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journal homepage: www.elsevier.com/locate/funbio

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

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ARTICLE INFO

Article history:
Received 21 April 2010
Received in revised form
5 September 2010
Accepted 15 September 2010
Available online 8 October 2010
Corresponding Editor:
Joseph W. Spatafora

Keywords:
Chrysoporthe deuterocubensis
Gene flow
Multigene phylogeny
PCR–RFLP
Population differentiation

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.*

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1878-6146/$ – see front matter
doi:10.1016/j.funbio.2010.09.007

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, 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* (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 & 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 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.

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