Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*

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Abstract

Chrysoporthe cubensis is one of the most important pathogens of *Eucalyptus*. Based on phylogenetic evidence and geographic origin, isolates of this fungus are known to reside in distinct 'South America' and 'Southeast Asia' clades. In this study, reproductive isolation amongst these isolates of *C. cubensis* was tested using gene flow statistics for 12 polymorphic loci, and to support these data, phylogenetic affiliations based on gene trees and a multigene phylogeny were used. Gene flow statistics between populations, and relative to the closely related *Chrysoporthe austroafricana*, were low and not significantly different ($P < 0.05$). Additionally, phylogenetic analyses of DNA sequence data for four gene regions convincingly distinguished the two subclades of *C. cubensis*. Isolates in the Southeast Asian subclade are described in the new species, *Chrysoporthe deuterocubensis*. *Chrysoporthe cubensis* and *C. deuterocubensis* represent closely related fungi that are thought to be native to South America and Southeast Asia, respectively. A technique is presented that allows for rapid differentiation between these species and that will aid in quarantine procedures to limit their spread to new environments.

Introduction

Chrysoporthe cubensis causes a serious stem canker disease of *Eucalyptus* (Myrtaceae, Myrtales), commonly known as *Chrysoporthe* canker (Hodges 1980; Gryzenhout *et al*. 2009). Until 2004, *C. cubensis* and the closely related *Chrysoporthe austroafricana* were treated as *Cryphonectria cubensis* (Gryzenhout *et al*. 2004). Their recognition as distinct species in the new genus *Chrysoporthe*, was facilitated by DNA sequence-based phylogenetic analyses. Despite the fact that both of these species are associated with Myrtalean hosts, their geographic distributions do not overlap. *Chrysoporthe cubensis* is considered native to South and Central America and Southeast Asia, due to its association with native woody Melastomataceae (Myburg *et al*. 1999a; Roux *et al*. 2005; Nakabonge *et al*. 2006) such as *Miconia* and *Melastoma* species (Gryzenhout *et al*. 2009). In contrast, disease surveys on the African continent revealed that *Syzygium* species in the Myrtales (Heath *et al*. 2006; Nakabonge *et al*. 2006) are commonly infected by *C. austroafricana*, which suggest an African origin for this fungus (Gryzenhout *et al*. 2009).

Previous phylogenetic studies based on the rRNA internal transcribed spacer (ITS) regions, β-tubulin and histone H3 genes have consistently separated *C. cubensis* into two well-supported

clades (Myburg *et al*. 1999b; Myburg *et al*. 2002; Myburg *et al*. 2003; Gryzenhout *et al*. 2004; Myburg *et al*. 2004; Gryzenhout *et al*. 2006a; Gryzenhout *et al*. 2006c). One of these, referred to as the South American clade, accommodates isolates from countries in South and Central America, as well as likely introductions into western African countries such as Cameroon, Congo and the Democratic Republic of the Congo (Myburg *et al*. 2002; Myburg *et al*. 2003; Roux *et al*. 2003; Gryzenhout *et al*. 2006b). The second clade accommodates isolates from Southeast Asian countries such as Indonesia and Thailand, as well as likely introductions into Australia, China, Hawaii (Myburg *et al*. 2002; Myburg *et al*. 2003; Gryzenhout *et al*. 2006b), and several countries in Eastern Africa (Myburg *et al*. 2003; Gryzenhout *et al*. 2006b; Nakabonge *et al*. 2006). Although isolates in these clades have distinct and nonoverlapping geographic distributions (Gryzenhout *et al*. 2004), they all include native hosts in the Melastomataceae. Where they have been found on trees in the Myrtaceae such as eucalypts and clove (*Syzygium aromaticum*), these are considered to be host shifts (Slippers *et al*. 2005) either arising from planting these trees in areas where the fungus occurs on related native Myrtales, or through accidental introductions associated with agriculture and forestry (Wingfield 2003; Gryzenhout *et al*. 2009). There are also no obvious morphological characters that have been shown to distinguish specimens or isolates representing the two phylogenetic clades of *C. cubensis* (Gryzenhout *et al*. 2004).

So-called cryptic species, or species that are distinct but indistinguishable based on morphology, began to emerge when phylogenetic inference arose as an effective means to characterise fungal taxa (Taylor *et al*. 1999). Well-known examples of taxa harbouring cryptic species include *Coccidioides immitis* (Burt *et al*. 1996; Koufopanou *et al*. 2001), *Aspergillus flavus* (Geiser *et al*. 1998), *Aspergillus fumigatus* (Pringle *et al*. 2005), *Fusarium subglutinans* (Steenkamp *et al*. 2002), *Amanita muscaria* (Geml *et al*. 2006), *Neofusicoccum parvum* and *Neofusicoccum ribis* ([Pavlic *et al*. 2008; 2009). These species are mainly separated based on DNA sequence comparisons, and in some cases diagnostic morphological characters have later been found to support their separation (Geiser *et al*. 2000; Taylor *et al*. 2000; O'Donnell *et al*. 2004; Pavlic *et al*. 2008).

In addition to making use of phylogenetic species recognition (Hudson and Coyne 2002; De Queiros 2007), specifically the genealogical concordance version of this approach (Taylor *et al*. 2000), cryptic species can be separated based on low levels of interspecific gene flow (Taylor *et al*. 2000; Sites and Marshall 2003). This is because continuous admixture between disparately distributed populations can be detected from discordant genealogies for multiple genetic loci and/or low levels of population differentiation and high numbers of migrants (Geiser *et al*. 1998; Fisher *et al*. 2002; Zhou *et al*. 2007; Milgroom *et al*. 2008). Conversely, concordance among genealogies for multiple loci and diminished gene flow due to ecological, geographical or historical processes are generally regarded as useful indicators of species divergence (Avise and Wollenberg 1997; Barraclough and Nee 2001).

Chrysoporthe cubensis is an economically important fungal pathogen of substantial quarantine importance. *Chrysoporthe* canker has had a significant impact on one of the most important sources of paper pulp in the world, and has distinctly shaped *Eucalyptus* forestry globally (Wingfield 2003). Regulations to control its movement are frustrated by a vague taxonomic definition and the fact that very obvious phylogenetic differences amongst isolates are overlooked due to isolates residing under a single name. The aim of this study was, therefore, to gain a refined understanding of isolates residing in the two phylogenetic clades of *C. cubensis*. This was achieved using a population genetic approach based on polymorphic marker data to recognize distinct species as well as multigene phylogenetic inference to study relationships among isolates and species.

Materials and Methods

Isolates and DNA extraction

Eight isolates of *Chrysoporthe cubensis* representing the two phylogenetic clades, as well as representatives for the other known species of *Chrysoporthe* (Table 1), were used to construct gene genealogies and a multigene phylogeny. Isolates used for population genetic comparisons included 112 *C. cubensis* isolates obtained from *Eucalyptus* trees and specifically chosen to represent a wide geographic distribution encompassing the largest possible level of diversity. Of these, a total of 79 isolates potentially represented the South American clade and were obtained from Cuba (10), Colombia (34), Mexico (32), and the Democratic Republic of Congo (3). Populations from Southeast Asia (33 isolates) were represented by 16 isolates from Indonesia and 17 from Vietnam. For comparative purposes, the isolates used for the population genetics analyses included a population of 97 *Chrysoporthe austroafricana* isolates from South and Eastern Africa and included those from *Eucalyptus* sp. in Zambia (5), Mozambique (10) and South Africa (29), *Syzygium* sp. in Mozambique (12) and South Africa (26), and *Tibouchina* sp. in South Africa (15). All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Table 1. Isolates of *Chrysoporthe* sp. used in the multigene phylogenetic studies, and the GenBank accession numbers for the sequences included in phylogenetic analyses.

