# CHAPTER 1

## Species concepts in *Calonectria* (*Cylindrocladium*)

### A Literature Review

## 1.0 INTRODUCTION

The genus *Calonectria* (*Ca.*) was erected in 1867 by De Notaris, based on *Ca. daldiniana* collected on leaves of *Magnolia grandiflora* (*Magnoliaceae*), in Daldini, Italy (Rossman 1979a). Rossman (1979a) later reduced *Ca. daldiniana* to synonymy under *Ca. pyrochroa*, and defined this nectrioid fungus as having an ascocarp wall structure that is brightly coloured, changing to blood-red in 3% KOH solution, warty to scaly and with a *Cylindrocladium* (*Cy.*) anamorph (Rossman 1993, Rossman *et al.* 1999). However, due to the restricted morphological characteristics of the teleomorph (Rossman 1979b, 1983), specimens can in many cases only be identified to species level if the anamorph is present (Schoch *et al.* 2000b, Crous 2002).

The anamorph genus *Cylindrocladium*, which is based on *Cy. scoparium,* was first described by Morgan (1892) in the U.S.A., where it was found growing as saprobe on a pod of *Gleditsia triacanthos*. Although Morgan (1892) failed to mention the stipe extension terminating in a vesicle of characteristic shape, he defined the genus as having branched conidiophores producing cylindrical conidia. This fungus has a wide distribution in sub-tropical and tropical regions of the world, and species are pathogenic to numerous plants (Crous 2002).

The aim of this review is to present an overview of published research on the genus *Calonectria*. More specifically, the application of three types of species concepts is considered as they pertain to the taxonomic history of this genus up to 2008. Although several species concepts (Mayden 1997) have been proposed, only the Morphological Species Concept (MSC), the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC) are treated, as these have been most widely applied to *Calonectria*. Several reviews (Rossman 1996, Brasier 1997, Harrington & Rizzo 1999, Taylor *et al.* 1999, 2000, Seifert *et al.* 2000; Kohn 2005) have treated the various species concepts applied to the taxonomy of fungi and this topic is not treated other than in the manner in which it applies to *Calonectria.*

## 2.0 TAXONOMIC HISTORY

*Calonectria* resides in the Nectriaceae, one of three families in Hypocreales, an order that has been reviewed extensively (Rogerson 1970, Rossman 1983, Rossman *et al.* 1996, 1999). The Nectriaceae is circumscribed as having uniloculate ascomata that are orange to purple and not immersed in well-developed stromata (Rossman *et al.* 1999). The family includes approximately 20 genera of socio-economic importance and of these, *Calonectria* are more clearly distinguished from the others by their *Cylindrocladium* anamorphs and relevance as plant pathogens.

The first monograph of *Cylindrocladium*, by Boedjin & Reitsma (1950), introduced seven *Cylindrocladium* species with a *Calonectria* connection to one of these species. Later, in her treatment of *Calonectria*, Rossman (1983) recognized five species including the novel *Ca. ophiospora*. However, this species description did not include the anamorph state. The circumscribed type, *Ca. pyrochoa*, was also incorrectly reduced to synonymy with several other species based only on the teleomorph morphology. Peerally (1991a) highlighted this in a monograph of *Cylindrocladium*, where he regarded the anamorph morphology as important in distinguishing species of *Calonectria*. He subsequently recognized 10 *Calonectria* species with their *Cylindrocladium* anamorphs, including an additional 16 *Cylindrocladium* species not associated with a teleomorph. However, he mistakenly reduced *Cylindrocladiella*, a genus that accommodates *Cylindrocladium*-like species with small conidia (Boesewinkel 1982), to synonymy with *Cylindrocladium*.

The monograph of *Cylindrocladium* by Crous & Wingfield (1994) entrenched the importance of anamorph characteristics in the taxonomy of *Calonectria* spp. In this monograph, 22 *Cylindrocladium* species and one variety were recognised, associated with 16 *Calonectria* species. Five species were assigned to the genus *Cylindrocladiella* based on morphological characters of the holomorph. The focus on anamorph characteristics is perpetuated in the most recent monograph (Crous 2002), which recognized 28 *Calonectria* species, all associated with *Cylindrocladium* anamorphs and an additional 18 *Cylindrocladium* species for which teleomorph states were not known. Of the latter group, seven taxa were of doubtful authenticity.

Presently, 32 *Calonectria* and 52 *Cylindrocladium* species are recognized (Table 1; Crous 2002, Crous *et al.* 2004b, 2006, Gadgil & Dick 2004).

A general search on MycoBank (www.mycobank.org; Crous *et al.* 2004a, Roberts *et al.* 2005) and Index Fungorum (www.indexfungorum.org) resulted in a total of 256 and 258 name records respectively for *Calonectria*. A similar search for *Cylindrocladium* species on both electronic databases indicated a total of 103 and 94 names respectively.

**3.0 NOMENCLATURE OF *CALONECTRIA***

The nomenclature of pleomorphic fungi has been a topic of substantial debate during the course of the past two decades (Gams 1991, Cannon & Kirk 2000, Hawksworth 2004, 2005). The separate naming of anamorphs (mitotic morphs) and teleomorphs (meiotic morphs) has resulted in confusion, especially for non-taxonomists (Cannon & Kirk 2000). This is especially evident where teleomorph species epithets are different to those of their anamorphs and also where more than one anamorph (synanamorph) is found. The naming of fungal morphs based on the International Code of Botanical Nomenclature (ICBN; McNeill *et al.* 2005) and in particular following strict interpretation of Article 59 of the Code has now been unsatisfactory for many fungal groups due to our ability to connect morphs using molecular evidence, and there are increasing calls for further changes to be made.

According to Article 59.4, the teleomorph name takes precedence over the anamorph name when both types belong to the same holomorph taxon. Further, the earliest available legitimate name typified (Article 59.1) should be regarded as the correct name after 1 January 2008 (Hawksworth 2004). Following these rules, the name *Calonectria* typified in 1867, takes precedence over *Cylindrocladium* typified in 1892 (Morgan 1892). Although there are several *Cylindrocladium* species without *Calonectria* connections (Crous 2002, Crous *et al.* 2004b, 2006), we believe that new species should be described in *Calonectria* irrespective of whether a teleomorph is known or not. This follows a clear view based on phylogenetic inference that *Cylindrocladium* spp. all are derived from the same common ancestor as the *Calonectria* spp. (Schoch *et al.* 1999, 2000a, 2000b, Crous 2002, Crous *et al.* 2004b,

2006). Thus, for taxonomic purposes, *Cylindrocladium* species with known teleomorph states are referred to as *Calonectria* in this review.

## 4.0 IMPORTANCE OF *CALONECTRIA*

The genus *Calonectria* was initially regarded as a saprobe as no disease symptoms could be induced by inoculating a suspected host (Graves 1915). The first proof of pathogenicity of these fungi was provided by Massey (1917), and subsequently by Anderson (1919), who proved pathogenicity of *Ca. morganii*. Subsequently, *Calonectria* species have been associated with a wide range of disease symptoms on a large number of hosts worldwide (Crous 2002; Table 2). In the past, several authors have indicated that *Calonectria* species cause disease on plants residing in approximately 30 plant families (Booth & Gibson 1973, French & Menge 1978, Peerally 1991a, Wiapara *et al.* 1996, Schoch *et al.* 1999). Upon closer inspection, the number of plant host families is actually closer to 100 (Table 2) that include approximately 335 plant species (Crous 2002). These hosts include important forestry, agricultural and horticultural crops. This suggests that the impact of these plant pathogens has been underestimated in the past.

The majority of disease reports associated with *Calonectria* species in forestry include hosts in 5 plant families, of which the most important are associated with Fabaceae (*Acacia* spp.), Myrtaceae (*Eucalyptus* spp.) and Pinaceae (*Pinus* spp.). Disease symptoms include cutting rot (Crous *et al.* 1991, Crous 2002), damping-off (Batista 1951, Cox 1953, Terashita & Itô 1956, Sharma & Mohanan 1982, Sharma *et al.* 1984, Crous *et al.* 1991, Brown & Ferreira 2000, Crous 2002, Taniguchi *et al.* 2008) leaf diseases (Cox 1953, Hodges & May 1972, Barnard 1984, Sharma *et al.* 1984, El-Gholl *et al.* 1986, Peerally *et al.* 1991a, Crous *et al.* 1993b, Crous & Wingfield 1994, Crous *et al.* 1998b, Schoch & Crous 1999, Schoch *et al.* 1999, Booth *et al.* 2000, Park *et al.* 2000, Crous & Kang 2001, Gadgil & Dick 2004), shoot blight (Sharma *et al.* 1984, Crous *et al.* 1991, Crous *et al.* 1998b, Crous & Kang 2001), stem cankers (Cox 1953, Sharma *et al.* 1984, 1985, Crous *et al.* 1991) and root rot (Cox 1953, Hodges & May 1972, Cordell & Skilling 1975, Mohanan & Sharma 1985, Crous *et al.* 1991). The majority of these diseases are associated with seedling and cutting production in forestry nurseries, but in a few cases *Cylindrocladium* species have also been reported

from commercial plantations. In these cases the pathogens have been reported to cause leaf diseases and shoot blight resulting in defoliation of trees leading to loss of growth vigour (Hodges & May 1972, Sharma *et al.* 1985, Booth *et al.* 2000, Park *et al.* 2000, Crous & Kang 2001, Crous 2002, Old *et al.* 2003, Rodas *et al.* 2005).

