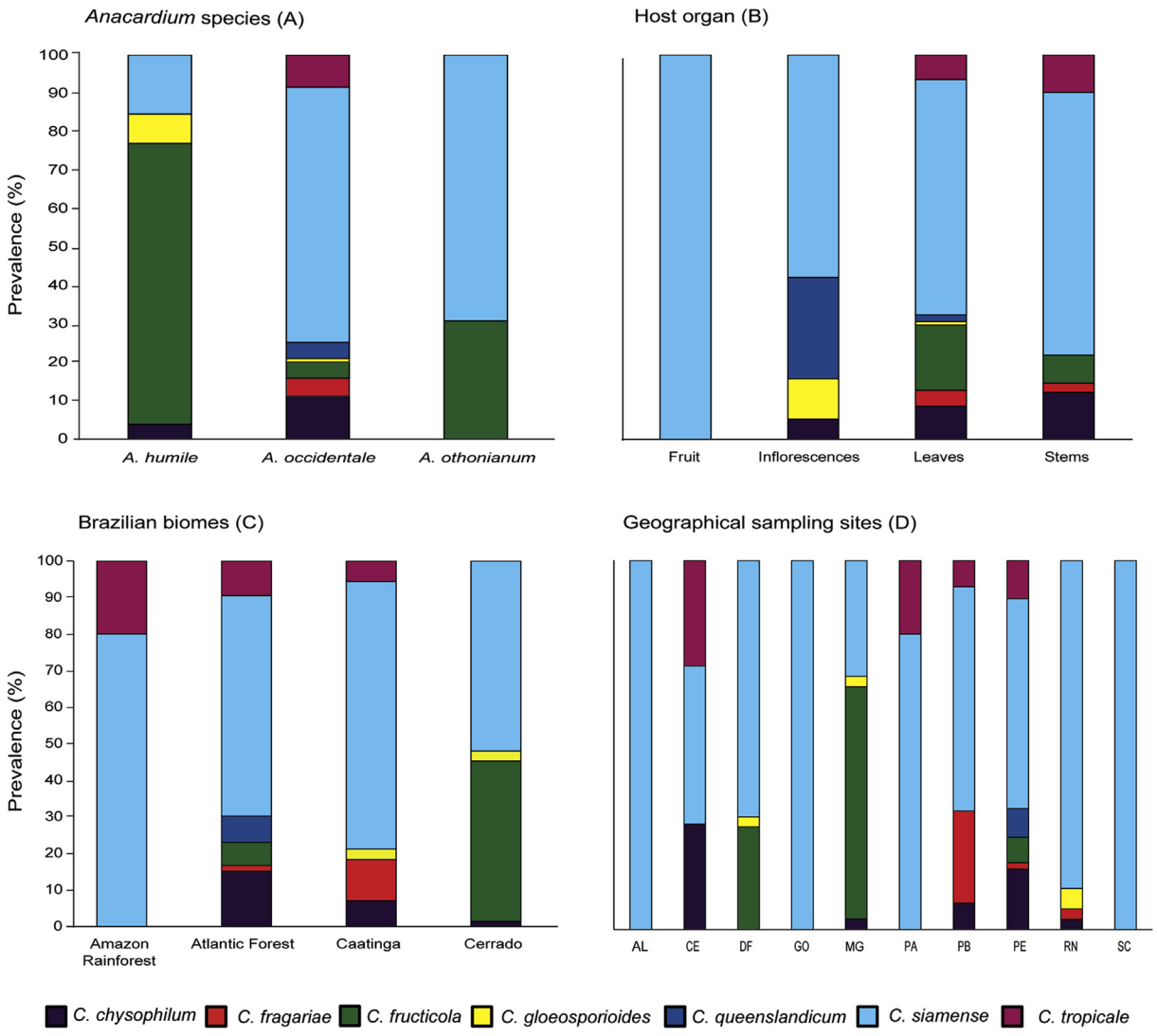

Fig. 2. Prevalence of *Colletotrichum* species associated with different *Anacardium* hosts (A), plant organs (B), Brazilian biomes (C), and 10 geographical sampling sites (D). AL = Alagoas, CE = Ceará, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, PA = Pará, PB = Paraíba, PE = Pernambuco, RN = Rio Grande do Norte, and SC = Santa Catarina.