Taxon	Origin	Isolate number[a]	ACT	BT1	BT2	EF-1α	ITS
Chrysoporthe austroafricana	South Africa	CMW9327	GQ290173	GQ290185	GQ290194	GQ290151	GQ290158
		CMW10192	GQ290163	GQ290176	GQ290187	GQ290138	AY214299
Chrysoporthe cubensis	Colombia	CMW10028	GQ290161	GQ290175	GQ290186	GQ290137	GQ290153
	Republic of Congo	CMW10669	GQ290171	GQ290177	GQ290188	GQ290140	GQ290154
	Brazil	CMW10778	GQ290165	GQ290178	GQ290189	GQ290141	GQ290155
	Mexico	CMW12734	GQ290159	DQ368791	GQ290191	GQ290146	DQ368769
Chrysoporthe deuterocubensis	Australia	CMW2631	GQ290174	GQ290184	AF543825	GQ290149	GQ290157
	Indonesia	CMW8650	GQ290172	AY084024	GQ290193	GQ290150	AY084001
	Singapore	CMW12745	GQ290160	GQ290183	DQ368781	GQ290147	DQ368764
	Thailand	CMW17178	GQ290164	DQ368785	GQ290192	GQ290148	DQ368766
Chrysoporthe doradensis	Ecuador	CMW11287	GQ290167	GQ290179	GQ290190	GQ290142	GQ290156
Chrysoporthella hodgesiana	Colombia	CMW9995	GQ290162	AY956978	AY956977	GQ290152	AY956969
		CMW10625	GQ290170	AY262391	AY956979	GQ290139	AY262399
Chrysoporthe inopina		CMW12727	GQ290169	GQ290180	DQ368805	GQ290143	DQ368777
		CMW12731	GQ290168	GQ290182	DQ368811	GQ290145	DQ368779
Amphilogia gyrosa[b]	Taiwan	BCRC34145	EF025600	EF025615	EF025615	–	EF026147

a CMW – culture collection of the FABI, University of Pretoria, Pretoria, South Africa; BCRC – Bioresource Collection and Research Center, Taiwan.

b *Amphilogia gyrosa* was used as an outgroup taxon (Ju *et al*. 2007).

Isolates were grown on 20 % w/v malt extract agar or inoculated into 800 µl malt extract broth in 1.5 ml microcentrifuge tubes. After 1 week of growth in the dark at 25 °C, fungal mycelium was harvested. Total genomic DNA was extracted using a previously published method based on hexadecyltrimethylammonium bromide (CTAB) and standard phenol–chloroform extractions (Steenkamp *et al*. 1999).

Phylogenetic analyses

Polymerase chain reactions (PCR) were used to amplify rRNA ITS and the intron or noncoding regions of the Actin (ACT), β-tubulin (Bt1 and Bt2 primer sets), and eukaryotic translation elongation factor 1-α (EF-1α) genes (White *et al*. 1990; Glass and Donaldson 1995; Carbone and Kohn 1999) for phylogenetic comparisons using a subset of isolates. Each PCR reaction contained 0.1 U SuperTherm Taq DNA polymerase enzyme (Southern Cross Biotechnology, South Africa), 25

mM $MgCl_2$, 2 µM of each primer, 200 µM of each dNTP, 25 ng genomic DNA and 1.5 µl 10× PCR buffer. Reaction volumes were adjusted to 15 µl using sterile deionised water. Reactions were performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, USA) with the cycling protocol described by Glass & Donaldson (1995). Reaction annealing temperatures were 55 °C for the ITS and EF-1α loci, and 62 °C for the ACT locus and two regions of the β-tubulin gene (BT1 and BT2). Amplicon sizes were visually confirmed using agarose gel (2 % w/v) electrophoresis, after which the PCR products were purified using polyethylene glycol precipitation (Steenkamp *et al*. 2006). PCR products were sequenced using BigDye® dye terminator chemistry (Applied Biosystems, USA) and an ABI™ Prism® 3500 automated sequencing machine (Applied Biosystems).

DNA sequences for each locus were aligned using Muscle 3.6 (Edgar 2004) and manually adjusted using SeaView 2.2 (Galtier *et al*. 1996). The alignments were amended with ACT, β-tubulin and ITS sequences from the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) nucleotide database for a closely related taxon, *Amphilogia gyrosa*, to serve as outgroup (Ju *et al*. 2007). The incongruence length difference (ILD) test (Farris *et al*. 1995; Cunningham 1997) implemented in PAUP* 4.0b10 (Swofford 2002), was used to test whether the resulting alignments represent homogenous partitions. In order to test for phylogenetic signal, the g_1 statistic (Hillis & Huelsenbeck 1992) for each data set was calculated using parsimony methods in PAUP* 4.0b10. Individual and combined gene alignments were subjected to maximum likelihood (ML) analyses using PhyML 2.4.5 (Guindon & Gascuel 2003), incorporating the GTR + G + I model of evolution as determined by jModelTest 0.1.1 (Posada 2008). The confidence in branches was tested using 1000 bootstrap replicates for each analysis. Phylogenic trees were displayed and annotated using MEGA4 (Tamura *et al*. 2007).

Population genetic analyses

In order to determine whether populations of isolates representing *Chrysoporthe austroafricana* and the two clades of *Chrysoporthe cubensis* were significantly different from each other, population differentiation and gene flow between these species were estimated. For this purpose 12 microsatellite markers (Table 2) were used following previously published methods (van der Merwe *et al*. 2003).

Table 2. Primers for polymorphic DNA markers used in this study.

Locus name[a]	Dye label	Bin size (bp)	Primer name	Primer sequence (5′–3′)
CcPMC	VIC	190–212	PMCF	ttgcgtatggaaatgacg
			PMCR	atggcgcttgtatagagca
CcPMG	6-FAM	197–297	PMGF	tgattcacgtctattgccac
			PMGR	gttaagtctctcggtgaatcg
COL6	6-FAM	260–270	COL6F	ggccagggcagaggtaaggcag
			COL6R	gctagagagtcaacatgatgtg
COL7	VIC	173–174	COL7F	gaaccccgactacgtgattatc
			COL7R	tggcactatatcaccatcactg
COL11	VIC	258–267	COL11F	ctcatgggtcctgcatgcgac
			COL11R	gtggcactaccagaacatacag
SA1	NED	300–320	SA1F	ggaatcaccaccactagcgtcc
			SA1R	gtgtctccgttaacgcagtggt
SA3	6-FAM	200–215	SA3F	tcaccaccactggcgtccagac
			SA3R	tcgttatcttggtgactgtaga
SA4	PET	150–200	SA4F	cagagcatgagatgaatagatg
			SA4R	agtcaggctcttcacgctctgt
SA6[b]	PET	209–221, 316–365	SA6F	atcgacgatcaggttctggatc
			SA6R	tattgcggtaacccaattttcg
SA9[b]	NED	190–200, 203–215	SA9F	gctcgggctgccaatccttaag
			SA9R	cgccgagtttctcgccaccatc

a Loci in bold were published in van der Merwe et al. (2003). CcPMC and CcPMG were developed according to previously described methods (van der Merwe et al. 2003).

b Primer pairs for markers SA6 and SA9 each amplify two polymorphic loci.

The computer programme MultiLocus 1.3b (Agapow & Burt 2001) was used for all allelic analyses. The population differentiation (θ) (Weir & Cockerham 1984) and theoretical number of migrants per generation $\widehat{M} = 1/2(\frac{1}{\theta} - 1)$ (Slatkin 1995) were estimated between a population of *C. cubensis* isolates from South America, from which the type of *C. cubensis* originates (Bruner 1917), and a population of the known species *C. austroafricana*. This was used as the expected statistic in further analyses, since the species represented by these populations are well defined. Pair wise θ-values were then calculated in all combinations between isolates representing the two clades of *C. cubensis* and *C. austroafricana*.

