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.

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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. 2005). 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. australasiana*, 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, 2003; Gryzenhout et al. 2004; Myburg et al. 2004; Gryzenhout et al. 2006a, 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, 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, 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* (Frisingle et al. 2005), *Fusarium subglutinans* (Steenkamp et al. 2002), *Amanita muscaria* (Geml et al. 2006), *Neofusisococcum parvum* and *Neofusisococcum 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 & 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 & 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 & Wollenberg 1997; Barraclough & 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 & 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 australasiana* 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.

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 & Donaldson 1995; Carbone & Kohn 1999) for phylogenetic
Isolates of Chrysoporthe sp. used in the multigene phylogenetic studies, and the GenBank accession numbers for the sequences included in phylogenetic analyses.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Origin</th>
<th>Isolate number</th>
<th>ACT</th>
<th>BT1</th>
<th>BT2</th>
<th>EF-1α</th>
<th>ITS</th>
</tr>
</thead>
<tbody>
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<td>GQ290194</td>
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<td>GQ290186</td>
<td>GQ290137</td>
<td>GQ290153</td>
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<td></td>
<td>Republic of Congo</td>
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<td>GQ290171</td>
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<td>Chrysoporthe deuterocubensis</td>
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<td>GQ290165</td>
<td>GQ290178</td>
<td>GQ290189</td>
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<td>AF543825</td>
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<td>GQ290193</td>
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<td>DQ368785</td>
<td>GQ290192</td>
<td>GQ290148</td>
<td>DQ368766</td>
</tr>
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<td>GQ290167</td>
<td>GQ290179</td>
<td>GQ290190</td>
<td>GQ290142</td>
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<td>GQ290152</td>
<td>AY956969</td>
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<td>GQ290169</td>
<td>GQ290180</td>
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<td>DQ368777</td>
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<tr>
<td>Amphiloiga gyrosa</td>
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<td>GQ290168</td>
<td>GQ290182</td>
<td>DQ368811</td>
<td>GQ290145</td>
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<tr>
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<td>BCRC34145</td>
<td>EF025600</td>
<td>EF025615</td>
<td>EF025615</td>
<td>—</td>
<td>EF026147</td>
</tr>
</tbody>
</table>

a CMW — culture collection of the FABI, University of Pretoria, Pretoria, South Africa; BCRC — Bioresource Collection and Research Center, Taiwan.
b Amphiloiga gyrosa was used as an outgroup taxon (Ju et al. 2007).

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 MgCl₂, 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 deionized 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, Amphiloiga 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 homogeneous partitions. In order to test for phylogenetic signal, the g2 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. Phylogenetic 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).

The computer programme MultiLocus 1.3b (Agapow & Burt 2001) was used for all allelic analyses. The population differentiation (θ) (Weir & Cockerman 1984) and theoretical number of migrants per generation (M = 1/2(1 − c)) (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 & Rohlf 1994), which is the ML statistical significance of deviation,

$$ G = 2 \sum O_i \ln \left( \frac{O_i}{E_i} \right) $$

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 the χ² distribution with (k − 1) degrees of freedom, where k is the number of loci.
Existence of a cryptic species within *C. cubensis*

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

<table>
<thead>
<tr>
<th>Locus namea</th>
<th>Dye label</th>
<th>Bin size (bp)</th>
<th>Primer name</th>
<th>Primer sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcPMc</td>
<td>VIC</td>
<td>190–212</td>
<td>PMCF</td>
<td>ttcgatgagaattgacg</td>
</tr>
<tr>
<td>CcPMg</td>
<td>6-FAM</td>
<td>197–297</td>
<td>PMCF</td>
<td>attgcgcctgtatagacg</td>
</tr>
<tr>
<td>COL6</td>
<td>6-FAM</td>
<td>260–270</td>
<td>COL6F</td>
<td>tgaacggccaggaaggcag</td>
</tr>
<tr>
<td>COL7</td>
<td>VIC</td>
<td>173–174</td>
<td>COL7F</td>
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</tr>
<tr>
<td>COL11</td>
<td>VIC</td>
<td>258–267</td>
<td>COL11F</td>
<td>gacccgcacactgttacg</td>
</tr>
<tr>
<td>SA1</td>
<td>NED</td>
<td>300–320</td>
<td>SA1F</td>
<td>ggatacaccaccctacgctc</td>
</tr>
<tr>
<td>SA3</td>
<td>6-FAM</td>
<td>200–215</td>
<td>SA3F</td>
<td>gcttcgcgtaaccctagctg</td>
</tr>
<tr>
<td>SA4</td>
<td>PET</td>
<td>150–200</td>
<td>SA4R</td>
<td>ttcaccacactgctagctc</td>
</tr>
<tr>
<td>SA6b</td>
<td>PET</td>
<td>209–221, 316–365</td>
<td>SA6F</td>
<td>atgcggatgattgcctgctg</td>
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<td>SA9b</td>
<td>NED</td>
<td>190–200, 203–215</td>
<td>SA9R</td>
<td>taacggagtccatcctgctg</td>
</tr>
</tbody>
</table>