the most diverse assemblage of species. However, when treating putatively independent lineages within *C. siamense* as distinct, *A. occidentale* is also more diverse even when accounting for relative abundance ($q = 1$) and dominance ($q = 2$). Similarly, leaves were the most diverse host organ, but were so on the basis of both species richness and accounting for relative abundance ($q = 1$). While the Atlantic Forest was still the most diverse on the basis of species richness, in contrast to treating *C. siamense* as a single lineage, the second most diverse biome (overlapping with the Atlantic Forest) was the Cerrado (Fig. 4C, Table 3). The Atlantic Forest and the Amazon Rainforest were the most diverse with respect to both Shannon and Simpson diversity ($q = 1, 2$; Fig. 4C, Table 3). Pernambuco was the most diverse location on the basis of species richness (Table 3) followed by Minas Gerais. However, Pará was the most diverse alongside Pernambuco based on Shannon diversity and was the most diverse location when focusing on dominant species ($q = 2$).

4. Discussion

In a broad phylogenetic analysis involving seven genes (Apn2/MAT-IGS, TUB2, CAL, GAP2-IGS, GAPDH, GS and APN2), the present study revealed a highly diverse group of *Colletotrichum* species associated with anthracnose on cultivated and wild species of cashew in Brazil. These included *C. chrysophilum*, *C. fragariae*, *C. fructicola*, *C. gloeosporioides s.s.*, *C. queenslandicum*, *C. siamense*, and *C. tropicale*. However, when *C. siamense* was treated as a species complex rather than a single species, the diversity pattern among and within some strata changed depending upon the order of Hill numbers ($q = 0$, 1 or 2). According to Chao et al. (2014), researchers using Hill numbers should report at least the diversity of all species ($q = 0$), of 'typical' species ($q = 1$), and of dominant species ($q = 2$), keeping in mind that inferences of diversity for $q \geq 1$ are more reliable. Based on $q = 1$, and considering *C. siamense* as a single species, the diversity of the assemblage harbored by *A. occidentale* was similar to that of *A. humile* and more diverse than *A. othonianum*, while no significant differences were observed based on $q = 2$. This result is supported by the prevalence analysis (Fig. 2), showing that the relative abundance ($q = 1$) of *C. fructicola* in *A. humile* is similar to that of *C. siamense* in *A. occidentale*, and that these *Colletotrichum* species were also dominant ($q = 2$) in *A. othonianum*. In contrast, when *C. siamense* was treated as a species complex with multiple independent lineages, *A. occidentale* was the host harboring the most diverse assemblage of *Colletotrichum* species regardless of q order.