In agriculture, *Calonectria* species have been reported to cause diseases on several economically important crops. Several plant families of agricultural importance are susceptible to *Calonectria* infections, of which the most significant fall in Fabaceae, and Solanaceae. Important diseases in these families include Cylindrocladium black rot of *Arachis hypogea* (peanut) and red crown rot of *Glycine max* (soybean) caused by *Ca. ilicicola* and *Ca. pyrochroa* in the USA (Bell & Sobers 1966, Beute & Rowe 1973, Rowe *et al.* 1973, Sobers & Littrell 1974, Rowe & Beute 1975, Phipps *et al.* 1976, Johnson 1985, Dianese *et al.* 1986, Berner *et al.* 1988, Berner *et al.* 1991, Culbreath *et al.* 1991, Porter *et al.* 1991, de Varon 1991, Hollowell *et al.* 1998, Kim *et al.* 1998) and Cylindrocladium tuber rot of *Solanum tuberosum* (potato) (Boedjin & Reitsma 1950, Bolkan *et al.* 1980, 1981) by *Cy. gracile* in Brazil. Other diseases associated with *Calonectria* species on agricultural crops include root rot and leaf diseases of fruit bearing and spice plants (Jauch 1943, Wormald 1944, Sobers & Seymour 1967, Nishijima & Aragaki 1973, Milholland 1974, Krausz & Caldwell 1987, Hutton & Sanewski 1989, Anandaraj & Sarma 1992, Risède 1994, Jayasinghe & Wijesundera 1996, Risède & Simoneau 2001, Vitale & Polizzi 2008), post-harvest diseases of fruits (Fawcett & Klotz 1937, Boedjin & Reitsma 1950, Sepiah 1990, Fitzell & Peak 1992, Vaidya & Roa 1992, Sivapalan *et al.* 1998), root and crown rot of *Medicago sativa* (alfalfa) (Ooka & Uchida 1982, Hwang & Flores 1987), and sheath net blotch of *Oryza sativa* (rice) (Crous 2002).

On horticultural crops, *Calonectria* species have been reported mostly from the Northern Hemisphere, especially in gardens and ornamental commercial nurseries in Europe and Asia (Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Pérez-Sierra *et al.* 2007, Polizzi *et al.* 2007a, 2007b, Hirooka *et al.* 2008). Hosts in this sector include ornamental trees, shrubs and cut-flowers in several plant families, most commonly in Arecaceae, Asteraceae, Ericaceae and Rosaceae. A wide range of disease symptoms are recorded including crown–, collar– and root rot, leaf spots, and cutting rot (Massey 1917, Anderson 1919, Aragaki *et al.* 1972, 1988,

Peerally 1991b, Uchida & Kadooka 1997, Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Henricot & Beales 2003, Poltronieri *et al.* 2004, Lane *et al.* 2006, Polizzi *et al.* 2006a, 2006b, 2007a, 2007b, Pérez-Sierra *et al.* 2007, Vitale & Polizzi 2007, Hirooka *et al.* 2008, Vitale *et al*. 2008).

## 5.0 MORPHOLOGY

Morphological or phenotypic characters have played a major role in the description of fungal species (Brasier 1997, Taylor *et al.* 2000) and form the basis of new fungal descriptions as required by the ICBN (McNeill *et al.* 2005). In recent years, the use of morphological characters alone to delimit new species has been set aside, to a large extent, with more focus being placed on biological and phylogenetic characters (Rossman 1996, Brasier 1997, Taylor *et al.* 2000). This trend is also evident in recent studies on *Calonectria* species (Crous *et al.* 2004b, 2006).

The morphology of *Calonectria* and to a greater extent its anamorph, *Cylindrocladium*, has been important in the taxonomic history of these fungi. Prior to the 1990's, identification of species was based on morphological characteristics and to a lesser extent on sexual compatibility using standardised media (Boedjin & Reitsma 1950, Peerally 1991a, Crous *et al.* 1992, Crous & Wingfield 1994, Crous 2002). This resulted in the establishment of several species complexes, as many *Cylindrocladium* species are morphologically very similar. These include the *Ca. scoparia* complex (Schoch *et al.* 1999), *Cy. gracile* complex (Crous *et al.* 2004b) and *Ca. kyotensis* complex (Crous *et al.* 2006). Characteristics (Fig. 1) of the anamorphs that are extensively employed in identifications include vesicle shape (Fig. 1C–F), stipe extension length (Fig. 1A–B) and macroconidial septation and dimensions (Fig. 1G–J; Boesewinkel 1982, Peerally 1991a, Crous & Wingfield 1994, Crous 2002). The morphological characteristics of the teleomorph that are important for identifications are ascospore (Fig. 1M–N) septation and dimensions. The perithecia of *Calonectria* species are morphologically very similar and these are typically not very useful in identifications (Crous & Wingfield 1994, Crous 2002).

Biochemical techniques can also be used in phenotypic characterization. These include substrate utilization and cell wall polysaccharide analysis. The use of aminopeptidase specificity (Stevens *et al.* 1990) and utilization of specific nitrogen

and carbon (Hunter & Barnett 1978, Sharma *et al*. 1992) have been used successfully to separate several *Cylindrocladium* species. The use of polysaccharides obtained from cell walls of *Cylindrocladium* positively identified linkages between asexual species and their respective *Calonectria* teleomorphs (Ahrazem *et al*. 1997). However, this method has been found to have a limited value as it does not distinguish between some species in complexes (Crous 2002).

**6.0 MATING COMPATIBILITY**

Mating strategies have been employed in the taxonomy of *Calonectria* and have played an important role in identifying new species of the genus (Schoch *et al.* 1999, Crous 2002). Based on these studies, there are approximately 18 homothallic and 34 heterothallic species of *Calonectria* (Crous 2002, Crous *et al.* 2004b, Gadgil & Dick 2004, Crous *et al.* 2006), with the heterothallic species showing a biallelic mating system (Schoch *et al.* 1999). Studies in the female fertility of *Cylindrocladium* by Schoch *et al.* (1999, 2000a, 2001a) have also shown that several species are self-sterile hermaphrodites requiring fertilization from an opposite mating type. This is typical of heterothallic ascomycetes (Leslie & Klein 1996).

Several difficulties associated with applying the BSC have been highlighted (Brasier 1997, Taylor *et al.* 1999, Taylor *et al.* 2000, Kohn 2005). The most relevant underlying problem occurs where genetically isolated fungal strains retain the ancestral ability to recombine to produce viable progeny (Brasier 1997). This phenomenon has also been found with several phylogenetic species that are closely related in *Calonectria*. Crous (2002), for example, showed that *Cy. hawksworthii*, *Ca. insularis* and *Ca. morganii* were capable of recombining, but that the progeny had low levels of fertility. Other mating studies done by Overmeyer *et al.* (1996) and Neubauer & Zinkernagel (1995) have found that induction of fertile perithecia requires the presence of an additional isolate that, however, does not contribute to the genetic make-up of the progeny. This clearly highlights the need for further studies regarding the mechanism of perithecial formation and recombination in *Calonectria*.

**7.0 PHYLOGENY**

Phylogenetic studies on *Calonectria*, and its *Cylindrocladium* anamorphs have substantially influenced the taxonomy of these genera. Application of molecular techniques and particularly DNA sequence comparisons to distinguish between species has resulted in the recognition of numerous cryptic species. Several molecular approaches have been employed that include total protein electrophoresis (Crous *et al.* 1993a, El-Gholl *et al.* 1993a), isozyme electrophoresis (El-Gholl *et al.* 1992, 1997, Crous *et al.* 1998a), random amplification of polymorphic DNA (RAPD) (Overmeyer *et al.* 1996, Victor *et al.* 1997, Schoch *et al.* 2000a, Riséde & Simoneau 2004) restriction fragment length polymorphisms (RFLP) (Crous *et al.* 1993b, Crous *et al.* 1995, Crous *et al.* 1997b, Jeng *et al.* 1997, Victor *et al.* 1997; Riséde & Simoneau 2001) and DNA hybridization (Crous *et al.* 1993b, 1995, 1997a, Victor *et al.* 1997). Although the above-mentioned techniques have been useful, DNA sequence comparisons and associated phylogenetic inference have had the most dramatic impact on the taxonomy of *Calonectria* and are most widely applied today.

In the first study using 5.8S ribosomal RNA gene and flanking internally transcribed spacers (ITS) sequences Jeng *et al.* (1997) were able to distinguish between *C. scoparium* and *C. floridanum* isolates. Subsequently, it was found that this gene region contains few informative characters (Crous *et al.* 1999, Schoch *et al.* 1999, Riséde & Simoneau 2001, Schoch *et al.* 2001b). Therefore, the β-tubulin (Schoch *et al.* 2001b) and histone H3 (Kang *et al.* 2001a) gene regions have been applied in order to allow for improved resolution in separating species.

The first complete DNA sequence-based phylogenetic study using partial β-tubulin gene sequences (Schoch *et al.* 2001b) compared phenotypic, biological and phylogenetic concepts used in the taxonomy of *Cylindrocladium*. This also highlighted the fact that *Calonectria* represents a monophyletic lineage (Schoch *et al.* 2000b, 2001b). Subsequently, combined DNA sequence data for the ITS, β-tubulin and histone H3 gene regions have been widely used in studies relating to taxonomic issues surrounding *Cylindrocladium* and *Calonectria* (Crous *et al.* 1999, Schoch *et al.* 2000a, 2000b, Crous & Kang 2001, Kang *et al.* 2001a, 2001b, Henricot & Culham 2002, Crous *et al.* 2004b, 2006). Other partial gene sequences recently used include

translation elongation 1-alpha (TEF-1α) and calmodulin (Crous *et al.* 2004b). However, insufficient data are currently available for these gene regions on GenBank (www.ncbi.nlm.nih.gov) to make them particularly valuable for comparative analysis.

In a search of GenBank, at total of 734 partial gene sequences was obtained for *Calonectria* and *Cylindrocladium*. These include 311 for β-tubulin, 177 for histone H3, 159 for ITS, 39 for calmodulin, 36 for TEF-1α, five for large subunit RNA gene (LSU), three each for the high mobility group (HMG) box and peptidase synthetase and one for the small subunit RNA (SSU) gene. For *Cylindrocladium* and *Calonectria*, there are only four studies (Kang *et al.* 2001a, 2001b; Crous *et al.* 2004b, 2006) that provide files on TreeBase (www.treebase.org).