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.

of $\chi^2$, 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 burn-in 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 lati 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 Axiosvision 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 $\beta$-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 $\beta$-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.

A partition homogeneity test revealed that all the DNA regions used in this study could be combined (*P* = 0.001) (Cummins *et al.* 1995). Inspection of the *g*₂ 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.

**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 (Μ; 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.

**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. Wingf., sp. nov.

Mycobank No.: MB516634

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

Ascosporas (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)
\[ \mu m; \text{position actinis } 475 \text{ (G, A); positions } \beta \text{-tubulinis } 546 \text{ (C, T), } 699 \text{ (T, C) } 729 \text{ (T, C), } 1477 \text{ (C, T), } 1488 \text{ (G, A), } 1572 \text{ (C, T)} \text{ (TreeBase SN4622); sitibus exceptionis pro Aval, fragmenta } 87 \text{ bp, } 113 \text{ bp, et } 337 \text{ bp ferentiibus, et uno pro HindIII fragmenta } 206 \text{ bp et } 331 \text{ bp ferenti.}

Ascosporae (5.5 – 6.5 – 7.5 – 8) \times 2.5 – 3.5 \mu m; conidio-
mata sienna to almost orange to fuscous-black, pyriform to clavate to pulvinate; conidia (3 – 3.5 – 4.5 – 5) × (1.5 – 2.5) \mu m; ACT position 475 (G, A); \beta \text{-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 Aval, yielding fragments of 87 bp, 113 bp, and 337 bp, and one restriction site for HindIII, yielding fragments of 206 bp and 331 bp.

Fig 1 – ML phylogenies obtained from (A) ACT, (B) \beta \text{-tubulin (BT1 and BT2 regions), (C) elongation factor 1-\alpha, 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 gyroa. 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; \textit{g1} statistic (Hillis & Huelsenbeck 1992) at a significance level of } P = 0.01. \]
Ascostromata semiimmersed erumpent, top of perithecial bases covered with cinnamon to orange, predominantly prosenchymatous, limited stromatic tissue, ascostroma 120–230 \( \mu \)m high above level of bark, 280–490 \( \mu \)m diam., perithecia valsoid, bases immersed in bark, fuscous-black, extending necks up to 240 \( \mu \)m long emerging through bark covered in umber stromatic tissue of textura porrecta, appearing fuscous-black. Ascii (19–)22–26.5(–28) \( \times \) (4.5–)5–6.5(–7) \( \mu \)m, fusoid to ellipsoidal, 8-spored. Ascospores (5.5–)6.5–7.5 (–8) \( \times \) 2–2.5(–3) \( \mu \)m, hyaline, 1-septate, fusoid to oval, ends tapered, with septum variously placed in the spore but usually central.

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 \( \mu \)m high, 100–950 \( \mu \)m diam, necks up to 230 \( \mu \)m long, 90–240 \( \mu \)m wide. Conidiomatal locules with even to convoluted inner surface,

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.