The significance of equality or positive deviation from the expected differentiation value was determined using a one-tailed G-test (Sokal and Rohlf 1994), which is the ML statistical significance of deviation,

$$G = 2\sum_{i} O_i \cdot \ln\left(\frac{O_i}{E_i}\right)$$

where G is the ML estimator, O is the observed value, and E is the expected value. The distribution of G is approximately that of χ^2, with the same degrees of freedom. Thus, if isolates representing the Southeast Asian clade of *C. cubensis* represent a distinct species, they should display population differentiation values that are equal to or higher than those obtained between the populations representing *C. cubensis* from South America and *C. austroafricana*. However, if G-test values were significantly lower than expected, the null hypothesis of equal or higher levels of differentiation could be rejected.

Population differentiation was further explored using the programme Structure 2.3.1 (Pritchard *et al*. 2000). Allelic data were subjected to an assignment test with the origin of an isolate dictating the prior to the Markov Chain analysis. Therefore, three populations, i.e., *C. austroafricana* and the two clades of *C. cubensis*, were used as priors. The run length was 1 100 000 with a burnin of 100 000 iterations. After analysis, the assignments were visualized using a bar plot constrained by the population priors, and a triangle plot to visualize possible admixture between populations. These plots depicted the estimated membership coefficients for each individual to each population prior (q) in two different ways (Pritchard *et al*. 2000). In the bar plot, each individual was represented by a vertical bar partitioned into K population priors that indicated the estimated membership of that individual to each prior. The triangle plot depicted Q, the probability of an individual's ancestry from population prior q, where each individual was indicated with a dot and the distance of the dot from each of the triangle's edges was proportionate to the ancestry vectors for the individual. Therefore, each of the individuals in this analysis would have had K = 3 ancestry vectors adding up to 1.

Morphology

In order to characterise and compare the morphology of representative specimens of the two clades of *Chrysoporthe cubensis*, dried herbarium specimens of *C. cubensis sensu lato* bearing fruiting structures (Gryzenhout *et al*. 2004) were reexamined microscopically. Some of these specimens are linked to isolates in the two subclades (Gryzenhout *et al*. 2004). Fruiting structures were mounted in Leica mountant (Setpoint Premier, South Africa) and sectioned at 12–16 µm using a Leica CM1100 cryostat (Setpoint Premier). Sections were mounted in lactophenol and examined using light microscopy and the measurement software Axiovision 4.8 (Carl Zeiss, GmbH). Fifty asci, ascospores, conidiophores and conidia were measured for each specimen, and a range was obtained for ascostromata and conidiomata. Measurements were represented as (minimum–) (mean–SD) (mean + SD) (–maximum) where SD is the standard deviation.

Results

Phylogenetic analyses

After sequencing and alignment of four gene regions from each isolate (Table 1), alignment lengths ranged from 273 bp for ACT to 830 bp for the two β-tubulin regions. The total alignment length when gene regions were combined was 1914 characters. These alignments are available in TreeBase (SN4622).

Sequence alignments revealed 13 fixed nucleotide polymorphisms across all gene regions that differentiate isolates representing the Southeast Asian clade of *Chrysoporthe cubensis* from the South American clade and *Chrysoporthe austroafricana* (Table 3). Three nucleotide polymorphisms in the β-tubulin gene differentiated Southeast Asian *C. cubensis* from the others, while three polymorphisms across the four genes were diagnostic for South American *C. cubensis*. Similarly, six polymorphisms across the four genes were characteristic of *C. austroafricana*. Therefore, these fixed nucleotide differences are diagnostic for the different species, either in combination or singly in the case of private polymorphisms.

Table 3. Nucleotide polymorphisms[a] associated with *C. austroafricana*, *C. cubensis* (South American clade) and *C. deuterocubensis* (Southeast Asian clade).

Species	Isolate	ACT	β-tubulin										EF-1α	ITS			
			*													*	
						1	1	1	1	1	1	1		2	2	2	2
		4	5	6	7	4	4	4	4	4	5	5	4	1	1	2	2
		7	5	9	2	7	8	9	9	9	5	7	1	0	7	0	1
		5	0	9	9	7	8	0	3	9	4	2	8	7	8	5	0
C. austroafricana	CMW9327	G	T	C	C	C	G	C	C	C	C	C	A	T	A	–	A
	CMW10192	G	T	C	C	C	G	C	C	C	C	C	A	T	A	–	A
C. cubensis	CMW10028	A	T	C	C	T	A	A	A	T	T	T	C	C	A	–	A
	CMW10669	A	T	C	C	T	A	A	A	T	T	T	C	C	A	–	A
	CMW10778	A	T	C	C	T	A	A	A	T	T	T	C	C	A	–	A
	CMW12734	A	T	C	C	T	A	A	A	T	T	T	C	C	A	–	A
C. deuterocubensis	CMW2631	G	C	T	T	C	G	A	A	T	T	C	C	C	G	T	G
	CMW8650	G	C	T	T	C	G	A	A	T	T	C	C	C	G	T	G
	CMW12745	G	C	T	T	C	G	A	A	T	T	C	C	C	G	T	G
	CMW17178	G	C	T	T	C	G	A	A	T	T	C	C	C	G	T	G
Unfixed polymorphisms		2	2										5	11			

a Nucleotide positions are relative to the start codon of the corresponding gene from *Neurospora crassa* or (*) alignment positions due to a corresponding gap in the *N. crassa* sequence. Shaded nucleotides differentiate *C. deuterocubensis* (Southeast Asian clade) from *C. austroafricana* and *C. cubensis* (South American clade), while nucleotides in bold were exploited in a PCR–RFLP diagnostic technique.

A partition homogeneity test revealed that all the DNA regions used in this study could be combined (P = 0.001) (Cummings *et al*. 1995). Inspection of the g1 statistic for each of the four gene regions, as well as the combined data set, revealed that all data sets contained useful phylogenetic signal (P = 0.01) (Fig 1). ML analysis of individual gene regions mostly recovered the two clades of *C. cubensis* as separate (Fig 1). Southeast Asian *C. cubensis* isolates formed a separate clade in the β-tubulin and ITS gene genealogies (Fig 1B and D). However, in the EF-1α genealogy, the Southeast Asian and South American *C. cubensis* were difficult to distinguish because they were present in the same clade with no bootstrap-supported partitions (Fig 1C). The ACT genealogy (Fig 1A) did not distinguish between Southeast Asian *C. cubensis* and *Chrysoporthe inopina*. ML analysis of the combined information for the four regions sequenced (Fig 1E) recovered two well-supported and separate clades for the Southeast Asian and South American *C. cubensis* isolates. Using these analyses, isolates representing the South American clade of *C. cubensis* were more closely related to *C. austroafricana* than to isolates in the Southeast Asian clade.