## 8.0 FUTURE RESEARCH

### 8.1 Population Biology

Most studies on *Calonectria* have focused on the taxonomy, phylogeny and pathology of species. There have in contrast been relatively few studies treating the population biology of these fungi. This is unfortunate as population dynamics contributes considerable knowledge to a better understanding of population structure, distribution of genetic diversity, gene flow, centres of origin and mating strategies (McDonald 1997, Linde *et al.* 2002, Grünwald *et al.* 2003). An understanding of the population dynamics of *Calonectria* would contribute in determining the natural spread of these fungi as well as assist in phytosanitary and quarantine regulations. Another important aspect surrounding knowledge of *Calonectria* population dynamics is that this would contribute to plant breeding programmes and thus control of the many diseases that are caused by these fungi (McDonald 1997, Wright *et al.* 2006, 2007).

Limited research has been conducted on the population dynamics of *Calonectria*. To date only two studies (Wright *et al.* 2006, 2007) have reported on the development of polymorphic markers to characterise simple sequence repeats (SSRs) in loci of *Ca. ilicicola* (Wright *et al.* 2006) and *Ca. pauciramosa* (Wright *et al.* 2007). However, no study has yet been published on the population biology of either of these important pathogens using these markers. There is clearly a gap in this area of research concerning *Calonectria* spp. and future research in this area should be encouraged.

## 8.2 Whole genome sequences

A relatively new and innovative technology employed in fungal genetics is the use of whole genome sequences of filamentous fungi. Whole genome sequencing has become relatively inexpensive and thus common in recent years. This revolutionary technology will promote our understanding of the mechanisms of gene function, conidiation, pathogenesis and sexual reproduction at the genotype level (Kupfer *et al.* 1997, Prade 1998, Yoder & Turgeon 2001, Foster *et al.* 2006, Cuomo *et al.* 2007). It is estimated that most filamentous fungi have a genome size of 30 to 40 Mb, containing approximately 8000 to 9000 genes (Kupfer *et al.* 1997, Prade 1998, Foster *et al.* 2006). There are currently several completed fungal genome sequences (http://www.broad.mit.edu/annotation/fungi/fgi/, Foster *et al.* 2006, Baker *et al.* 2008) that include the model yeast *Saccharomyces cerevisiae* (Goffeau *et al.* 1996), plant pathogens and spoilage fungi such as *Aspergillus flavus* (Payne *et al.* 2006), *Fusarium graminearum* (http://www.broad.mit.edu, Cuomo *et al.* 2007), *Magnaporthe grisea* (Dean *et al.* 2005) and the model filamentous fungus *Neurospora crassa* (Galagan *et al.* 2003). Although there are currently over 300 ongoing filamentous fungal genome sequencing projects (http://www.genomesonline.org, Baker *et al.* 2008, Liolios *et al.* 2008), none include species of *Calonectria*.

The most closely related plant pathogen to *Calonectria* species currently being sequenced is *Haematonectria haematococca* (http://www.ncbi.nlm.gov). When the first *Calonectria* species is selected for whole genome sequencing, comparisons with *H. haematococca* could help to identify some important genes in pathogenesis and sexual reproduction. Some *Calonectria* species that could be considered for genome sequencing include *Ca. pauciramosa*, based on its pathogenicity and importance on several plant hosts worldwide (Crous 2002), and *Ca. reteaudii*, one of the most important forest pathogens of South East Asia (Booth *et al.* 2000, Old *et al.* 2003).

## 9.0 CONCLUSIONS

Early studies on the taxonomy of *Calonectria* and *Cylindrocladium* focused on the use of MSC in combination with BSC. More recently, the wide availability of molecular techniques and particularly DNA sequence data have revolutionised the taxonomy of *Calonectria* and *Cylindrocladium.* Today, it is well accepted that the

morphology of the *Cylindrocladium* state contributes most information to naming species and that these fungi all reside in *Calonectria*.

The first study to combine MSC, BSC and PSC concepts by Schoch *et al.* (1999) resulted in the identification of four species within a single species complex. Subsequently, several studies including the MSC, BSC and PSC have elucidated cryptic species in the genus (Kang *et al.* 2001a, 2001b; Henricot & Culham 2002; Crous *et al.* 2004b, 2006). Application of the BSC in the taxonomy of *Calonectria* has been found to be unreliable in some instances (Crous 2002). However, the implementation of MSC and PSC in combination provides powerful tool for taxonomic studies of these genera and it is likely that this will continue in future studies. Although several species complexes have been identified in *Calonectria*, more research is needed on the population level in order to study the gene flow between populations. Additional to this, more gene regions need to be identified and widely used in PSC. With the identification of several new species since 2002, an updated monograph is required to facilitate ease of identification.

**LITERATURE SITED**

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**Table 1.** List of recognized *Calonectria* species and their respective *Cylindrocladium* anamorph species.

| Teleomorph | Reference | Anamorph | Reference |
|---|---|---|---|
| *Calonectria acicola* Gadgil & Dick | Gadgil & Dick 2004 | *Cylindrocladium acicola* Gadgil & Dick | Gadgil & Dick 2004 |
| *Calonectria asiatica* Crous & Hywel-Jones | Crous *et al.* 2004b | *Cylindrocladium asiaticum* Crous & Hywel-Jones | Crous *et al*. 2004b |
| *Calonectria avesiculata* T.S. Schubert, Ell-Gholl, Alfieri & Schoult. | Schubert *et al.* 1989 | *Cylindrocladium avesiculatum* D.L. Gill, Alfieri & Sobers | Gill *et al.* 1971 |
| *Calonectria clavata* Alfieri, El-Gholl, & E.L. Barnard | El-Gholl *et al*. 1993b | *Cylindrocladium flexuosum* Crous | Crous *et al.* 1995 |
| *Calonectria colhounii* Peerally | Peerally 1973 | *Cylindrocladium colhounii* Peerally | Peerally 1973 |
| *Calonectria colombiensis* Crous | Crous *et al*. 2004b | *Cylindrocladium colombiense* Crous | Crous *et al.* 2004b |
| *Calonectria gracilipes* Crous & G.R.A. Mchau | Crous *et al.* 1997a | *Cylindrocladium graciloideum* Crous & G.R.A. Mchau | Crous *et al.* 1997a |
| *Calonectria gracilis* Crous, M.J. Wingf. & Alfenas | Crous *et al.* 1997b | *Cylindrocladium pseudogracile* Crous M.J. Wingf. & Alfenas | Crous *et al.* 1997b |
| *Calonectria hederae* C. Booth & J.S. Murray | Booth & Murray 1960 | *Cylindrocladium hederae* Peerally | Peerally 1991a |
| *Calonectria hongkongensis* Crous | Crous *et al*. 2004b | *Cylindrocladium hongkongense* Crous | Crous *et al*. 2004b |

**Table 1.** (Continued)

| Teleomorph | Reference | Anamorph | Reference |
|---|---|---|---|
| *Calonectria ilicicola* Boedjin & Reitsma | Boedjin & Reitsma 1950 | *Cylindrocladium parasiticum* Crous, M.J. Wingf. & Alfenas | Crous *et al.* 1993d |
| *Calonectria indusiata* Crous | Crous 2002 | *Cylindrocladium theae* (Petch) Alfieri & Sobers | Alfieri *et al.* 1972 |
| *Calonectria insularis* C.L. Schoch & Crous | Schoch *et al.* 1999 | *Cylindrocladium insulare* C.L. Schoch & Crous | Schoch *et al.* 1999 |
| *Calonectria kyotensis* Terashita | Terashita 1968 | *Cylindrocladium floridanum* Sobers & C.P. Seymour | Sobers & Seymour 1967 |
| *Calonectria leguminum* Crous | Crous 2002 | *Cylindrocladium leguminum* Crous | Crous 2002 |
| *Calonectria macroconidialis* Crous | Crous *et al.* 1999 | *Cylindrocladium macroconidiale* Crous | Crous *et al.* 1999 |
| *Calonectria madagascariensis* Crous | Crous 2002 | *Cylindrocladium madagascariense* Crous | Crous 2002 |
| *Calonectria mexicana* C.L. Schoch & Crous | Schoch *et al.* 1999 | *Cylindrocladium mexicanum* C.L. Schoch & Crous | Schoch *et al.* 1999 |
| *Calonectria morganii* Crous, Alfenas & M.J. Wingf. | Crous *et al.* 1993a | *Cylindrocladium scoparium* Morgan | Morgan 1892 |
| *Calonectria multiseptata* Crous & M.J. Wingf. | Crous *et al.* 1998b | *Cylindrocladium multiseptatum* Crous & M.J. Wingf. | Crous *et al.* 1998b |

**Table 1.** (Continued)

| **Teleomorph** | **Reference** | **Anamorph** | **Reference** |
|---|---|---|---|
| *Calonectria naviculata* Crous & M.J. Wingf. | Crous *et al*. 1994a | *Cylindrocladium naviculatum* Crous & M.J. Wingf. | Crous *et al*. 1994a |
| *Calonectria ovata* D. Victor & Crous | Victor *et al.* 1997 | *Cylindrocladium ovatum* El-Gholl , Alfenas, Crous & T.S. Schubert | El-Gholl *et al.* 1993a |
| *Calonectria pauciramosa* C.L. Schoch & Crous | Schoch *et al.* 1999 | *Cylindrocladium pauciramosum* C.L. Schoch & Crous | Schoch *et al*. 1999 |
| *Calonectria pseudospathiphylli* J.C. Kang, Crous & C.L. Schoch | Kang *et al.* 2001b | *Cylindrocladium pseudospathiphylli* J.C. Kang, Crous & C.L. Schoch | Kang *et al*. 2001b |
| *Calonectria pteridis* Crous, M.J. Wingf. & Alfenas | Crous *et al.* 1993c | *Cylindrocladium pteridis* F.A. Wolf | Wolf 1926 |
| *Calonectria pyrochroa* Saccardo | Rossman 1979a | *Cylindrocladium ilicicola* Boedjin & Reitsma | Boedjin & Reitsma 1950 |
| *Calonectria reteaudii* C. Booth | Booth 1966 | *Cylindrocladium reteaudii* Boesewinkel | Boesewinkel 1982 |
| *Calonectria rumohrae* El-Gholl & Alfenas | El-Gholl *et al.* 1997 | *Cylindrocladium rumohrae* El-Gholl & Alfenas | El-Gholl *et al.* 1997 |
| *Calonectria scoparia* Ribeiro & Matsuoka ex Peerally | Peerally 1991 | *Cylindrocladium candelabrum* Viegas | Crous 2002 |