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>CcPMC</th>
<th>CcPMG</th>
<th>COL6</th>
<th>COL7</th>
<th>COL11</th>
<th>SA1</th>
<th>SA3</th>
<th>SA4</th>
<th>SA6-1</th>
<th>SA6-2</th>
<th>SA9-1</th>
<th>SA9-2</th>
<th>( \bar{H} )a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysoporthe austroafricana</td>
<td>97</td>
<td>0.7782</td>
<td>0.2368</td>
<td>0.5029</td>
<td>0.0204</td>
<td>0.3119</td>
<td>0.3700</td>
<td>0.7397</td>
<td>0.6862</td>
<td>0.2610</td>
<td>1.0811</td>
<td>0.6791</td>
<td>0.3981</td>
</tr>
<tr>
<td>Chrysoporthe cubensis</td>
<td>79</td>
<td>0.7713</td>
<td>0.0496</td>
<td>0.5435</td>
<td>0.0000</td>
<td>0.1647</td>
<td>0.0759</td>
<td>0.4995</td>
<td>0.5188</td>
<td>0.7152</td>
<td>0.0251</td>
<td>0.2279</td>
<td>0.4619</td>
</tr>
<tr>
<td>Chrysoporthe deuterocubensis</td>
<td>33</td>
<td>0.3177</td>
<td>0.1140</td>
<td>0.7907</td>
<td>0.2187</td>
<td>0.3801</td>
<td>0.5933</td>
<td>0.5951</td>
<td>0.4115</td>
<td>0.7400</td>
<td>0.0000</td>
<td>0.4628</td>
<td>0.5841</td>
</tr>
</tbody>
</table>

a \( \bar{H} \) is the average gene (allele) diversity for a population over all loci.
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. deuterocebensis (given as the gene name, the nucleotide position relative to the start codon of the corresponding

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**Fig 3** – Fruiting structures of *Chrysoporthe deuterocebensis*. (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 ascostromata. (E) Ascus. (F) Ascospores. (G) Conidioma on bark. (H) Vertical section through conidiomata. (I) Tissue of textura globulosa for the conidiomatal 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.
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 SN46622). 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**


**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, 2003; Roux et al. 2003; Gryzenhout et al. 2004, 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 *Ava*I and *Hind*III. Separate digests with these enzymes revealed that *C. austroafricana*, *C. cubensis* and *C. deuterocubensis* could easily be distinguished from each other (Fig 4). When *Ava*I 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, *Hind*III did not cut for *C. austroafricana* but produced two fragments (206 bp and 331 bp) for each of the other two species. Therefore, *Hind*III 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.

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 & Avise 1998; Dettman et al. 2003) that is expected to be present when closely related species are considered (Hudson & 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. australoafricana 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. australoafricana (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. australoafricana 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 australoafricana 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. australoafricana, C. cubensis and C. deuterocubensis occur on a wide range of hosts in the Myrtaceae, their native hosts in their areas of origin are different. While C. australoafricana 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.

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

<table>
<thead>
<tr>
<th>Continent of origin</th>
<th>Country of origin</th>
<th>Host species</th>
<th>Number of isolates</th>
<th>Assigned species name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>Democratic Republic of Congo</td>
<td>Eucalyptus sp.</td>
<td>12</td>
<td>Chrysoporthe cubensis</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Eucalyptus grandis</td>
<td>39</td>
<td>Chrysoporthe australoafricana</td>
</tr>
<tr>
<td>Malawi</td>
<td>Eucalyptus sp.</td>
<td>1</td>
<td>Chrysoporthe cubensis</td>
<td></td>
</tr>
<tr>
<td>Mozambique</td>
<td>Eucalyptus saligna</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanzania</td>
<td>Eugenia cariophillus</td>
<td>1</td>
<td>Chrysoporthe deuterocubensis</td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td>Eucalyptus sp.</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>Eucalyptus sp.</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South America</td>
<td>Eucalyptus sp.</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colombia</td>
<td>Eucalyptus sp.</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vaccinium floribundum</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuba</td>
<td>Eucalyptus sp.</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>Eucalyptus sp.</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venezuela</td>
<td>Eucalyptus sp.</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast Asia</td>
<td>Indonesia</td>
<td>Eucalyptus grandis</td>
<td>23</td>
<td>Chrysoporthe deuterocubensis</td>
</tr>
<tr>
<td></td>
<td>Eugenia aromatica</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vietnam</td>
<td>Eucalyptus sp.</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
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. austroaficana, 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. austroaficana, 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 & 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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References