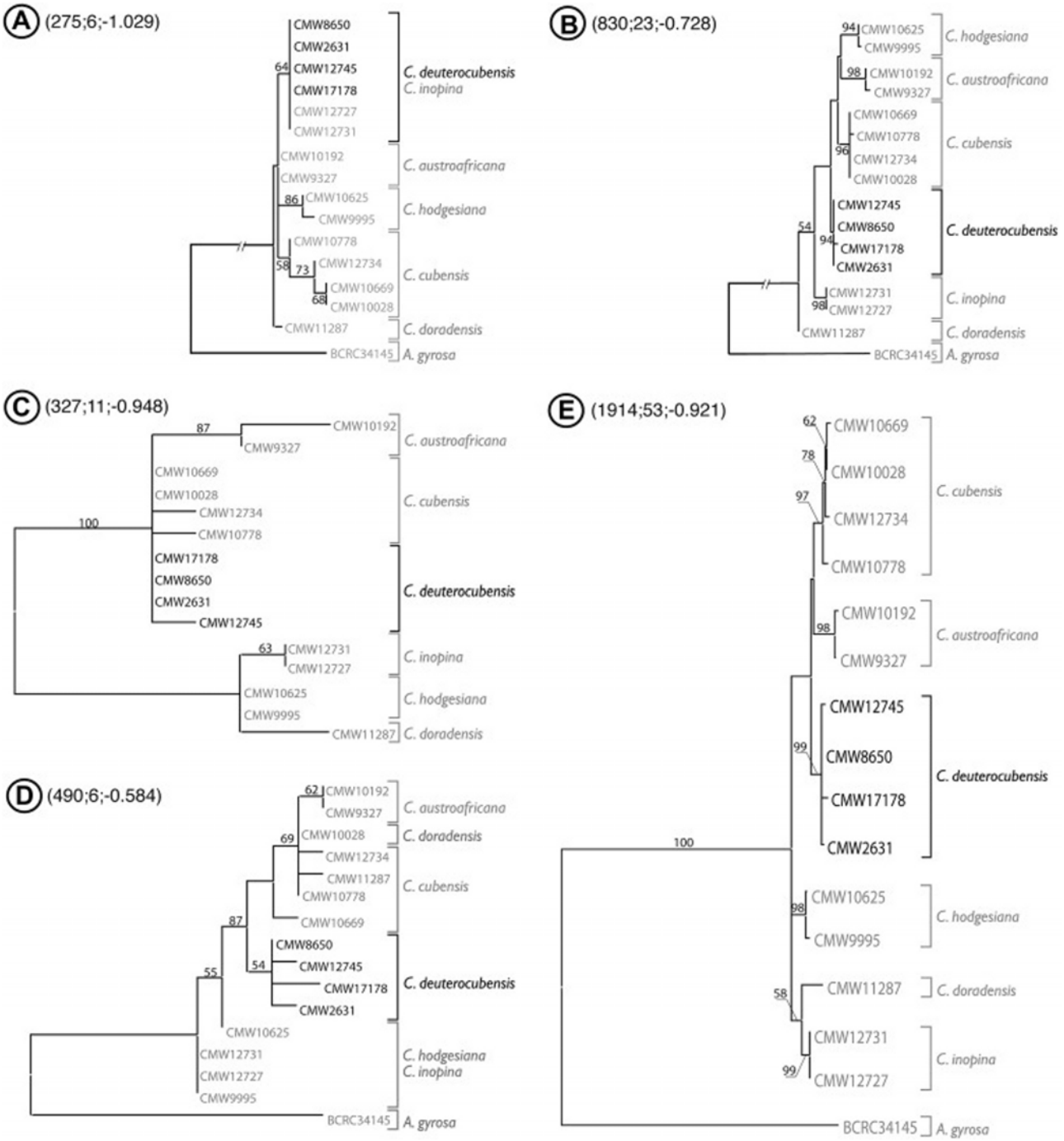

Fig 1. ML phylogenies obtained from (A) ACT, (B) β-tubulin (BT1 and BT2 regions), (C) elongation factor 1-α, and (D) ITS gene sequences. (E) The multilocus phylogeny when all genes were combined and analyzed with ML (C) was midpoint rooted, while the other phylogenies were rooted with *Amphilogia gyrosa*. Bootstrap values above 50 % (1000 replicates) are indicated above each branch. For each genealogy, relevant tree statistics are indicated in parentheses in the order: alignment length in base pairs; number of parsimony-informative characters; g1 statistic (Hillis & Huelsenbeck 1992) at a significance level of P = 0.01.

Population differentiation analysis

Differentiation between *Chrysoporthe austroafricana* and South American *Chrysoporthe cubensis* populations (i.e., the expected level of differentiation between two distinct species), was 0.30 (Fig 2C). Analyses using a G-test showed that there were no significant differences ($P < 0.05$) in the levels of differentiation among the three populations. It was thus possible to reject the null hypothesis that these populations are not significantly different, because the theoretical number of

migrants per generation ($\widehat{M}$; calculated from the θ value) between the different populations were comparable and similar

levels of differentiation were observed among them. Similarly, the results of population assignment tests suggested that populations of *C. austroafricana* and South American and Southeast Asian *C. cubensis* can be readily separated (Fig 2A and B). These data highlighted the fact that the three populations were each characterized by markedly different allelic compositions, although a low level of admixture was detected (Fig 2A, Table 4). However, the genetic distance between the two populations of *C. cubensis* was comparable to those between the *C. austroafricana* population and the respective *C. cubensis* populations (Fig 2B). This was evident from the reciprocal presence of alleles and nearly identical ancestry vectors for all three populations.

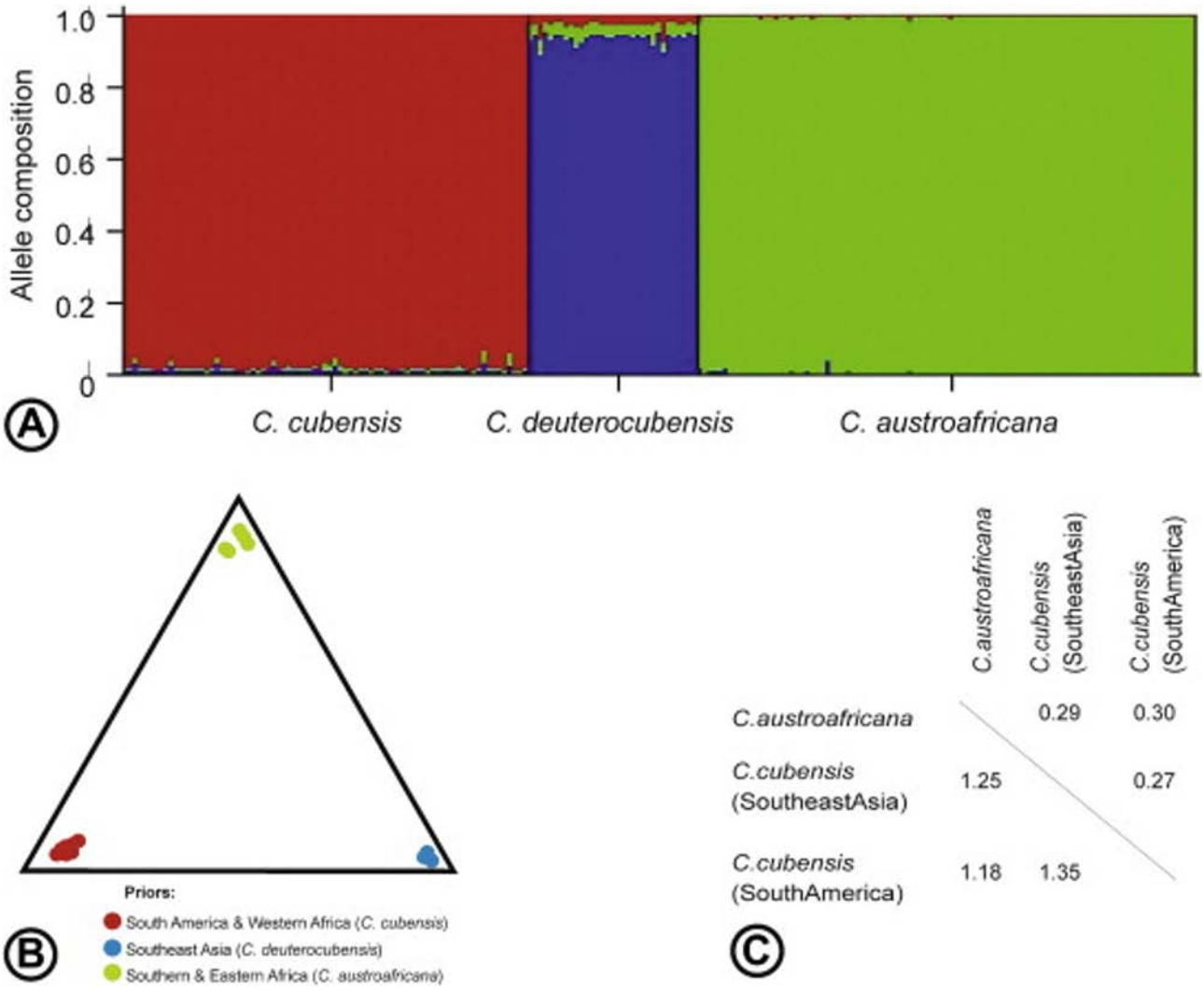

Fig 2. Population assignment and gene flow statistics for the *Chrysoporthe austroafricana* (green; southern and western Africa), *C. cubensis* (red; South America) and *C. deuterocubensis* (blue; Southeast Asia) genotypes observed in this study. (A) Visualization of population assignment tests for allelic data and (B) a bar plot displaying individuals (X-axis) and the percentage of alleles from each of the priors that each individual possesses (Y-axis), as revealed by structure. (C) Population differentiation values (θ) and the estimated numbers of migrants per generation View the MathML source appear above and below the diagonal, respectively, and show the general absence of genotype admixture between species.