**Table 1.** (Continued)

| Teleomorph | Reference | Anamorph | Reference |
|---|---|---|---|
| *Calonectria spathiphylli* El-Gholl, J.Y. Uchida, Alfenas, T.S. Schubert, Alfieri & A.R. Chase | El-Gholl *et al.* 1992 | *Cylindrocladium spathiphylli* Schoulties, El-Gholl & Alfieri | Schoulties *et al.* 1982 |
| *Calonectria spathulata* El-Gholl, Kimbr. & E.L. Barnard | Crous & Wingfield 1994 | *Cylindrocladium spathulatum* El-Gholl, Kimbr. & E.L. Barnard | Crous & Wingfield 1994 |
| *Calonectria variabilis* Crous, B.J.H. Janse, D. Victor, G.F. Marais & Alfenas | Crous *et al.* 1993b | *Cylindrocladium variabile* Crous, B.J.H. Janse, D. Victor, G.F. Marais & Alfenas | Crous *et al.* 1993b |
| | | *Cylindrocladium angustatum* Crous & El-Gholl | Crous *et al.* 2000 |
| | | *Cylindrocladium australiense* Crous & K.D. Hyde | Crous *et al.* 2006 |
| | | *Cylindrocladium canadense* J.C. Kang, Crous & C.L. Schoch. | Kang *et al.* 2001b |
| | | *Cylindrocladium chinense* Crous | Crous *et al.* 2004b |
| | | *Cylindrocladium citri* Boedjin & Reitsma | Boedjin & Reitsma 1950 |
| | | *Cylindrocladium curvatum* Boedjin & Reitsma | Boedjin & Reitsma 1950 |

**Table 1.** (Continued)

| Teleomorph | Reference | Anamorph | Reference |
|---|---|---|---|
| | | *Cylindrocladium curvisporum* Crous & D. Victor | Victor *et al.* 1997 |
| | | *Cylindrocladium ecuadoriae* Crous & M.J. Wingf. | Crous *et al.* 2006 |
| | | *Cylindrocladium gordoniae* Leahy, T.S. Schubert & El-Gholl | Leahy *et al.* 2000 |
| | | *Cylindrocladium gracile* Boesewinkel | Boesewinkel 1982 |
| | | *Cylindrocladium hawksworthii* Peerally | Peerally 1991b |
| | | *Cylindrocladium hurae* (Linder & Whetzel) Crous | Crous 2002 |
| | | *Cylindrocladium indonesiae* Crous | Crous *et al.* 2004b |
| | | *Cylindrocladium leucothoes* El-Gholl, Leahy & T.S. Schubert | El-Gholl *et al.* 1989 |
| | | *Cylindrocladium malesianum* Crous | Crous *et al.* 2004b |
| | | *Cylindrocladium multiphialidicum* Crous, P. Simoneau & J-M. Riséde | Crous *et al.* 2004b |

**Table 1.** (Continued)

| Teleomorph | Reference | Anamorph | Reference |
|---|---|---|---|
| | | *Cylindrocladium pacificum* J.C. Kang, Crous & C.L. Schoch | Kang *et al*. 2001b |
| | | *Cylindrocladium penicilloides* Tubaki | Tubaki 1958 |
| | | *Cylindrocladium pseudonaviculatum* Crous | Crous *et al.* 2002 |
| | | *Cylindrocladium sumatrense* Crous | Crous *et al.* 2004b |

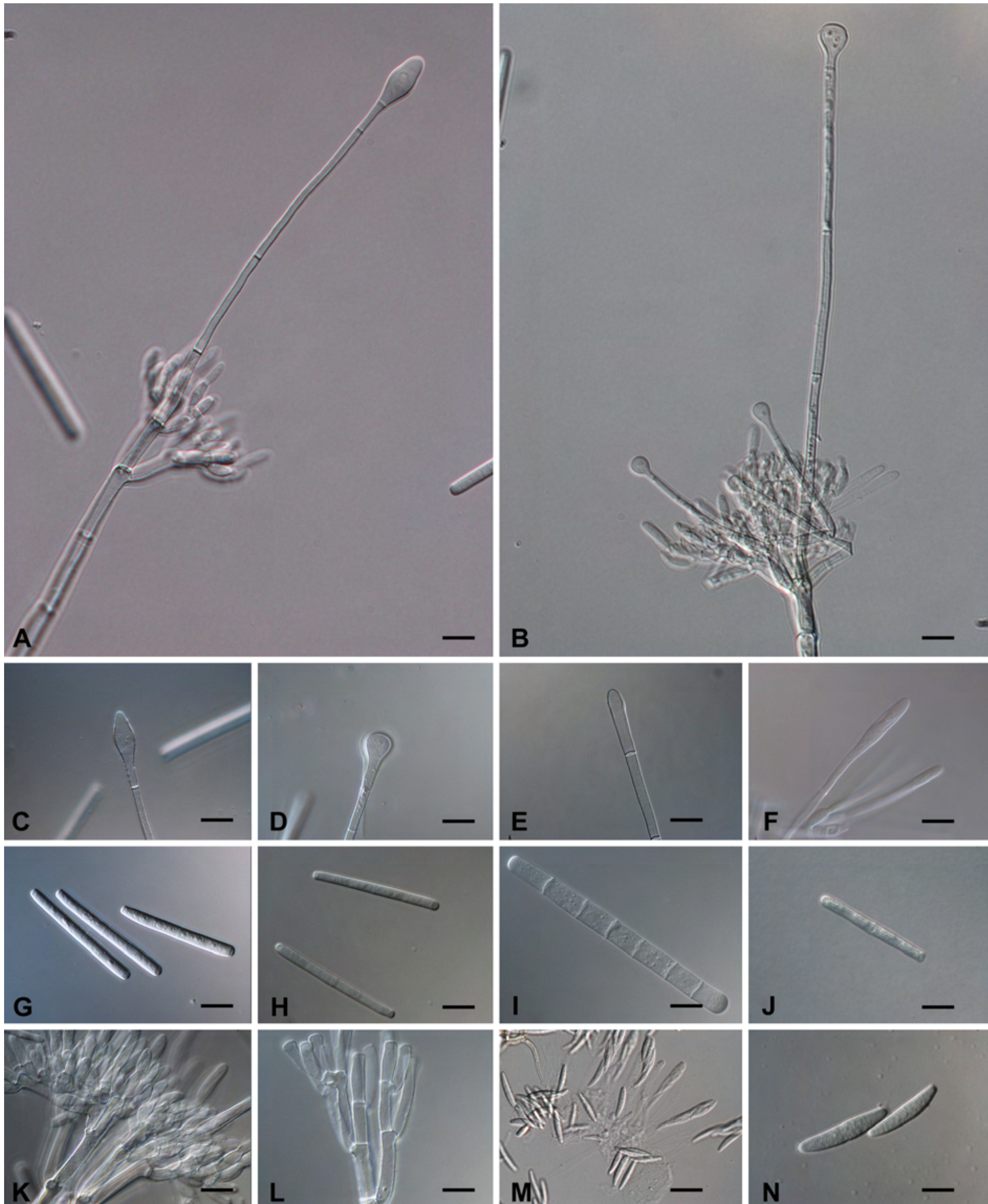
**Table 2.** Plant families that are hosts to *Calonectria* species and number of plant host species in each family.

| Host Plant family | Host species | Host Plant family | Host species | Host Plant family | Host species | Host Plant family | Host species |
|---|---|---|---|---|---|---|---|
| Actinidiaceae | 2 | Cornaceae | 1 | Malipighiaceae | 2 | Polypodiaceae | 1 |
| Altingiaceae | 1 | Crassulaceae | 1 | Malvaceae | 6 | Proteaceae | 7 |
| Anacardiaceae | 3 | Cupressaceae | 4 | Meliaceae | 2 | Pteridaceae | 1 |
| Annonaceae | 4 | Curcurbitaceae | 3 | Moraceae | 2 | Rhamnaceae | 1 |
| Aparagaceae | 1 | Cycadaceae | 1 | Musaceae | 2 | Rhizophoraceae | 1 |
| Apiaceae | 1 | Davalliaceae | 1 | Myristicaceae | 1 | Rosaceae | 10 |
| Apocynaceae | 2 | Dennstaedtiaceae | 1 | Myrsinaceae | 1 | Rubiaceae | 2 |
| Aquifoliaceae | 4 | Dilleniaceae | 1 | Myrtaceae | 31 | Ruscaceae | 1 |
| Araceae | 5 | Dipterocarpaceae | 1 | Nelumbonaceae | 1 | Rutaceae | 3 |
| Araliaceae | 2 | Dryopteridaceae | 2 | Nepenthaceae | 1 | Salicaceae | 3 |
| Arecaceae | 20 | Ebenaceae | 1 | Nothofagaceae | 1 | Sapindaceae | 4 |
| Armacariaceae | 2 | Ericaceae | 14 | Nymphaeaceae | 1 | Sapotaceae | 3 |
| Aspleniaceae | 1 | Euphorbiaceae | 6 | Oleaceae | 1 | Sarraceniaceae | 1 |

**Table 2.** (Continued).

| Host Plant family | Host species | Host Plant family | Host species | Host Plant family | Host species | Host Plant family | Host species |
|---|---|---|---|---|---|---|---|
| Asteraceae | 5 | Fabaceae | 57 | Onagraceae | 2 | Saxifragaceae | 1 |
| Berberidaceae | 2 | Fagaceae | 4 | Orchidaeae | 1 | Solanaceae | 4 |
| Betulaceae | 1 | Ginkgoaceae | 1 | Oryzeae | 1 | Sterculiaceae | 2 |
| Bixaceae | 1 | Juglandaceae | 2 | Phoeniceae | 1 | Strelilziaceae | 2 |
| Bromeliaceae | 3 | Lauraceae | 6 | Phytolaccaceae | 1 | Theaceae | 1 |
| Buxaceae | 1 | Laxmanniaceae | 1 | Pinaceae | 17 | Ulmaceae | 1 |
| Caricaceae | 2 | Lecythidaceae | 1 | Piperaceae | 1 | Verbenaceae | 1 |
| Caryophyllaceae | 1 | Leeaceae | 1 | Platanaceae | 1 | Vitaceae | 2 |
| Celastraceae | 1 | Linaceae | 1 | Plumbaginaceae | 1 | Vochysiaceae | 1 |
| Chenopodiaceae | 1 | Lomariopsidaceae | 1 | Poaceae | 5 | Xanthorrhoeaceae | 1 |
| Combretaceae | 3 | Lythraceae | 1 | Polygalaceae | 1 | Zingiberaceae | 1 |
| Convolvulaceae | 1 | Magnoliaceae | 2 | Polygonaceae | 3 | | |