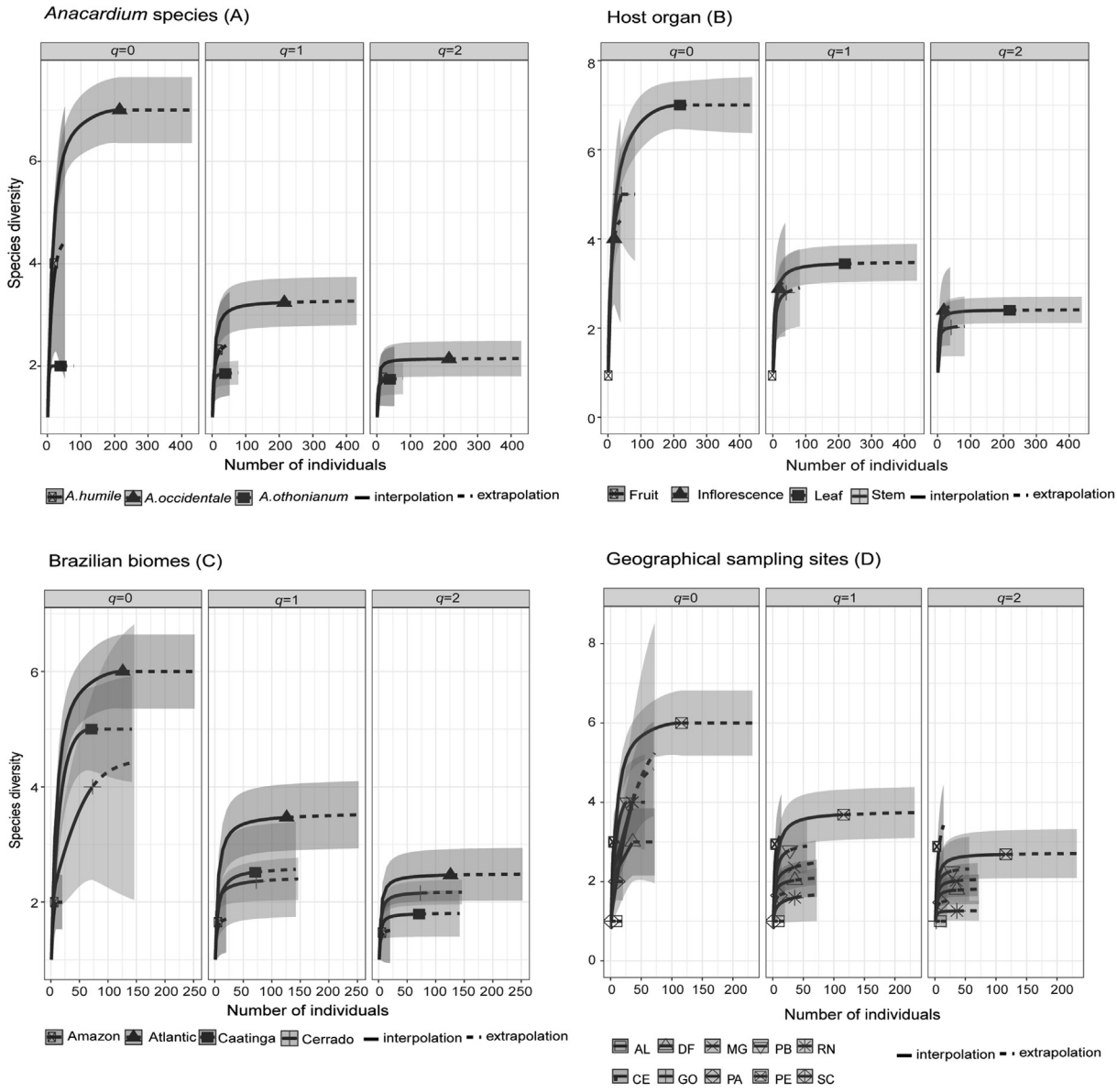

Fig. 3. Rarefaction (solid lines) and extrapolation (dashed lines) curves of *Colletotrichum* species diversity (considering *C. siamense* as a single species) based on the Hill numbers ($q = 0, 1, 2$) for each *Anacardium* species, host organ, Brazilian biome and geographical sampling site. The 95% confidence intervals (gray-shaded regions) were obtained by a bootstrap method based on 200 replications. Reference samples are denoted by different symbols. AL = Alagoas, CE = Ceará, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, PA = Pará, PB = Paraíba, PE = Pernambuco, RN = Rio Grande do Norte, and SC = Santa Catarina.

Considering *C. siamense* as a single species or as a species complex leads to the conclusion that the Atlantic Forest is the most diverse biome, however the rank order of diversity among the remaining biomes changes depending on approaches to species delimitation. While the Amazon Rainforest is the least diverse when treating *C. siamense* as a single species, it is comparable in diversity to the Atlantic Forest ($q = 1$) or the most diverse biome ($q = 2$) when treating it as a species complex. Variation in the diversity rank among strata is also observed with geographical sampling sites and the divergence in diversity among host organs is similarly impacted by choices with respect to species delimitation. The seeming instability of results across strata reflects the fact that *C. siamense* treated as a single species is represented by individuals of 15 lineages (*C. communis*, *C. endomangiferae*, *C. dianesei* and 12 undetermined lineages), whereas its consideration as a species complex treats these lineages as independent species with lower relative abundance. These results suggest taking an inclusive view and delimiting species more broadly may mask important differences in community composition among sampling strata. When two communities are viewed as comparable in diversity, future studies may ignore continued sampling in one or the other expecting inferences drawn from one to be transferable to another. However, our results indicate that one may want to consider alternate delimitation scenarios before drawing conclusions and planning future sampling efforts.

Despite a multilocus dataset with the most informative loci available for the *C. gloeosporioides* complex, some lineages within the *C. siamense* species complex were not recovered. It is possible these lineages diverged recently and retain ancestral polymorphisms, indicative of incomplete lineage sorting (Carstens and Knowles, 2007; Choleva et al., 2014; Hudson, 1992; Stewart et al., 2014). However, recombination or hybridization is another potential source of conflicting phylogenetic signal (Crouch et al., 2006; Dettman et al., 2003; Stewart et al., 2013; Taylor et al., 2000). Our