Table 4. Allele frequency distributions expressed as Nei's gene (allele) diversity (Nei 1973) per locus and per species, in representative populations of *Chrysoporthe austroafricana*, *C. cubensis* and *C. deuterocubensis*.

	Number of isolates	CcPMC	CcPMG	COL6	COL7	COL11	SA1	SA3	SA4	SA6-1	SA6-2	SA9-1	SA9-2	$\overline{H}$[a]
Chrysoporthe austroafricana	97	0.7782	0.2368	0.5029	0.0204	0.3119	0.3700	0.7397	0.6862	0.2610	0	0.1911	0.6791	0.3981
Chrysoporthe cubensis	79	0.7713	0.0496	0.5435	0	0.1647	0.0759	0.4995	0.5188	0.7152	0.0251	0.2279	0.4619	0.3379
Chrysoporthe deuterocubensis	33	0.3177	0.1140	0.7907	0.2187	0.3801	0.5933	0.5951	0.4115	0.7400	0	0.4628	0.5841	0.4340

a $\overline{H}$ is the average gene (allele) diversity for a population over all loci.

Taxonomy

Phylogenetic and population genetic analyses in this study have provided robust justification to treat the Southeast Asian and South American isolates of *Chrysoporthe cubensis* as distinct taxa. *Chrysoporthe cubensis* was first described from Cuba (Bruner 1917) and this name should be reserved for South American isolates related to those from Cuba. Gryzenhout *et al*. (2006a) designated an epitype for *C. cubensis* based on an isolate from Cuba and residing in the South American clade of the fungus. Isolates representing the Southeast Asian clade represent a distinct taxon described as follows:

Chrysoporthe deuterocubensis Gryzenh & M.J. Wingf., sp. nov. MycoBank No.: MB516634 Fig 3.

Etymology: The name reflects the fact that the fungus is different yet closely related to *Chrysoporthe cubensis*.

Ascospores (5.5–)6.5–7.5(–8) × 2–2.5(–3) µm; conidiomata subaurantiaca, brunnea Siennae vel atrofusca, pyriformia, clavata vel pulvinata; conidia (3–)3.5–4.5(–5) × (1.5)2(–2.5) µm; position actinis 475 (G, A); positions β-tubulinis 546 (C, T), 699 (T, C) 729 (T, C), 1477 (C, T), 1488 (G, A), 1572 (C, T) (TreeBase SN4622); sitibus exceptionis pro AvaI, fragmenta 87 bp, 113 bp, et 337 bp ferentibus, et uno pro HindIII fragmenta 206 bp et 331 bp ferenti.

Ascospores (5.5–)6.5–7.5(–8) × 2–2.5(–3) µm; conidiomata sienna to almost orange to fuscous-black, pyriform to clavate to pulvinate; conidia (3–)3.5–4.5(–5) × (1.5–)2(–2.5) µm; ACT position 475 (G, A); β-tubulin positions 546 (C, T), 699 (T, C) 729 (T, C), 1477 (C, T), 1488 (G, A), 1572 (C, T) (TreeBase SN4622). Two restriction sites for AvaI, yielding fragments of 87 bp, 113 bp, and 337 bp, and one restriction site for HindIII, yielding fragments of 206 bp and 331 bp.

Ascostromata semiimmersed erumpent, top of perithecial bases covered with cinnamon to orange, predominantly prosenchymatous, limited stromatic tissue, ascostroma 120–230 µm high above level of bark, 280–490 µm diam., perithecia valsoid, bases immersed in bark, fuscous-black, extending necks up to 240 µm long emerging through bark covered in umber stromatic tissue of textura porrecta, appearing fuscous-black. Asci (19–)22–26.5(–28) × (4.5–)5–6.5(–7) µm, fusoid to ellipsoidal, 8-spored. Ascospores (5.5–)6.5–7.5(–8) × 2–2.5(–3) µm, hyaline, 1-septate, fusoid to oval, ends tapered, with septum variously placed in the spore but usually central.