**Fig. 1.** Morphological characteristic used for identification of *Calonectria* (= *Cylindrocladium*) species. A, B. Macroconidiophores. A. Macroconidiophore of *Ca. pauciramosa*. B. Macroconidiophore of *Ca. hongkongensis*. C–F. Vesicles. C. Obpyrifrom vesicle of *Ca. pauciramosa*. D. Sphaeropenduculate vesicle of *Ca. hongkongensis*. E, F. Clavate vesicle of *Cy. gracile*. G–I. Macroconidia. G. Macroconidia of *Ca. pauciramosa*. H. Macroconidia of *Ca. hongkongensis*. I. Macroconidia of *Ca. reteaudii*. J. Microconidia of *Ca. reteaudii*. K, L. Fertile branches. K. Fertile branches with doliiform to reniform phialides of *Ca. pauciramosa*. L. Fertile branches of *Ca. reteaudii* with cylindrical to allantoid phialides. M. Asci of *Ca. hongkongensis* with ascospore. N. Ascospores of *Ca. hongkongensis*. Scale bars A, B, M = 20 µm, C–L, N = 10 µm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# CHAPTER 2

## *Calonectria* (*Cylindrocladium*) species associated with dying *Pinus* cuttings

Published as: Lombard L, Rodas CA, Crous PW, Wingfield BD, Wingfield MJ. (2009). *Calonectria* (*Cylindrocladium*) species associated with dying *Pinus* cuttings. *Persoonia* **23**: 41–47.

**ABSTRACT**

*Calonectria* (*Ca.*) species and their *Cylindrocladium* (*Cy.*) anamorphs are well-known pathogens of forest nursery plants in subtropical and tropical areas of the world. An investigation of the mortality of rooted *Pinus* cuttings in a commercial forest nursery in Colombia led to the isolation of two *Cylindrocladium* anamorphs of *Calonectria* species. The aim of this study was to identify these species by using DNA sequence data and morphological comparisons. Two species were identified, namely one undescribed species, and *Cy. gracile*, which is allocated to *Calonectria* as *Ca. brassicae*. The new species, *Calonectria brachiatica* sp. nov., resides in the *Ca. brassicae* species complex. Pathogenicity tests with *Ca. brachiatica* and *Ca. brassicae* showed that both are able to cause disease on *Pinus maximinoi* and *P. tecunumanii*. An emended key is provided to distinguish between *Calonectria* species with clavate vesicles and 1-septate macroconidia.

**Taxonomic novelties:** *Calonectria brassicae* (Panwar & Bohra) L. Lombard, M.J. Wingf. & Crous comb. nov., *Calonectria brachiatica* L. Lombard, M.J. Wingf. & Crous. sp. nov.

**INTRODUCTION**

Species of *Calonectria* (anamorph *Cylindrocladium*) are plant pathogens associated with a large number of agronomic and forestry crops in temperate, sub-tropical and tropical climates, worldwide (Crous & Wingfield 1994, Crous 2002). Infection by these fungi gives rise to symptoms including cutting rot (Crous *et al.* 1991), damping-off (Sharma *et al.* 1984, Ferreira 1995), leaf spot (Sharma *et al.* 1984, Ferreira *et al.* 1995, Crous *et al.* 1998), shoot blight (Crous *et al.* 1991, Crous *et al.* 1998), stem cankers (Sharma *et al.* 1984, Crous *et al.* 1991) and root disease (Mohanan & Sharma 1985, Crous *et al.* 1991) on various forest trees species.

The first report of *Ca. morganii* (as *Cy. scoparium*) infecting *Pinus* species was by Graves (1915), but he failed to re-induce the symptoms and assumed that it was a saprobe. There have subsequently been several reports of *Cylindrocladium* spp. infecting *Pinus* and other conifers, leading to root rot, stem cankers and needle blight (Jackson 1938, Cox 1953, Thies & Patton 1970, Sober & Alfieri 1972, Cordell & Skilling 1975, Darvas *et al.* 1978, Crous *et al.* 1991, Crous 2002). Most of these reports implicated *Ca. morganii* and *Ca. pteridis* (as *Cy. macrosporum* or *Cy. pteridis*) as the primary pathogens (Thies & Patton 1970, Ahmad & Ahmad 1982). However, as knowledge of these fungi has grown, together with refinement of their taxonomy applying DNA sequence comparisons (Crous *et al.* 2004, 2006), several additional *Cylindrocladium* species have been identified as causal agents of disease on different conifer species. These include *Ca. acicola*, *Ca. colhounii*, *Ca. kyotensis* (= *C. floridanum*), *Ca. pteridis, Cy. canadense*, *Cy. curvisporum, Cy. gracile* and *Cy. pacificum* (Hodges & May 1970, Crous 2002, Gadgil & Dick 2004, Taniguchi *et al.* 2008).

In a recent survey, wilting, collar and root rot symptoms were observed in Colombian nurseries generating *Pinus* spp. from cuttings. Isolations from these diseased plants consistently yielded *Cylindrocladium* anamorphs of *Calonectria* species, and hence the aim of this study was to identify them, and to determine if they were the causal agents of the disease in Colombian nurseries.

## MATERIAL AND METHODS

### Isolates

*Pinus maximinoi* and *P. tecunumanii* rooted cutting plants showing symptoms of collar and root rot (Fig. 1) were collected from a nursery close to Buga in Colombia. Isolations were made directly from lesions on the lower stems and roots on fusarium selective medium (FSM; Nelson *et al.* 1983) and malt extract agar (MEA, 2 % w/v; Biolab, Midrand, South Africa). After 5 d of incubation at 25 °C, fungal colonies of *Calonectria* spp. were transferred on to MEA and incubated further for 7 d. For each isolate, single conidial cultures were prepared on MEA, and representative strains are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

### Taxonomy

For morphological identification of *Calonectria* isolates, single conidial cultures were prepared on MEA and synthetic nutrient-poor agar (SNA; Nirenburg 1981). Inoculated plates were incubated at room temperature and examined after 7 d. Gross morphological characteristics were assessed by mounting fungal structures in lactic acid. Thirty measurements at ×1 000 magnification were made for each isolate. The 95 % confidence levels were determined for the pooled measurements of the respective species studied and extremes for structure sizes are given in parentheses. Optimal growth temperatures were determined between 6–36 ºC at 6 ºC intervals in the dark on MEA for each isolate. Colony reverse colours were determined after 7 d on MEA at 24 ºC in the dark, using the colour charts of Rayner (1970) for comparison.

### DNA phylogeny

*Calonectria* isolates were grown on MEA for 7 d. Mycelium was then scraped from the surfaces of the cultures, freeze-dried, and ground to a powder in liquid nitrogen, using a mortar and pestle. DNA was extracted from the powdered mycelium as described by Lombard *et al.* (2008). A fragment of the β-tubulin gene region was amplified and sequenced using primers T1 (O'Donnell & Cigelnik 1997) and CYLTUB1R (Crous *et al.* 2004) and a fragment for the histone H3 (HIS3) gene region was sequenced using primers CYLH3F and CYLH3R (Crous *et al.* 2004).

The PCR reaction mixture used to amplify the different loci consisted of 2.5 units FastStart *Taq* polymerase (Roche Applied Science, USA), 10× PCR buffer, 1–1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 µm of each primer and approximately 30 ng of fungal genomic DNA, made up to a total reaction volume of 25 µL with sterile distilled water.

Amplified fragments were purified using High Pure PCR Product Purification Kit (Roche, USA) and sequenced in both directions. For this purpose, the BigDye terminator sequencing kit v. 3.1 (Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on an Eppendorf Mastercycler Personal PCR (Eppendorf AG, Germany) with cycling conditions as described in Crous *et al.* (2006) for each locus.

Sequences generated were added to other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) and were assembled and aligned using Sequence Navigator v. 1.0.1 (Applied Biosystems) and MAFFT v. 5.11 (Katoh *et al.* 2005), respectively. The aligned sequences were then manually corrected where needed.

PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2002) was used to analyse the DNA sequence datasets. A partition homogeneity test (Farris *et al.* 1994) and a 70 % reciprocal bootstrap method (Mason-Gamer & Kellog 1996) were applied to evaluate the feasibility of combining the data sets. Phylogenetic relationships were estimated by heuristic searches based on 1 000 random addition sequences and tree bisection-reconnection, with the branch swapping option set on 'best trees' only.

All characters were weighted equally and alignment gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analysis (Hillis & Bull 1993) was based on 1 000 replications. All sequences for the isolates studied were analysed using the Basic Local Alignment Search Tool for Nucleotide sequences (BLASTN, Altschul *et al.* 1990). The phylogenetic analysis included 19 partial gene sequences per gene, representing eight *Calonectria* species (Table 1) closely related to the isolates studied. *Calonectria colombiensis* was used as the outgroup taxon. All sequences were deposited in GenBank and the alignments in TreeBASE (http://treebase.org).

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). Models of nucleotide substitution for each gene were determined using Mrmodeltest (Nylander 2004) and included for each gene partition. Four MCMC chains were run simultaneously from random trees for one million generations and sampled every 100 generations. The first 800 trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees.

