The forgotten *Calonectria* collection: Pouring old wine into new bags

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Abstract: The genus *Calonectria* with its *Cylindrocladium* asexual morphs has been subject to several taxonomic revisions in the past. These have resulted in the recognition of 116 species, of which all but two species (*C. hederae* and *C. pyrochroa*) are supported by ex-type cultures and supplemented with DNA barcodes. The present study is based on a large collection of unidentified *Calonectria* isolates that have been collected over a period of 20 years from various substrates worldwide, which has remained unstudied in the basement of the CBS-KNAW Fungal Biodiversity Centre. Employing a polyphasic approach, the identities of these isolates were resolved and shown to represent many new phylogenetic species. Of these, 24 are newly described, while *C. uniseptata* is reinstated at species level. We now recognise 141 species that include some of the most important plant pathogens globally.

Key words: *Cylindrocladium*, Cryptic species, Phylogeny, Taxonomy.

Taxonomic novelties: New species: *Calonectria amazonica* L. Lombard & Crous, *C. amazoniensis* L. Lombard & Crous, *C. brasiliana* L. Lombard & Crous, *C. brassicicola* L. Lombard & Crous, *C. brevistipitata* L. Lombard & Crous, *C. cliffordiicola* L. Lombard & Crous, *C. ericae* L. Lombard & Crous, *C. indonesiana* L. Lombard & Crous, *C. lageniformis* L. Lombard & Crous, *C. machaerinae* L. Lombard & Crous, *C. multilateralis* L. Lombard & Crous, *C. paracolhounii* L. Lombard & Crous, *C. parva* L. Lombard & Crous, *C. plurilateralis* L. Lombard & Crous, *C. pseudoecuadoriae* L. Lombard & Crous, *C. pseudouxmalensis* L. Lombard & Crous, *C. putriramosa* L. Lombard & Crous, *C. stipitata* L. Lombard & Crous, *C. syzygiicola* L. Lombard & Crous, *C. tereticornis* L. Lombard & Crous, *C. terricola* L. Lombard & Crous, *C. tropicalis* L. Lombard & Crous, *C. uxmalensis* L. Lombard & Crous, *C. venezuelana* L. Lombard Crous.

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INTRODUCTION

The genus *Calonectria*, first introduced in 1867 (Rossman 1979), has been the subject of numerous taxonomic studies since the 1990s (Crous & Wingfield 1994, Crous 2002, Lombard *et al.* 2010b, 2015a, Alfenas *et al.* 2015). These studies have resulted in the recognition of 116 species, of which all but two (*C. hederae* and *C. pyrochroa*) are supported by ex-type cultures and supplemented by DNA barcodes (Crous 2002, Lechat *et al.* 2010, Lombard *et al.* 2010b). This large number of species has arisen mainly due to the introduction of DNA sequence data and subsequent phylogenetic inference enabling delimitation of numerous previously unrecognised cryptic taxa. These species often share the same plant hosts, informing knowledge of the epidemiology and fungicide resistance (Graça *et al.* 2009, Vitale *et al.* 2013, Gehesquière *et al.* 2016).

Calonectria spp. are characterised by sexual morphs that have yellow to dark red perithecia, with scaly to warty ascocarp walls, and *Cylindrocladium* asexual morphs in which the cylindrical and septate conidia are produced from phialides clustered below and surrounding a stipe extention terminating in variously shaped vesicles (Rossman 1993, Crous 2002, Lombard *et al.* 2010b,c). For many years these fungi were best known by their *Cylindrocladium* names associated with important plant diseases (Crous & Wingfield 1994, Crous 2002, Lombard *et al.* 2010c). Following convention that only one scientific name should be used for a fungal species (Hawksworth 2011, 2012, Hawksworth *et al.* 2011, McNeill *et al.* 2012), *Calonectria* has been chosen (Rossman *et al.* 2013). This newly adopted convention should resolve confusion regarding their names (Wingfield *et al.* 2011). However, it is important to recognise that the asexual *Cylindrocladium* morph represents the life phase most commonly found in nature and many species are known only in this form, which also plays a major role in the dissemination of *Calonectria* spp.

Calonectria spp. cause important diseases in numerous plant hosts worldwide. This includes leaf blight, cutting rot, damping-off and root rot (Crous 2002, Lombard *et al.* 2010c, 2015a, Vitale *et al.* 2013, Alfenas *et al.* 2015). The majority of the diseases caused by *Calonectria* spp. are associated with forestry-related plants (see Lombard *et al.* 2010c), where Calonectria leaf blight (CLB) is an important constraint to plantation productivity in South America (Rodas *et al.* 2005, Alfenas *et al.* 2015) and Southeast Asia (Crous & Kang 2001, Old *et al.* 2003, Chen *et al.* 2011, Lombard *et al.* 2015a). In other regions, such as southern Africa and Australia, *Calonectria* spp. appear mostly to be limited to forestry nurseries (Crous 2002, Lombard *et al.* 2009, 2010a,b,c). In agricultural and horticultural crops, *Calonectria* spp. have chiefly been reported only from South America and the Northern Hemisphere, where they are mostly associated with nursery diseases (Lombard *et al.* 2010c, Vitale *et al.* 2013), Cylindrocladium black rot of peanut (Bell & Sobers 1966, Beute & Rowe 1973, Hollowell *et al.* 1998) and box blight of *Buxus* spp. (Henricot *et al.* 2000, Crepel & Inghelbrecht 2003, Brand 2005, Saracchi *et al.* 2008, Saurat *et al.* 2012, Mirabolfathy *et al.* 2013, Gehesquière *et al.* 2016).