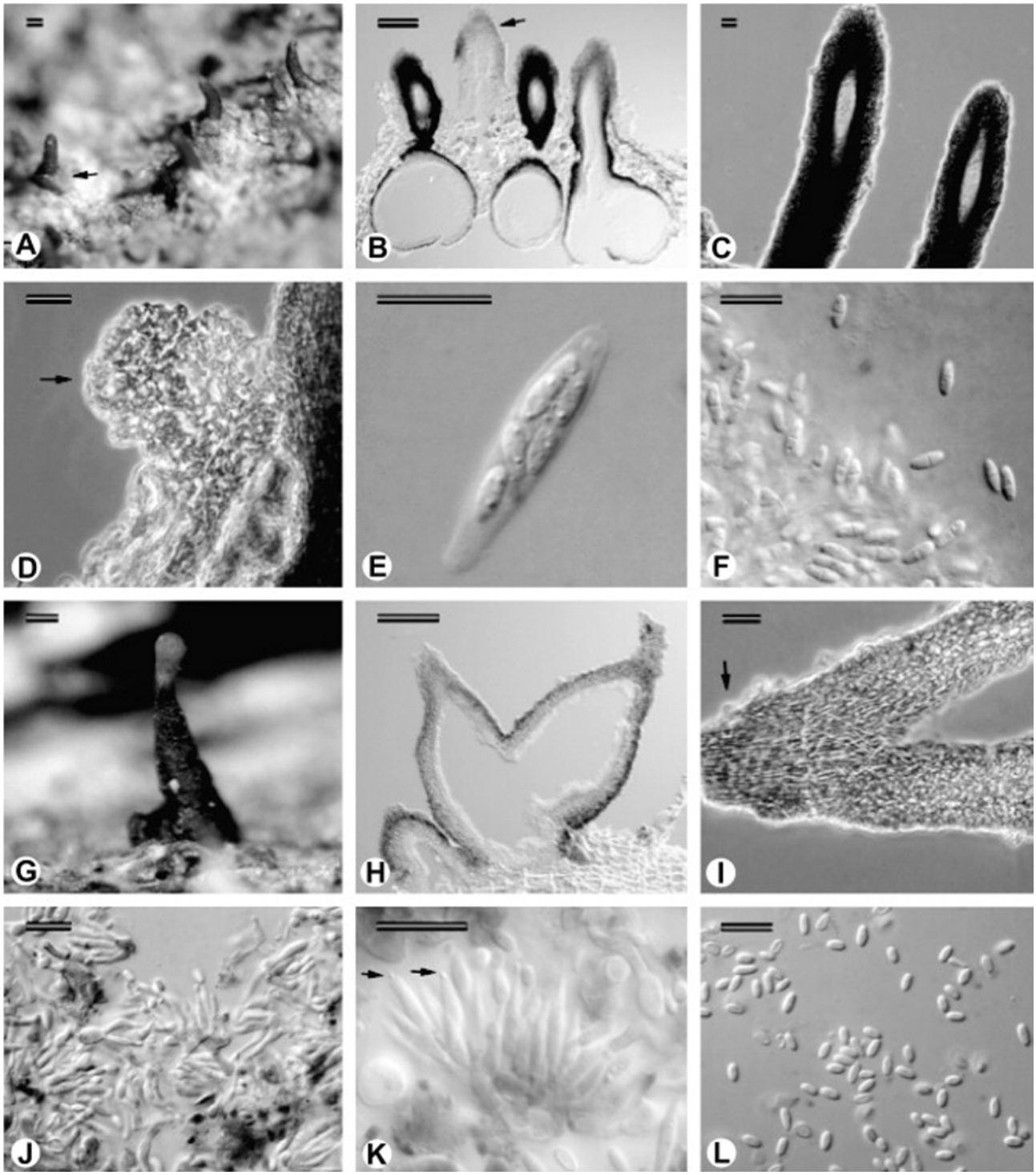

Fig 3. Fruiting structures of *Chrysoporthe deuterocubensis*. (A) Ascostromata on bark (arrow indicates stromatic tissue). (B) Longitudinal section through ascostroma. (C) Black perithecial necks covered with stromatic tissue. (D) Prosenchymatous stromatic tissue (arrow) of ascostroma. (E) Ascus. (F) Ascospores. (G) Conidioma on bark. (H) Vertical section through conidiomata. (I) Tissue of textura globulosa for the conidiomal base and of textura porrecta for the neck (arrow). (J–K) Conidiophores. (L) Conidia. Scale bars A–B, G–H = 100 μm; C–D, I = 20 μm; E–F, J–L = 10 μm.

Conidiomata occurring on the surface of the ascostroma or as separate structures, superficial to slightly immersed, sienna to almost orange to fuscous-black, with an umber interior when young, pyriform to clavate, sometimes pulvinate, with one to four attenuated necks per structure, conidiomatal base above the bark surface 130–740 μm high, 100–950 μm diam, necks up to 230 μm long, 90–240 μm wide. Conidiomatal locules with even to convoluted inner surface, occasionally

multilocular, single locule connected to one or several necks. Stromatic tissue at base of textura globulosa with walls of outer cells thickened, neck cells of textura porrecta. Conidiophores hyaline, with globose to rectangular basal cells that are (2.5–)4–7(–8.5) × (2–)3–4.5(–5.5) µm, branched irregularly at the base or above into cylindrical cells, cells delimited by septa or not, total length of conidiophore (12–)13.5–19(–24.5) µm, conidiogenous cells cylindrical to flask-shaped with attenuated apices, (1.5–)2–2.5(–3) µm wide. Conidia (3–)3.5–4.5(–5) × (1.5–)2(–2.5) µm, hyaline, oblong, aseptate, exuded as bright luteous tendrils or droplets.

Cultures white with cinnamon to hazel patches on malt extract agar, fluffy, margin smooth, fast-growing, covering a 90 mm diam plate after a minimum of 5 d at the optimum temperature of 30 °C (Gryzenhout *et al*. 2004).

The following nucleotide characters are differentially fixed for *C. deuterocubensis* (given as the gene name, the nucleotide position relative to the start codon of the corresponding aligned gene for *Neurospora crassa*, and in parentheses, the nucleotides fixed for *C. deuterocubensis* and *C. cubensis*, respectively): ACT position 475 (G, A); β-tubulin positions 546 (C, T), 699 (T, C) 729 (T, C), 1477 (C, T), 1488 (G, A), 1572 (C, T) (TreeBase SN4622). Additionally, the Bt1 region of the β-tubulin gene of *C. deuterocubensis*, amplified using primers Bt1a and Bt1b (Glass & Donaldson 1995), contains two restriction sites for AvaI, yielding fragments of 87 bp, 113 bp, and 337 bp, and one restriction site for HindIII, yielding fragments of 206 bp and 331 bp.

Specimens examined

INDONESIA, Sumatra, Lake Toba, *Melastoma malabathricum*, May 2005, M.J. Wingfield, holotype PREM 58799, living extype culture CMW 18515 = CBS 118651 shown to group in the Southeast Asian clade (Gryzenhout et al. 2006a), Lake Toba, Aek Nauli, *M. malabathricum*, Feb. 2004, M.J. Wingfield, PREM 58798, living culture CMW 16192 = CBS 119474; Sulawesi, *Syzygium aromaticum*, 2001, M.J. Wingfield, PREM 57470, cultures CMW 8650 = CBS 115719, CMW 8651 = CBS 115718; Sulawesi, *S. aromaticum*, 2003, M.J. Wingfield, PREM 58018, PREM 58019; Sulawesi, Utard, *S. aromaticum*, 2003, M.J. Wingfield, PREM 58020; Bankals, Selindung, *Eugenia* sp., C.P.A. Bennett, IMI 231648; Sumatra, Kurai, Taji, *Eugenia* sp., C.P.A. Bennett, IMI 231649; Sumatra, *Eucalyptus* sp., 2001, M.J. Wingfield, PREM 57297, cultures from the same area CMW 11288 = CBS 115736, CMW 11289 = CBS 115737, CMW 11290 = CBS 115738; Sumatra, Sei Kabaro, *Eucalyptus* sp., 2001, M.J. Wingfield, PREM 58021, cultures from same area CMW 11289, CMW 11290. MALAYSIA, Johar, Kluang, *Eucalyptus aromatica*, 1986, Loh Chow Fong, IMI 304273; Serdang, Fe. Exp. Stn., *S. aromaticum* (as *Eugenia caryophyllata*), 1954, A. Johnston, IMI 58569; *Eugenia* sp., 1954, A. Johnston, IMI 58567, IMI 58568; Jelok Bahang, *S. aromaticum* (as *E. caryophyllata*), 1954, A. Johnston, IMI 58388. SINGAPORE, Istana grounds, *S. aromaticum*, 1991, C.P. Yik, dried culture IMI 350626; *Tibouchina urvilleana*, April 2003, M.J. Wingfield, PREM 58797, living culture CMW 12745 = CBS 117837.

Distribution

Countries where the identity has been confirmed based on DNA sequence comparisons: U.S.A. (Hawaii), Tanzania (Zanzibar), Kenya, Malawi, Mozambique, Indonesia, Singapore, Thailand, China (Hong Kong-ITS only), Australia (Myburg *et al*. 1999a; Myburg *et al*. 2003; Roux *et al*. 2003; Gryzenhout *et al*. 2004; Gryzenhout *et al*. 2006b; Nakabonge *et al*. 2006). Isolates from Vietnam, although previously reported as *C. cubensis* (Old *et al*. 2003), are also shown for the first time to represent *C. deuterocubensis* based on DNA sequence data. *Chrysoporthe cubensis sensu lato* reported from India, Malaysia and Western Samoa (Hodges *et al*. 1979; Sharma *et al*. 1985; Hodges *et al*. 1986; Old *et al*. 2003) most likely also reside in *C. deuterocubensis* although sequence data for these isolates are not available.

Restriction enzyme-based DNA diagnostic

To facilitate routine differentiation among *Chrysoporthe cubensis*, *Chrysoporthe deuterocubensis* and *Chrysoporthe austroafricana*, the β-tubulin Bt1 region was subjected to PCR–RFLP (restriction fragment length polymorphism) analysis. For this purpose we used two restriction enzymes AvaI and HindIII. Separate digests with these enzymes revealed that *C. austroafricana*, *C. cubensis* and *C. deuterocubensis* could easily be distinguished from each other (Fig 4). When AvaI was used, three bands (87 bp, 113 bp and 337 bp) were observed for *C. austroafricana* and *C. deuterocubensis*, while two bands (87 bp and 440 bp) were observed for *C. cubensis*. Therefore, this enzyme could distinguish *C. cubensis* from *C. austroafricana* and *C. deuterocubensis*. In contrast, HindIII did not cut for *C. austroafricana* but produced two fragments (206 bp and 331 bp) for each of the other two species. Therefore, HindIII could distinguish *C. austroafricana* from *C. cubensis* and *C. deuterocubensis*.