**Pathogenicity tests**

In order to test the pathogenicity of the *Calonectria* spp. collected in this study, profusely sporulating isolates CMW 25293, representing *Ca. brachiatica*, CMW 25296 and CMW 25297, both representing *Ca. brassicae*, were used for inoculations onto rooted cuttings of *P. maximinoi.* Isolate CMW 25299, representing *Ca. brassicae* and isolates CMW 25302 and CMW 25307 representing *Ca. brachiatica* were used for inoculations onto rooted cuttings of *P. tecunumanii*. Trees used for inoculation were between 0.5–1 m in height and 10–50 mm diam at the root collar. Trees were maintained in a greenhouse under controlled conditions prior to inoculation, so that they could become acclimatised and to ensure that they were healthy. Sixty trees for each *Pinus* spp. were used and an additional 60 trees were used as controls. This resulted in a total of 180 trees in the pathogenicity tests.

Inoculations were preformed in the greenhouse by making a 5 mm diam wound on the main stems of plants with a cork borer to expose the cambium. The cambial discs were replaced with an MEA disc overgrown with the test fungi taken from 7 d old cultures. The inoculum discs were placed mycelium side facing the cambium and the inoculation points were sealed with Parafilm to reduce contamination and desiccation. Control trees were treated in a similar fashion but inoculated with a sterile MEA plug.

Six weeks after inoculation, lesion lengths on the stems of the plants were measured. The results were subsequently analysed using SAS Analytical Programmes v. 2002. Re-isolations were made from the edges of lesions on the test trees to ensure the presence of the inoculated fungi.

**RESULTS**

**DNA phylogeny**

For the β-tubulin gene region, approx. 580 bases were generated for each of the isolates used in the study (Table 1). The adjusted alignment included 19 taxa with the outgroup, and 523 characters including gaps after uneven ends were removed from the beginning of each sequence. Of these characters, 459 were constant and uninformative. For the analysis, only the 64 parsimony informative characters were included. Parsimony analysis of the aligned sequences yielded five most parsimonious trees (TL = 231 steps; CI = 0.870; RI = 0.799; RC = 0.695; results not shown).

Sequences for the histone gene region consisted of approx. 460 bases for the isolates used in the study and the adjusted alignment of 19 taxa including the outgroup, consisted of 466 characters including gaps. Of these characters, 391 were excluded as constant and parsimony uninformative and 79 parsimony informative characters included. Analysis of the aligned data yielded one most parsimonious tree (TL = 290 steps; CI = 0.845; RI = 0.807; RC = 0.682; results not shown).

The partition homogeneity test showed that the β-tubulin and histone data set could be combined (P = 0.245). The 70 % reciprocal bootstrap method indicated no conflict in tree topology among the two partitions, resulting in a combined sequence data set consisting of 993 characters including gaps for the 19 taxa (including outgroup). Of these, 850 characters were constant and parsimony uninformative and excluded from the analysis. There were 143 characters in the analysis that were parsimony informative. Parsimony analysis of the combined alignments yielded one most parsimonious tree (TL = 526 steps; CI = 0.848; RI = 0.791; RC = 0.670), which is presented in Fig. 2 (TreeBase S2568).

All the isolates obtained from the *Pinus* spp. used in this study grouped in the *Ca. brassicae* species complex with a bootstrap (BP) value of 96 and a low Bayesian posterior probability (PP) of 0.70. This clade was further subdivided into two clades. The first clade (BP = 64, PP below 0.70) representing *Ca. brassicae*, included the type of *Cy. gracile* and *Cy. clavatum*. It also included three isolates (CMW 25297, CMW 25296 and CMW 25299) from *P. maximinoi* and *P. tecunumanii*. The second clade (BP = 98, PP = 0.82) accommodated *Calonectria* isolates (CMW 25293, CMW 25298, CMW 25302 and CMW 25307), representing what we recognize as a distinct species. The consensus tree obtained with Bayesian analysis showed topographical similarities with the most parsimonious tree as indicated in Fig. 2.

### Pathogenicity tests

All plants inoculated with *Calonectria* spp. in this study developed lesions. Lesions included discolouration of the vascular tissue with abundant resin formation, 6 wk after inoculation. Lesions on the control trees were either non-existent or small, representing wound reactions. There were significant ($p < 0.0001$) differences in lesion lengths associated with individual isolates used on *P. maximinoi* (Fig. 3). Comparisons of the lesion lengths clearly showed that *Ca. brassicae* (CMW 25297) produced the longest average lesions (av. = 30.04 mm) compared to the undescribed *Calonectria* sp. (CMW 25293) (av. = 14.41mm). The other *Ca. brassicae* isolate (CMW 25296) produced an average lesion length of 15.30 mm. Lesions on the control trees were an average of 8.84 mm and significantly ($p < 0.0001$) smaller than those on any of the trees inoculated with the test fungi (Fig. 5).

Results of inoculations on *P. tecunumanii* were similar to those on *P. maximinoi*. Thus, *Ca. brassicae* (CMW 25299) (av. = 20.64 mm) produced the longest lesions compared with the undescribed *Calonectria* sp. (CMW 25302; av. = 18.63 mm and CMW 25307; av. = 15.20 mm). The lesions on the *P. tecunumanii* control trees were also significantly ($p < 0.0001$) smaller (av. = 8.82 mm) than those on any of the trees inoculated with the test fungi. Re-isolations from the test trees consistently yielded the inoculated fungi and no *Calonectria* spp. were isolated from the control trees.

### Taxonomy

Isolates CMW 25296, CMW 25297 and CMW 25299 clearly represent *Ca. brassicae* based on morphological observations (Crous 2002) and comparisons of DNA sequence data. Isolates CMW 25293, CMW 25298, CMW 25302 and CMW 25307 represent an undescribed species closely related to *Ca. brassicae* but morphologically distinct. Species of *Cylindrocladium* (1892) represent anamorph states of *Calonectria* (1867) (Rossman *et al.* 1999), and therefore this fungus is described as a new species of *Calonectria,* which represents the older generic name for these holomorphs:

***Calonectria brachiatica*** L. Lombard, M.J. Wingf. & Crous, **sp. nov.** MycoBank MB512998. Fig. 4

*Etymology*: Name refers to the lateral branches on the conidiophore stipes .

Stipa extensiones septatum, hyalinum, 134–318 µm, in vesiculam clavatum, 5–7 µm diam terminans. Conidia cylindrica, hyalina, 1–2-septata, utrinque obtusa, (37–)40–48(–50) × 4–6 µm.

*Teleomorph* unknown. *Conidiophores* with a stipe bearing penicillate suites of fertile branches, stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 32–67 × 6–8 µm; stipe extensions septate, straight to flexuous, 134–318 µm long, 4–5 µm wide at the apical septum, terminating in a clavate vesicle, 5–7 µm diam; lateral stipe extensions (90° to the axis) also present. *Conidiogenous apparatus* 40–81 µm long, and 35–84 µm wide; primary branches aseptate or 1-septate, 15–30 × 4–6 µm; secondary branches aseptate, 10–23 × 3–5 µm; tertiary branches and additional branches (–5), aseptate, 10–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 10–15 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. *Macroconidia* cylindrical, rounded at both ends, straight, (37–)40–48(–50) × 4–6 µm (av. = 44 × 5 µm), 1(–2)-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. *Mega-* and *microconidia* not seen.

*Specimens examined* : **Colombia**, Valle del Cauca, Buga, from *Pinus maximinoi*, July 2007, M.J. Wingfield, Herb. PREM 60197, **holotype** of *Ca. brachiatica*, culture ex-type CMW 25298 = CBS 123700; Valle del Cauca, Buga, from *P. tecunumanii*, July 2007, M.J. Wingfield, culture CMW 25303 = CBS 123699; Valle del Cauca, Buga, from *P. tecunumanii,* July 2007, M.J. Wingfield, (Herb. PREM 60198) culture CMW 25341 = CBS 123703.

*Cultural characteristics*: Colonies fast growing with optimal growth temperature at 24 ºC (growth at 12–30 ºC) on MEA, reverse amber (13k) to sepia (13i) brown after 7 d; abundant white aerial mycelium with moderate to extensive sporulation; chlamydospores extensive throughout the medium.

*Substrate*: *Pinus maximinoi*, *P. tecunumanii*.

*Distribution*: Colombia.

*Notes*: The anamorph state of *Ca. brachiatica* can be distinguished from *C. gracile*, *C. pseudogracile* and *C. graciloideum* by its shorter macroconidia. Another characteristic distinguishing *Ca. brachiatica* is the formation of lateral branches not reported for *C. gracile* or other closely related species.

***Calonectria brassicae*** (Panwar & Bohra) L. Lombard, M.J. Wingf. & Crous, **comb. nov.** MycoBank MB513423. Fig. 5

*Basionym*. *Cylindrocladium brassicae* Panwar & Bohra, Indian Phytopathology 27: 425. 1974.

= *Cylindrocarpon gracile* Bugnic., Encycl. Mycologique 11: 162. 1939.

≡ *Cylindrocladium gracile* (Bugnic.) Boesew., Trans. Brit. Mycol. Soc. 78: 554. 1982.

= *Cylindrocladium clavatum* Hodges & L.C. May, Phytopathology 62: 900. 1972.

*Notes*: Both the names *Ca. clavata* and *Ca. gracilis* and are already occupied, hence the oldest available epithet is that of *Cy. brassicae* (Crous 2002).

**DISCUSSION**

Results of this study show that *Calonectria* species are important pathogens in pine cutting nurseries in Colombia. In this case, two species were discovered, the one newly described here as *Ca. brachiatica* and the other representing *Ca. brassicae*. Both of the species were pathogenic on *P. maximinoi* and *P. tecunumanii*.

The description of *Ca. brachiatica* from *P. maximinoi* and *P. tecunumanii* adds a new species to the *Ca brassicae* species complex, which already includes six other *Calonectria* species (Crous 2002, Crous *et al.* 2006). This species can be distinguished from the other species in the complex by the formation of lateral branches on the macroconidiophores and the presence of a small number of 2-septate macroconidia. Macroconidial dimensions (av. = 44 × 5 µm) are also smaller then those of *Ca. brassicae* (av. 53 × 4.5 µm).

A recent study of *Calonectria* spp. with clavate vesicles by Crous *et al.* (2006) attempted to resolve the taxonomic status of these species, and added two new species to the group. Crosses among isolates of *Ca. brachiatica* and isolates of *Ca. brassicae*, did not result in sexual structures in the present study, and teleomorphs are rarely observed in this species complex.