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The present study is based on a large collection of unidentified *Calonectria* isolates that were collected over a period of 20 years from various substrates worldwide. This collection of isolates, deposited in the CBS-KNAW culture collection in 2002 has remained unstudied in the basement of the institute and hence, the title of this study "the forgotten basement collection". The large majority of these isolates were initially identified based solely on morphology and at a time when robust and multigene DNA sequence data were not commonly available. This implied that cryptic species could not be resolved (Lombard *et al.* 2010b, 2015a, Alfenas *et al.* 2015). The aim of the present study was to employ a polyphasic approach to identify these isolates.

MATERIALS AND METHODS

Isolates

Calonectria strains were obtained from the culture collection of the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands and the working collection of the senior author (CPC) housed at the CBS (Table 1).

Phylogeny

Total genomic DNA was extracted from 7-d-old axenic cultures, grown on MEA at room temperature, using the UltraClean™ Microbial DNA isolation kit (Mo Bio Laboratories, Inc., California, USA) following the protocols provided by the manufacturer. Based on previous studies (Lombard *et al.* 2010b, 2015b, Alfenas *et al.* 2015), partial gene sequences were determined for β-tubulin (*tub2*), calmodulin (*cmdA*), and the translation elongation factor 1-alpha (*tef1*) regions as these regions provided the best phylogenetic signal at species level for the genus *Calonectria*. Therefore, the primers and protocols described by Lombard *et al.* (2015b) were used to determine these regions.

To ensure the integrity of the sequences, the amplicons were sequenced in both directions using the same primers used for amplification. Consensus sequences for each locus were assembled in MEGA v. 7 (Kumar *et al.* 2016) and compared with representative sequences from Alfenas *et al.* (2013a,b, 2015), Chen *et al.* (2011) and Lombard *et al.* (2010a,b, 2011, 2015a). Subsequent alignments for each locus were generated in MAFFT v. 7.110 (Katoh & Standley 2013) and the ambiguously aligned regions of both ends were truncated. Congruency of the three loci was tested using the 70 % reciprocal bootstrap criterion (Mason-Gamer & Kellogg 1996) following the protocols of Lombard *et al.* (2015b).

Phylogenetic analyses of the individual gene regions and the combined dataset were based on Bayesian inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP). For BI and ML, the best evolutionary models for each locus were determined using MrModeltest (Nylander 2004) and incorporated into the analyses. MrBayes v. 3.2.1 (Ronquist & Huelsenbeck 2003) was used for BI to generate phylogenetic trees under optimal criteria for each locus. A Markov Chain Monte Carlo (MCMC) algorithm of four chains was initiated in parallel from a random tree topology with the heating parameter set at 0.3. The MCMC analysis lasted until the average standard deviation of split frequencies was below 0.01 with trees saved every 1 000 generations. The first 25 % of saved trees were discarded as the "burn-in" phase and posterior probabilities (PP) were determined from the remaining trees.

The ML analyses were preformed using RAxML v. 8.0.9 (randomised accelerated (sic) maximum likelihood for high performance computing; Stamatakis 2014) through the CIPRES website (http://www.phylo.org) to obtain another measure of branch support. The robustness of the analysis was evaluated by bootstrap support (BS) with the number of bootstrap replicates automatically determined by the software.

For MP, analyses were done using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2003) with phylogenetic relationships estimated by heuristic searches with 1 000 random addition sequences. Tree-bisection-reconnection was used, with branch swapping option set on "best trees" only. All characters were weighted equally and alignment gaps treated as fifth state. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analyses (Hillis & Bull 1993) were based on 1 000 replications. All new sequences generated in this study were deposited in GenBank (Table 1) and alignments and trees in TreeBASE.

Taxonomy

Axenic cultures were transferred to synthetic nutrient-poor agar (SNA; Nirenburg 1981) and incubated at room temperature for 7 d. Gross morphological characteristics were studied by mounting the fungal structures in 85 % lactic acid and 30 measurements were made at ×1 000 magnification for all taxonomically informative characters using a Zeiss Axioscope 2 microscope with differential interference contrast (DIC) illumination. The 95 % confidence levels were determined for the conidial measurements with extremes given in parentheses. For all other fungal structures measured, only the extremes are provided. Colony colour was assessed using 7-d-old cultures on MEA incubated at room temperature and the colour charts of Rayner (1970). All descriptions, illustrations and nomenclatural data were deposited in MycoBank (Crous *et al.* 2004a).

RESULTS

Phylogenetic analyses

Approximately 500−550 bases were determined for the three gene regions included in this study. The congruency analyses revealed no conflicts in tree topologies, with only minor differences in branch support. Therefore, the sequences of the three loci determined here were combined in a single dataset for analyses. For the BI and ML analyses, a HKY+I+G model was selected for all three gene regions and incorporated into the analyses. The ML tree topology confirmed the tree topologies obtained from the BI and MP analyses, and therefore, only the ML tree is presented.

The combined *cmdA*, *tef1* and *tub2* sequences dataset included 278 ingroup taxa and *Curvicladiella cignea* (CBS 109167) as outgroup taxon. This dataset consisted of 1 680