In order to test the robustness of this new identification technique, 400 putative isolates of *C. austroafricana*, *C. cubensis* and *C. deuterocubensis* were obtained from the CMW collection at FABI (Table 5). Isolates were randomized, renumbered and subjected to a blind test using the β-tubulin PCR–RFLP technique. After obtaining the restriction profile for each isolate, it was given a putative species name and compared to the place of origin recorded for that isolate number in the culture collection. All isolates from South Africa were identified as *C. austroafricana*, while *C. cubensis* isolates originated from South America and *C. deuterocubensis* isolates originated from Southeast Asia.

Discussion

Results of this study have shown that isolates representing the South American and Southeast Asian clades of *Chrysoporthe cubensis* represent distinct species. Those residing in the Southeast Asian clade have consequently been provided with the name *Chrysoporthe deuterocubensis*. Recognition of these two taxa as distinct species is supported by phylogenetic analyses of sequences for four variable gene regions that separated representative isolates of the two species. Both species are also associated with a number of differentially fixed polymorphisms in the five regions examined. Populations linked to these two species from different geographic regions also showed significant differentiation from each other as their distributions do not overlap.

Based on morphology, *C. cubensis* and *C. deuterocubensis* are virtually indistinguishable (Gryzenhout *et al*. 2004) and perceived differences are usually variable or due to environmental conditions (Gryzenhout *et al*. 2009). However, the sienna to sometimes orange colour of especially young conidiomata observed in some *C. deuterocubensis* specimens is not common in *C. cubensis*, although mature conidiomata are usually similar in appearance. An alternative and robust approach to distinguish the species is to use variation in the gene encoding β-tubulin, which can either be evaluated directly through sequencing or using the PCR–RFLP procedure described in this study. The latter approach is rapid and will be useful for quarantine purposes where a simple diagnostic is typically required.

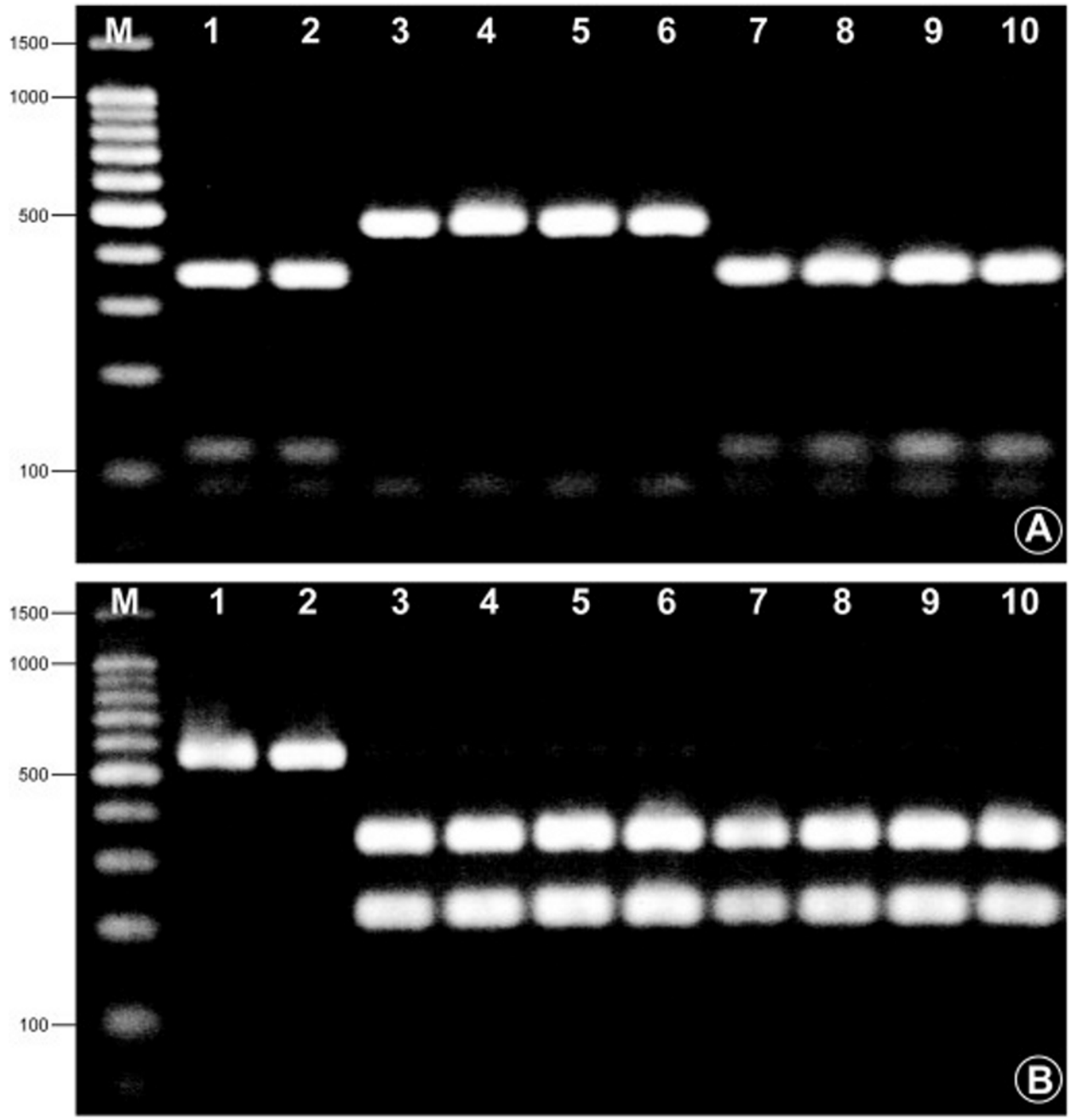
M 1 2 3 4 5 6 7 8 9 10
1500
1000
500
100
A
M 1 2 3 4 5 6 7 8 9 10
1500
1000
500
100
B
1298
1321
1470
1529
1812
1835
Bt1a
EXON6
EXON7
Bt1b
C
AvaI
87 337 113 C.austroafricana
87 440 C.cubensis
87 337 113 C.deuterocubensis
HindIII
537 C.austroafricana
206 331 C.cubensis
206 331 C.deuterocubensis

Fig 4. Digests of β-tubulin gene PCR products (primers Bt1a to Bt1b) for *C. austroafricana* (lanes 1 and 2), *C. cubensis* (lanes 3–6) and *C. deuterocubensis* (lanes 7–10). Lane 1, CMW9327; 2, CMW10192; 3, CMW10028; 4, CMW10669; 5, CMW10778; 6, CMW12734; 7, CMW2631; 8, CMW8650; 9, CMW12745; 10, CMW17178. Lane M is a 100 bp DNA ladder. (A) Restriction profile using AluI; (B) restriction profile using HindIII; (C) restriction maps showing restriction site differences between the three fungal species. The gene map displays coding sequence as grey boxes and intron sequence as a solid line. Primer positions are indicated with arrows on opposite strands of the expected amplicon of 537 bp. Position numbers start at 1 for the first coding base of the Bml gene for *Neurospora crassa* (Glass & Donaldson 1995).

Table 5. Results from a blind PCR–RFLP test showing nonoverlapping distributions for the three species of *Chrysoporthe*.