Hodges & May (1972) reported *Ca. brassicae* (as *Cy. clavatum*) from several *Pinus* species in nurseries and plantations in Brazil. Subsequent studies based on comparisons of DNA sequence data revealed *Cy. clavatum* to be a synonym of *Cy. gracile* (Crous *et al.* 1995, 1999, Schoch *et al.* 2001). *Calonectria brassicae* (*as Cy. gracile*) is a well-known pathogen of numerous plant hosts in subtropical and tropical areas of the world. However, in Colombia,

this plant pathogen has been isolated only from soil (Crous 2002, Crous *et al.* 2006). This study thus represents the first report of *Ca. brassicae* infecting *Pinus* spp. in Colombia.

Pathogenicity tests with isolates of *Ca. brachiatica* and *Ca. brassicae* clearly showed that they are able to cause symptoms similar to those observed in naturally infected plants. Both *P. maximinoi* and *P. tecunumanii* were highly susceptible to infection by *Ca. brassicae*. This supports earlier work of Hodges & May (1972) in Brazil, where they reported a similar situation. In their study, seven *Pinus* spp. were wound-inoculated with *Ca. brassicae* and this resulted in mortality of all test plants within 2 wk. Although they did not include *P. maximinoi* and *P. tecunumanii* in the study, they concluded that the pathogen is highly virulent and regarded it as unique in causing disease symptoms in established plantations of *Pinus* spp. No disease symptoms associated with *C. brachiatica* or *Ca. brassicae* were seen in established plantations in the present study and we primarily regard these fungi as nursery pathogens, of which the former species is more virulent than the latter.

The use of SNA (Nirenburg 1981) rather than carnation leaf agar (CLA; Fisher *et al.* 1982) for morphological descriptions of *Calonectria* species represents a new approach employed in this study. Previously, species descriptions for *Calonectria* have typically been conducted on carnation leaf pieces on tap water agar (Crous *et al.* 1992). However, carnation leaves are not always readily available for such studies and SNA, a low nutrient medium, also used for the related genera *Fusarium* and *Cylindrocarpon* species identification (Halleen *et al.* 2006, Leslie & Summerell 2006), provides a useful medium for which the chemical components are readily available. Another advantage of using SNA is its transparent nature, allowing direct viewing through a compound microscope as well as on mounted agar blocks for higher magnification (Leslie & Summerell 2006). In this study, it was found that the *Calonectria* isolates sporulate profusely on the surface of SNA and comparisons of measurements for structures on SNA and those on CLA showed no significant difference. However, CLA remains important to induce the formation of teleomorph structures in homothallic isolates or heterothallic isolates for which both mating types are present.

**Key to *Calonectria* species with clavate vesicles and predominantly 1-septate macroconidia** (To be inserted in Crous 2002, p. 56, couplet no. 2)

2. Stipe extention thick-walled; vesicle acicular to clavate ............................. ***Ca. avesiculata***

2. Stipe extention not thick-walled; vesicle clavate ........................................................ 3

3. Teleomorph unknown ........................................................................................ 4
3. Teleomorph readily formed ............................................................................... 7

4. Macroconidia always 1–(–2)-septate .................................................................. 5
4. Macroconidia 1–(–3)-septate ............................................................................ 6

5. Macroconidia 1-septate, (38–)40–55(–65) × (3.5–)4–5(–6) µm, av. = 53 × 4.5 µm; lateral stipe extensions absent ......................................................................... ***Ca. brassicae***
5. Macroconidia 1–(–2)-septate, (37–)40–48(–50) × 4–6 µm, av. = 44 × 5 µm; lateral stipe extensions present .......................................................................... ***Ca. brachiatica***

6. Macroconidia (48–)57–68(–75) × (6–)6.5(–7) µm, av. = 63 × 6.5 µm ***Cy. australiense***
6. Macroconidia (45–)48–55(–65) × (4–)4.5(–5) µm, av. = 51 × 4.5 µm .. ***Cy. ecuadoriae***

7. Macroconidial state absent; megaconidia and microconidia present .... ***Ca. multiseptata***
7. Macroconidial state present ............................................................................. 8

8. Teleomorph homothallic..................................................................................... 9
8. Teleomorph heterothallic................................................................................... 10

9. Perithecia orange; macroconidia av. size = 45 × 4.5 µm .......................... ***Ca. gracilipes***
9. Perithecia red; macroconidia av. size = 56 × 4.5 µm ................................... ***Ca. gracilis***

10. Perithecia orange; macroconidia av. size = 32 × 3 µm .............................. ***Ca. clavata***
10. Perithecia red-brown; macroconidia av. size = 30 × 3 µm ......................... ***Ca. pteridis***

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**Table 1.** Strains of *Calonectria* (*Cylindrocladium*) species included in the phylogenetic analyses (TreeBase S2568)

| Species | Isolate number[1] | β–tubulin[2] | Histone H3[2] | Host | Origin | Collector |
|---|---|---|---|---|---|---|
| *Ca. avesiculata* | CBS 313.92[T] | AF333392 | DQ190620 | *Ilex vomitoria* | USA | S.A. Alfieri |
| *Ca. brachiatica* sp. nov. | CMW 25293 | FJ716710 | FJ716714 | *P. maximinoi* | Colombia | M.J. Wingfield |
| | CMW 25298 (= CBS 123700)[T] | FJ696388 | FJ696396 | *P. maximinoi* | Colombia | M.J. Wingfield |
| | CMW 25302 | FJ716708 | FJ716712 | *P. tecunumanii* | Colombia | M.J. Wingfield |
| | CMW 25307 | FJ716709 | FJ716713 | *P. tecunumanii* | Colombia | M.J. Wingfield |
| *Ca. brassicae* com. nov. | CBS 111869[T] | AF232857 | DQ190720 | *Argyreia* sp. | South East Asia | |
| | CBS 111478 | DQ190611 | DQ190719 | Soil | Brazil | A.C. Alfenas |
| | CMW 25296 | FJ716707 | FJ716711 | *P. maximinoi* | Colombia | M.J. Wingfield |
| | CMW 25297; CBS123702 | FJ696387 | FJ696395 | *P. maximinoi* | Colombia | M.J. Wingfield |
| | CMW 25299; CBS123701 | FJ696390 | FJ696398 | *P. tecunumanii* | Colombia | M.J. Wingfield |
| *Ca. clavata* | CBS 114557[T] | AF333396 | DQ190623 | *Callistemon viminalis* | USA | N.E. El-Gholl |
| | CBS 114666 | DQ190549 | DQ190624 | *Pinus caribaea* | Brazil | C.S. Hodges |

**Table 1.** (Continued)

| Species | Isolate number[1] | β–tubulin[2] | Histone H3[2] | Host | Origin | Collector |
|---|---|---|---|---|---|---|
| *Cy. clavatum* (= *Cy. gracile*) | CBS111776[T] | AF232850 | DQ190700 | *Pinus caribaea* | Brazil | C.S. Hodges |
| *Ca. colombiensis* | CBS 12221 | AY725620 | AY725663 | Soil | Colombia | M.J. Wingfield |
| *Cy. ecuadoriae* | CBS 111406[T] | DQ190600 | DQ190705 | Soil | Ecuador | M.J. Wingfield |
| *Ca. gracilipes* | CBS 111141[T] | DQ190566 | DQ190644 | *Eucalyptus* sp. | Colombia | M.J. Wingfield |
| | CBS 115674 | AF333406 | DQ190645 | Soil | Colombia | M.J. Wingfield |
| *Ca. gracilis* | CBS 111284 | DQ190567 | DQ190647 | *Manilkara* sp. | Brazil | P.W. Crous |
| | CBS 111807[T] | AF232858 | DQ190646 | | Brazil | |

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria Pretoria, South Africa; [2]GenBank accession numbers. [T]Ex-type culture.

**Fig. 1.** Collar and root rot on *Pinus maximinoi* and *P. tecunumanii*. A. Girdled stem of *P. maximinoi*. B. Exposed *P. maximinoi* root collar showing discolouration and resin exudation. C, D. Exposed *P. tecunumanii* root collars showing girdling and discolouration of the cambium.

A
B
C
D

**Fig. 2.** The most parsimonious tree obtained from a heuristic search with 1000 random addition of the combined β-tubulin and Histone H3 sequence alignments. Scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown above the branches. Bayesian posterior probabilities are indicated below the branches. Red lines indicates bootstrap support value of 100 and posterior probability value of 1.00. Bold lines indicate branches present in the Bayesian consensus tree. The tree was rooted with *Calonectria colombiensis* (CBS 112221).