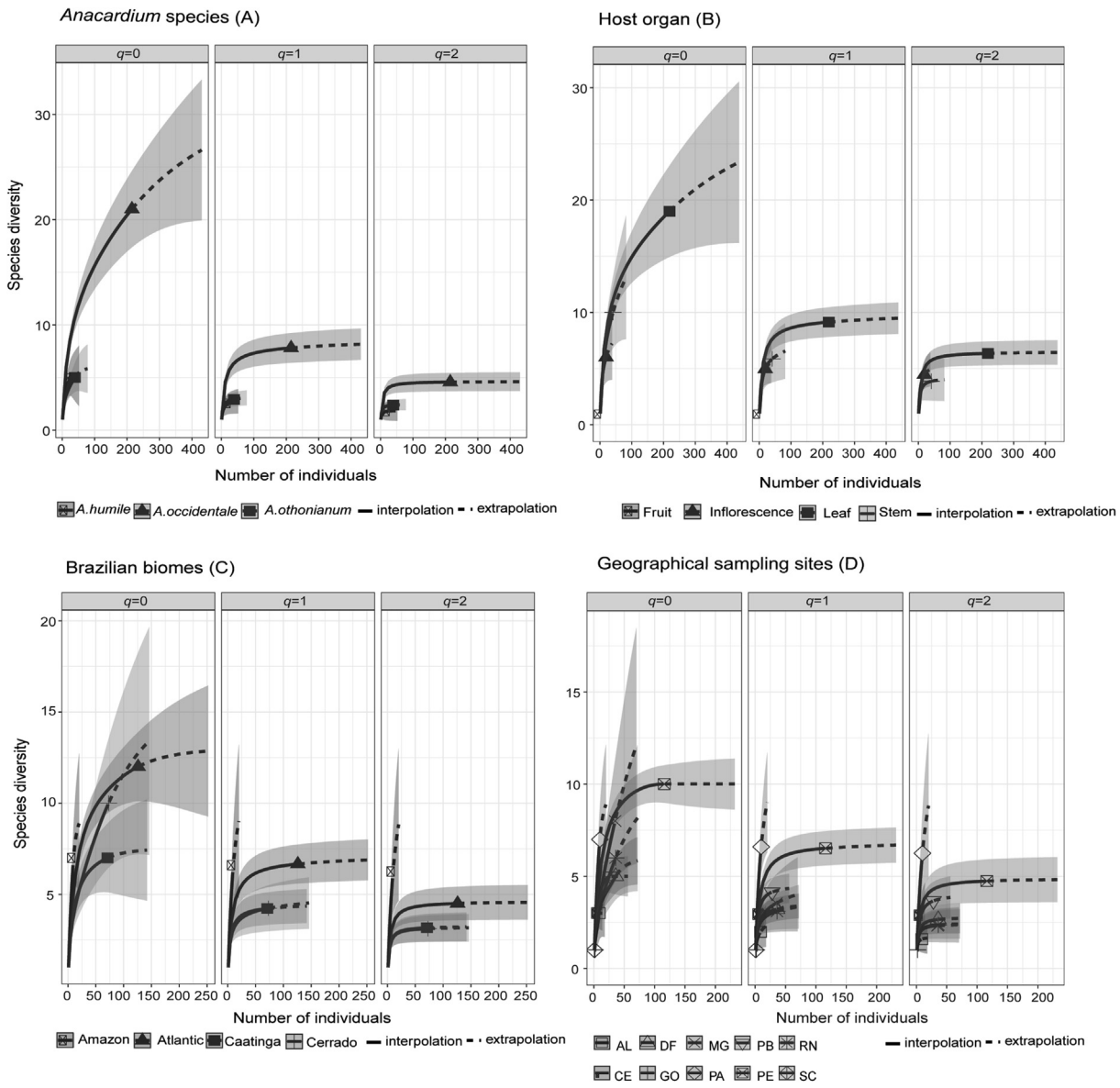

Fig. 4. Rarefaction (solid lines) and extrapolation (dashed lines) curves of *Colletotrichum* species diversity (considering *C. siamense* as a complex species) based on the Hill numbers (q = 0, 1, 2) for each *Anacardium* species, host organ, Brazilian biome and geographical sampling site. The 95% confidence intervals (gray-shaded regions) were obtained by a bootstrap method based on 200 replications. Reference samples are denoted by different symbols. AL = Alagoas, CE = Ceará, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, PA = Pará, PB = Paraíba, PE = Pernambuco, RN = Rio Grande do Norte, and SC = Santa Catarina.

findings are in agreement with other studies showing that incomplete lineage sorting and/or recombination may be the cause of significant incongruence among closely-related groups, such as *Fusarium oxysporum* (Laurence et al., 2014), *Alternaria alternata* (Stewart et al., 2014), and *C. siamense* (Liu et al., 2016). In addition to incomplete lineage sorting and recombination, secondary gene flow and hybridization may lead to misleading phylogenetic inferences (Crouch et al., 2006; Degnan and Rosenberg, 2009; Liu et al., 2016). However, it is very difficult to distinguishing the influence of these processes.