Continent of origin	Country of origin	Host species	Number of isolates	Assigned species name
Africa	Democratic Republic of Congo	*Eucalyptus* sp.	12	*Chrysoporthe cubensis*
	South Africa	*Eucalyptus grandis*	39	*Chrysoporthe austroafricana*
		Syzygium sp.	47	
		Tibouchina sp.	22	
	Malawi	*Eucalyptus* sp.	1	
		Syzygium cordatum	2	
	Mozambique	*Eucalyptus saligna*	11	
		Syzygium cordatum	21	
	Tanzania	*Eugenia cariophillus*	1	*Chrysoporthe deuterocubensis*
	Zambia	*Eucalyptus* sp.	11	*Chrysoporthe austroafricana*
Australia	Australia	*Eucalyptus* sp.	1	*Chrysoporthe deuterocubensis*
South America	Brazil	*Eucalyptus* sp.	20	*Chrysoporthe cubensis*
	Colombia	*Eucalyptus* sp.	46	
		Vaccinium floribundum (Mortiño)	38	
	Cuba	*Eucalyptus* sp.	25	
	Hawaii		17	
	Mexico		32	
	Venezuela		13	
Southeast Asia	Indonesia	*Eucalyptus grandis*	23	*Chrysoporthe deuterocubensis*
		Eugenia aromatica	1	
	Vietnam	*Eucalyptus* sp.	17	

The multigene phylogeny presented in this study showed clear separation of *C. deuterocubensis* from *C. cubensis* (Fig 1). However, analyses of the individual regions suggested that the EF-1α region is not sufficiently variable to allow separation of *C. deuterocubensis* from *C. cubensis* (Fig 1C). This was also true for the ACT sequences that did not allow separation of *C. deuterocubensis* and *Chrysoporthe inopina* (Fig 1A). This is probably due to the relatively recent divergence of species in *Chrysoporthe*, and different rates of mutation in the gene regions analyzed. Furthermore, the ITS region failed to recover a statistically supported monophyletic *C. cubensis*. This may be due to incomplete lineage sorting (Hare and Avise 1998; Dettman *et al*. 2003) that is expected to be present when closely related species are considered (Hudson and Coyne 2002; Rosenberg 2003).

Population genetic analysis of alleles for 12 loci in *C. cubensis*, *C. deuterocubensis* and *Chrysoporthe austroafricana* isolates showed that the levels of differentiation between the populations were not significantly different ($P < 0.05$). Although the inferred number of migrants between species is relatively high, they are typical for fungi and may be an artefact of close relatedness or incomplete lineage sorting (Stukenbrock *et al*. 2006; Liu *et al*. 2009). The allele diversity of the *C. deuterocubensis* population was also higher than that for *C. austroafricana* and *C. cubensis* (Table 4). These data, therefore, confirmed the observation based on multigene phylogenetic inference that *C. deuterocubensis* represents a distinct species. Additionally, the level of population differentiation reported in this study can be used in future studies considering species delineations in *Chrysoporthe*, assuming that it is possible to obtain populations of adequate size. Separate species in *Chrysoporthe* display differentiation values (θ) of c. 0.27–0.3, while the

corresponding number of migrants is c. 1.1–1.3. When new species are considered and the population differentiation increases above 0.3, the likelihood of complete lineage sorting increases and subsequently, new species can be described based on population genetic data, particularly where phylogenetic data are inconclusive or confusing.

Population assignment tests showed that *C. deuterocubensis* isolates represent a well defined assemblage. However, *C. deuterocubensis* isolates harboured higher frequencies of some alleles that were assigned to *C. cubensis* or *C. austroafricana* (Fig 2). It is, therefore, possible that *C. deuterocubensis* represents an ancestral species, and that the other species are derived from it. This notion is supported by the fact that *C. deuterocubensis* appears basal to *C. cubensis* and *C. austroafricana* when the joint phylogeny of five gene regions is considered (Fig 1E), and also by the higher allele diversity observed in this species (Table 4).

Africa is the only continent besides South America that harbours different species of *Chrysoporthe*. *Chrysoporthe austroafricana* has a wide geographic range and is thought to be native because it occurs on native *Syzygium* sp. and has not been found outside of Africa (Heath *et al*. 2006; Nakabonge *et al*. 2006). *Chrysoporthe cubensis* has been found in western African countries such as Cameroon, Congo and the Democratic Republic of Congo, while *C. deuterocubensis* is found in the eastern African countries of Zanzibar (Tanzania), Kenya, Malawi and Mozambique (Nakabonge *et al*. 2006). *Chrysoporthe cubensis* and *C. deuterocubensis* have not been found on any native African hosts in recent surveys (Roux *et al*. 2003; 2005; Nakabonge *et al*. 2006) and *C. deuterocubensis* was also shown to have a low genetic diversity (Nakabonge *et al*. 2007). These facts suggest that *C. deuterocubensis* was introduced into Africa. The same could be true for *C. cubensis* in Africa, although population level studies would be necessary to show this conclusively. If this should be true, *C. cubensis* and *C. deuterocubensis* do not occur naturally in Africa.

Although *C. austroafricana*, *C. cubensis* and *C. deuterocubensis* occur on a wide range of hosts in the Myrtales, their native hosts in their areas of origin are different. While *C. austroafricana* was originally found on *Eucalyptus* trees in South Africa (Wingfield *et al*. 1989) and where it caused widespread damage, two native hosts, *Syzygium cordatum* and *Syzygium guineense* (Myrtaceae), were later discovered (Heath *et al*. 2006). In contrast, *C. cubensis* infects native *Miconia rubiginosa* and *Miconia theaezans* (Melastomataceae) in South America (Rodas *et al*. 2005), while *C. deuterocubensis* was discovered on *S. aromaticum* (Myrtaceae) and *Melastoma melabathricum* (Melastomataceae) in Southeast Asia (Myburg *et al*. 2003; Gryzenhout *et al*. 2009). Therefore, even though the host ranges of the three fungal species overlap, the native hosts are distinct in the areas where the fungi are thought to be native.

Description of *C. deuterocubensis* now extends the number of known species in the genus to eight, including *Chrysoporthe zambiensis*, *Chrysoporthe syzygiicola* (Chungu *et al*. 2009), *C. austroafricana*, *C. cubensis*, *Chrysoporthe doradensis*, *C. inopina* and *Chrysoporthella hodgesiana* (Gryzenhout *et al*. 2009). Four of these have an apparent Central and South American distribution. Together with *C. cubensis* these include *C. doradensis*, *C. inopina* and *C. hodgesiana*, which are currently known only from Colombia and adjacent Ecuador (Gryzenhout *et al*. 2004; 2005; 2006b). All of these species, except *C. doradensis*, have been found on native trees (Gryzenhout *et al*. 2004; 2005; 2006b). This suggests that these species occur naturally in South America, with this continent currently harbouring the most species of *Chrysoporthe*.

The segregation of *C. cubensis* and *C. deuterocubensis* has important quarantine implications. Where these fungi were previously linked to *Chrysoporthe* canker on *Eucalyptus* with a single species as the causal agent (Gryzenhout *et al*. 2004; 2009), the name now encompasses three species including *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*, with geographical ranges that do not overlap. The pathogenicity of *C. cubensis* has been well established in inoculation trials

on *Eucalyptus* (Boerboom and Maas 1970; Hodges *et al*. 1976; Wingfield 2003). *Chrysoporthe deuterocubensis* is associated with symptoms in Southeast Asia, which are very similar to those caused by *C. cubensis* in South America. For example, pathogenicity tests on *Eucalyptus* have been conducted with C. deuterocubensis in Indonesia with results very similar to those for *C. cubensis* (Wingfield 2003). Pegg *et al*. (2010) have also conducted inoculation trials on *Eucalyptus* with *C. deuterocubensis* although the fungus in that study was treated as *C. cubensis*. Both species also have the ability to infect native woody plants in the Melastomataceae and Myrtaceae and could thus cause serious damage if they were accidentally introduced into new environments with native Myrtaceae or Melastomataceae (Gryzenhout *et al*. 2009).

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