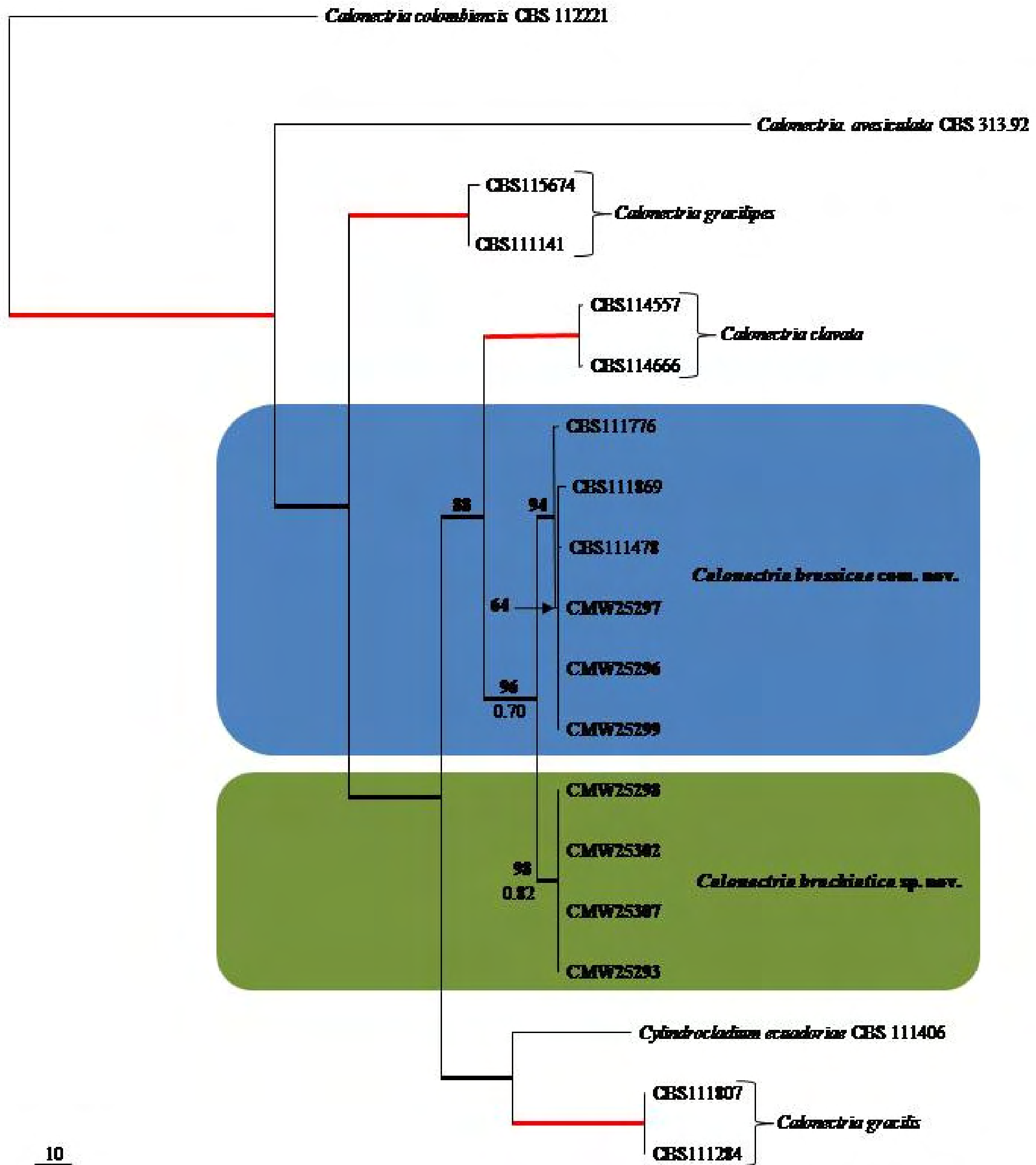

Calonectria colombiensis CBS 112221
Calonectria avesiculata CBS 313.92
CBS115674
CBS111141
Calonectria gracilipes
CBS114557
CBS114666
Calonectria clavata
CBS111776
CBS111869
CBS111478
CMW25297
CMW25296
CMW25299
Calonectria brassicae comb. nov.
88
94
64
96
0.70
CMW25298
CMW25302
CMW25307
CMW25293
98
0.82
Calonectria brachiatica sp. nov.
Cylindrocladium ecuadoriae CBS 111406
CBS111807
CBS111284
Calonectria gracilis
10

**Fig. 3.** Histogram showing mean lesion lengths induced by each isolate on *P. maximinoi* and *P. tecunumanii*. *Calonectria brassicae* is represented by CMW 25296, CMW 25297 and CMW 25299. *Ca. brachiatica* is represented by CMW 25293, CMW 25302 and CMW 25307.

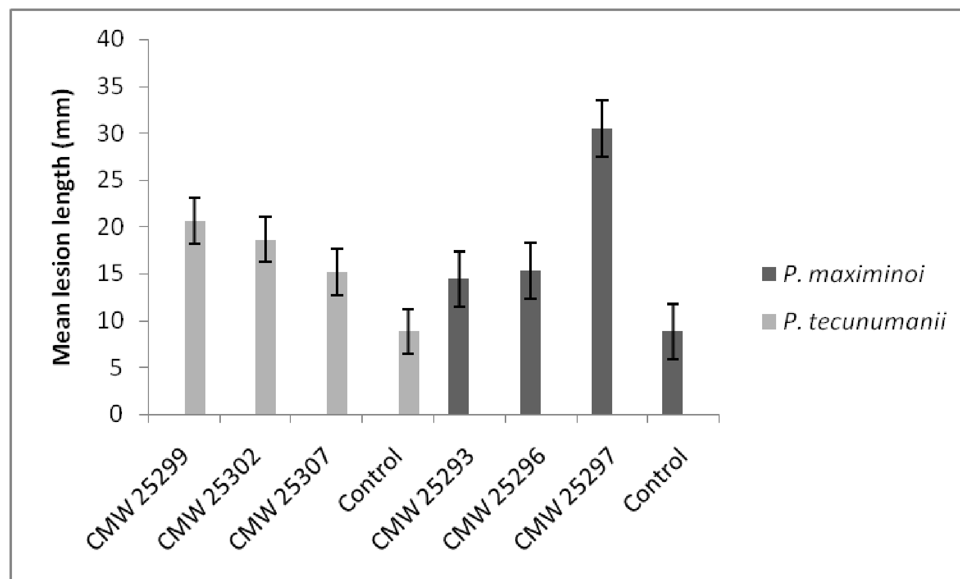

Mean lesion length (mm)
40
35
30
25
20
15
10
5
0
CMW 25299
CMW 25302
CMW 25307
Control
CMW 25293
CMW 25296
CMW 25297
Control
P. maximinoi
P. tecunumanii

**Fig. 4.** *Calonectria brachiatica*. A. Macroconidiophore with lateral branching stipe extentions. B, C. Clavate vesicles. D. Fertile branches. E. Macroconidia. Scale bars A = 20 µm, B–E = 10 µm.

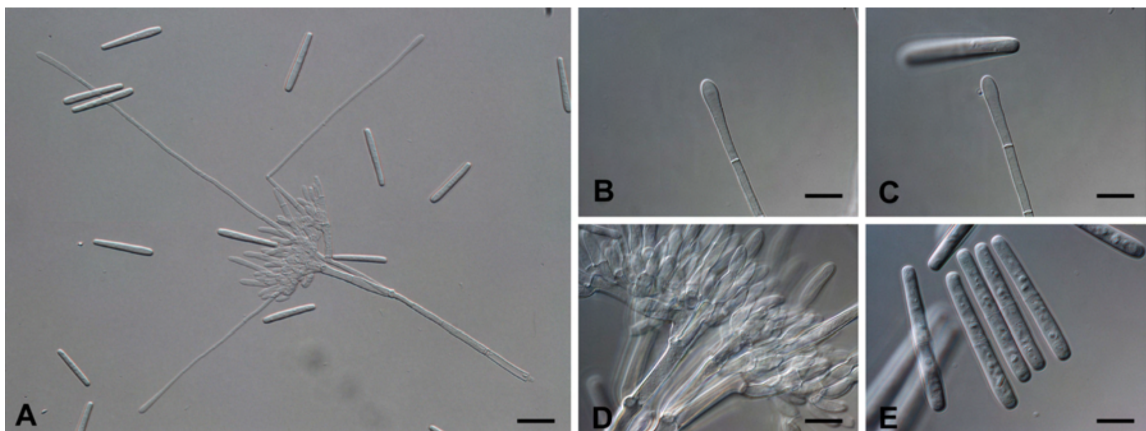
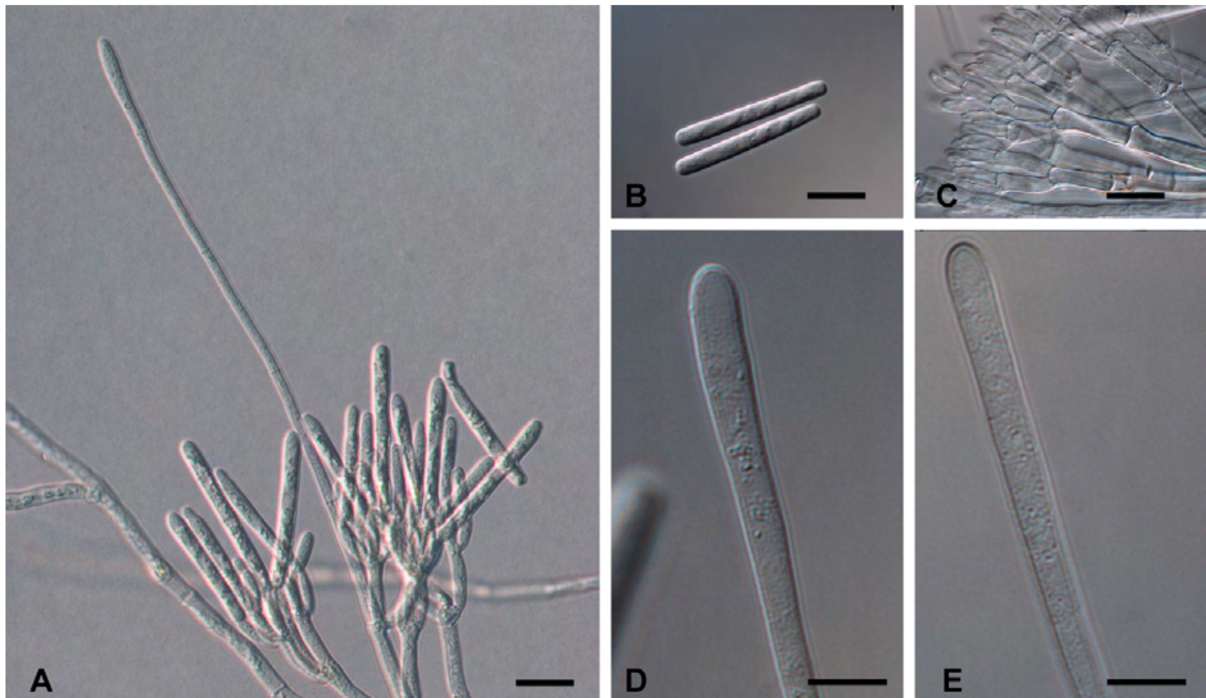

A
B
C
D
E

**Fig. 5.** *Calonectria brassicae*. A. Macroconidiophore on SNA. B. Macroconidia. C. Fertile branches. D, E. Clavate vesicles. Scale bars A = 20 µm, B–E = 10 µm.


A
B
C
D
E