Gazis et al. (2011) highlighted the inadequacy of the internal transcribed spacer (ITS) for delimiting species and making biogeographical and ecological inferences for endophytic fungi, including *Colletotrichum*. Our results support their conclusions, but also highlight the impact that different choices about how to delimit species even in the presence of strong phylogenetic data can have on downstream inferences. Collectively, our results indicate that different approaches to species recognition may affect our understanding of how *Colletotrichum* communities are structured with respect to host, plant organ and geographical distribution. Given the global distribution of *Colletotrichum* and its prevalence as a plant pathogen, this has important implications for improving our understanding of the biology and life history of the genus.

The observed diversity of *Colletotrichum* species associated with cashew anthracnose in Brazil was not a surprise for two reasons. First, Brazil is a primary center of diversity for *Anacardium* spp. (Agostini-Costa et al., 2006; Barros et al., 2002) reflecting a long evolutionary history in this part of the world. This history allows for the potential coevolution of *Anacardium* with both beneficial and pathogenic organisms. Second, prior investigations related to cashew anthracnose reported lower levels of diversity presumably because their estimates were based on morphological characterization and/or phylogenetic analyses using few genes with limited

phylogenetic signal (Lopez and Lucas, 2010; Serra et al., 2011; Uaciquete et al., 2013). These reports had ascribed cashew anthracnose exclusively to *C. gloeosporioides*, but this species represented only 1.4 % of the 280 isolates evaluated in the present study, while *C. siamense* and *C. fructicola* were the most common species associated with anthracnose on cashew plants in Brazil. Our findings are consistent with other studies showing that *C. gloeosporioides s.s.* is not a common pathogen of tropical fruits (Phoulivong et al., 2010). Similarly, *C. fructicola* and *C. tropicale* were collected from symptomatic (Lima et al., 2013) and asymptomatic (Vieira et al., 2014) mango organs in northeastern Brazil, while *C. gloeosporioides s.s.* was neither isolated as a pathogen nor as an endophyte.

Most of the *Colletotrichum* species identified in the present study represent the first report on cashew, and it seems like their occurrence throughout multiple Brazilian biomes may be associated with their host distribution and environmental conditions. All species were found on *A. occidentale*, a host from which samples were taken across all sampled biomes. In contrast, six *Colletotrichum* species were associated with *A. humile* and *A. othonianum*, both of which are restricted to the Cerrado. While structural and chemical variation among *Anacardium* spp. may contribute to differences in diversity among host species, given that anthracnose incidence on cashew orchards correlates with rainfall (Freire et al., 2002; Uaciquete et al., 2013) we expect that environmental conditions also influences the distribution of *Colletotrichum* spp. across the sampled biomes. While precipitation is regular throughout the y ($mm \cdot y^{-1}$) in the Amazon Rainforest (generally > 2000 mm y^{-1} [Ronchail et al., 2002]) and the Atlantic Forest (ca. 1300–1900 mm y^{-1} [Forti et al., 2003]), distinct dry and wet seasons characterize the climatic conditions of the Caatinga and Cerrado (usually < 750 mm y^{-1} within three months [Leal et al., 2005; Prado, 2003], and 800–2000 mm y^{-1} within six to seven months [Pivello, 2011; Ratter et al., 1997], respectively). Our isolates were collected during the rainy season, which is coincident with peak growth and the highest incidence/severity of cashew anthracnose. *Colletotrichum* spp. are also influenced by temperature (Baroncelli et al., 2015; Fernando et al., 2000; Zhang et al. 2014), another environmental factor that may influence their geographical distribution. A recent epidemiological study revealed that the *Colletotrichum* species associated with cashew anthracnose identified in the present study displayed optimum mycelial growth and conidial germination at temperatures ranging between 25–30 °C and 27–37 °C, respectively (Veloso et al. in review). These temperatures are typical for tropical zones, which may explain the widespread occurrence of cashew anthracnose throughout Brazil.

The greatest diversity of *Colletorichum* spp. on *A. occidentale* may have been influenced by the fact that the largest proportion of sampling effort was concentrated on this host (as suggested by both interpolation and extrapolation curves in Fig. 3, but see Fig. 4), but it may also reflect its domestication and the cropping system in which most cashew orchards are cultivated in Brazil. The transition from natural habitats to agricultural environments may have reduced the bioactivity of some compounds originally used for chemical defense against plant pathogens. In addition, cashew orchards are typically cultivated with few or without managed cropping practices (Cardoso et al., 1999; Freire et al., 2002), meaning that *A. occidentale* plants are usually grown without artificial limiting forces such as chemical spraying. Also, Brazilian smallholder farmers commonly exploit multiple fruit species in the same orchard, an approach that may enhance cross-infections among *Colletotrichum* spp. on a variety of host plants. This may particularly be the case for *C. fructicola* and *C. tropicale* which are also reported among those responsible for anthracnose on mango fruits in northeastern Brazil (Lima et al., 2013). Likewise, a recent study revealed *C. fragariae*, *C. siamense* and *C. tropicale* among those associated with anthracnose on banana (Vieira et al., 2018), another common fruit cultivated on small farms and urban backyards in Brazil. These results suggest that anthropogenic activities may represent a major driving force impacting the diversity of *Colletotrichum* species in both wild and agricultural habitats.

Other phylogenetic studies have investigated the relationships among *Colletotrichum* species associated with cultivated and wild plants. Based on genealogical concordance phylogenetic species recognition (GCPSR) and a multilocus analysis involving four genes, Doyle et al. (2013) identified seven different species within *C. gloeosporioides s.l.* associated with five host species from wild and commercial cranberry bogs (*Vaccinium macrocarpon* Aiton) in North America. Four of them, namely *C. temperatum*, *C. melanocaulon*, *C. rhexiae* and *C. fructivorum*, were found on *V. macrocarpon*, the latter of which is the principle fruit-rot pathogen of cultivated cranberry but is also capable of infecting alternative hosts such as *Vaccinium oxycoccos* and *Rhexia virginica* in commercial cranberry bogs. A multilocus analysis was also applied by Udayanga et al. (2013) to identify which *Colletotrichum* species were associated with anthracnose on commercially available cultivated and wild fruits in northern Thailand. They demonstrated that *C. gloeosporioides s.s.* was associated with *Citrus aurantifolia* and *Syzygium samarangense*, while *C. fructicola* was isolated from *Hylocereus undatus* and *Ziziphus* sp., and *C. endophytica* from an unknown wild fruit. However, the most predominant species in wild fruit was *C. siamense*, which was also identified as a pre- and/or postharvest pathogen in *Coffea arabica*, *Annona reticulata*, *Ficus racemosa*, *Azadirachta indica*, *Carica papaya* and *Musa* sp. A comparative study performed with five *Colletotrichum* species collected from symptomatic mango fruits in northeastern Brazil revealed them as pathogenic to banana, guava, mango and papaya fruit, although varying in aggressiveness (Lima et al. 2015). These data are consistent with previous reports that a single *Colletotrichum* species may infect a broad range of hosts while a single host species may harbor several *Colletotrichum* species (Bragança et al. 2016; Freeman et al., 1998; Lima et al. 2015; Phoulivong et al. 2012). These findings highlight the importance of species delimitation in managing plant diseases. Knowing the etiological agent is essential for building on our collective knowledge about the life history, dispersal strategies, and other epidemiological attributes in order to develop informed and adequate management tactics against any plant disease. Different fungal species may show differential responses to management practices or may be carried by different vectors (Lima et al., 2015). This means the first step in dealing with cashew anthracnose, or other plant diseases, must be reliable species identification to develop precise and effective control measures.

The research presented here shows high levels of *Colletotrichum* diversity associated with cashew anthracnose in Brazil, including species previously described as endophytes and others responsible for pre- and/or postharvest diseases on other cultivated and wild plant species. Most of the *Colletotrichum* isolates associated with cashew anthracnose nested within *C. siamense*, which was revealed as the most common species. *Colletotrichum siamense* was collected across all four sampled Brazilian biomes, across all sampled host species, and across all sampled plant organs, which reflects its capacity to be a generalist pathogen. Phylogenetic studies involving *Colletotrichum* species from different hosts and geographical areas are crucial to better understand the host and geographical distribution in order to gain insight into the biotic and abiotic factors that have shaped their evolutionary history and diversification. Since the correct identification of the causal agent is essential to define effective and adequate disease control measures, the present study will contribute to improving the management of cashew anthracnose.

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Conflicts of interest

The authors declare they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funbio.2018.03.005.

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