The enigma of Calonectria species occurring on leaves of Ilex aquifolium in Europe

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Abstract: Species of Calonectria are common saprobes and plant pathogens on a wide range of hosts occurring in subtropical to tropical regions of the world. The aim of the present study was to resolve the status of new Calonectria collections obtained on Ilex leaves from France. Based on DNA sequence data of their β-tubulin and histone gene regions, as well as morphology, the new collections matched the ex-type strain of Cylindrocladium ilicicola. On the host and in culture, yellow to brownish-yellow perithecia were observed that did not strain red in 3 % KOH. Based on these results, C. ilicicola and its purported teleomorph, Ca. pyrochroa, were shown to represent two distinct species, as the latter has bright red perithecia that strain purple in KOH. A new combination, Ca. lauri, based on Tetracytum lauri, is subsequently proposed for C. ilicicola. Calonectria lauri is distinct from Ca. ilicicola, a pathogen commonly associated with Cylindrocladium black rot of peanut. Finally, Ca. canadiana is proposed as new name for Cy. canadiense, which is a nursery pathogen involved with root rot of several tree genera in Quebec, Canada.

INTRODUCTION

Species of Calonectria are members of the Nectriaceae (Hypocreales, Ascomycetes) (Lombard 2010a–c). The Nectriaceae is characterised by having uniloculate, orange to purple, superficial ascomata (Rossman et al. 1999). Calonectria is easily distinguished from other members of the family based on its Cylindrocladium anamorphs. Formerly Cylindrocladium also included members of Cylindrocladiella, a genus that accommodates Cylindrocladium-like species with small conidia (Boesewinkel 1982, Victor et al. 1998) and Nectriciella telemorphs (Schoch et al. 2000). Other morphologically similar genera that have also since been separated from this complex include Xenocylinododium (Decock et al. 1997), Curvicladiella (Crous et al. 2006a) and Dematiocladium (Crous et al. 2005). Following the approach of Crous et al. (2006b, 2008, 2009a, b) with other fungal groups, Lombard et al. (2009, 2010a–d) chose to use the older Calonectria name for the genus, irrespective whether the teleomorph or Cylindrocladium anamorph, unnamed microconidial, megaconidial, or chlamydospore-like synanamorph was observed. All taxa are since accommodated in Calonectria, which is a monophyletic genus (Lombard et al. 2010a–c).

Most species of Calonectria occur commonly in soil, especially in subtropical to tropical regions of the world. Although the genus was originally regarded as saprobic (Graves 1915), taxa have since been proven to be important plant pathogens, associated with a wide host range of plants, causing disease symptoms ranging from leaf spots to stem cankers, damping off, cutting rot, root and fruit rot (Crous et al. 2004b, 2006a, Lombard et al. 2009, 2010a, d). Major diseases attributed to Calonectria infections include Cylindrocladium black rot of Arachis hypogea (peanut), and red crown rot of Glycine max (soybean) (Crous et al. 1993, Wright et al. 2010), as well as root rot and leaf diseases of numerous diverse hosts (Crous et al. 2004b, 2006a).

Over the past few years, a species of Calonectria was collected from leaves of Ilex aquifolium in France. Presently four species of Calonectria have been described from Ilex (Aquifoliaceae), namely Calonectria morganii on Ilex paraguayensis in Argentina, and Ilex vomitoria in Florida (USA); Calonectria avesiculata on Ilex spp. in Georgia and Florida (USA). Cylindrocladium ilicicola (as Calonectria pyrochroa) on Ilex aquifolium on Clare...
Isolates Single ascospore isolates were obtained from leaves of *Ilex aquifolium* as explained in Crous & Wingfield (1994). Isolates were incubated on plates of 2 % malt extract agar (MEA), 2 % potato-dextrose agar (PDA) and oatmeal agar (OA) (Crous *et al*. 2009c) for 7 d at 25 °C under continuous near-UV light, to promote sporulation. Reference strains are maintained in the CBS-KNAW Fungal Biodiversity Centre (CBS) Utrecht, The Netherlands.

DNA isolation, amplification and analyses Genomic DNA was isolated from fungal mycelium grown on MEA, using the UltraCleanTM Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA, U.S.A.) according to the manufacturer’s protocol. Two loci were amplified and sequenced as explained in Crous *et al*. (2004b) and Lombard *et al*. (2010c), namely, part of the β-tubulin gene (TUB), amplified with primers T1 (O’Donnell & Cigelnik 1997) and CYLTUB1R (Crous *et al*. 2004b); and part of the histone H3 gene (HIS) using primers CYLH3F and CYLH3R (Crous *et al*. 2004b). Part of the nuclear rDNA operon spanning the 3’ end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the 5’ end of the 28S rRNA gene (LSU) was amplified for some isolates as explained in Lombard *et al*. (2010c). The generated sequences were compared with other fungal DNA sequences from NCBI’s GenBank sequence database using a blastn search; TUB sequences with high similarity were added to the alignment and the result of sequences of the other loci were used as confirmation (not shown). The additional GenBank sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Phylogenetic analyses of the aligned sequence data were performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) and consisted of neighbour-joining analyses with the uncorrected (“p”), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analyses, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random (ITS) or simple (LSU) taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated. Sequences derived in this study were lodged at GenBank (www.ncbi.nlm.nih.gov), the alignment in TreeBASE (www.treebase.org/treebase/index.html), and taxonomic novelties in MycoBank (www.Mycobank.org; Crous *et al*. 2004a).

Morphology Characteristics in culture were determined after 7 d on MEA. PDA and OA (Crous *et al*. 2009c). Morphological descriptions were based on sporulating cultures on synthetic nutrient-poor agar (SNA) (Nirenburg 1981, Lombard *et al*. 2009) and carnation leaf agar (CLA) (Crous *et al*. 2009c). Slide preparations were made from sporulating cultures (SNA for anamorph, CLA for teleomorph) in clear lactic acid, with 30 measurements determined per structure, and observations made with a Nikon SMZ1500 dissecting microscope, and with a Zeiss Axioscope 2 microscope using differential interference contrast (DIC) illumination. Colony characters and pigment
production were noted after 7 d of growth on MEA, PDA and OA (Crous et al. 2009c) incubated at 25 ºC. Colony colours (surface and reverse) were rated according to the colour charts of Rayner (1970).

RESULTS

Phylogeny

Approximately 600, 480 and 680 bases were determined for the isolates indicated in Table 1 for TUB, HIS and ITS, respectively. Of the β-tubulin gene, 522 bases were used for phylogenetic analyses in the manually adjusted alignment containing 32 isolates (including the outgroup sequence). Of these 522 characters (including alignment gaps), 180 were parsimony-informative, 47 were variable and parsimony-uninformative, and 295 were constant. Neighbour-joining analysis using the three substitution models, as well as the parsimony analysis, yielded trees with exactly the same topologies. Parsimony analysis of the alignment yielded a single most parsimonious tree (TL = 381 steps; CI = 0.816; RI = 0.953; RC = 0.778), which is shown in Fig. 1.

Taxonomy

Calonectria lauri (Vanderw.) Lechat & Crous, comb. nov. — MycoBank MB517423; Fig. 2. Basionym: Tetractyum lauri Vanderw., Parasitica 1: 145. 1945. (as “lauri”).


= Cylindrocladium ilicicola (Hawley) Boedijn & Reitsma, Reinwardtia 1: 57. 1950.

Ascomata perithecial, solitary, scattered, subglobose to ovoid, 450–550 µm high × 380–420 µm diam, superficial, not obviously stromatic but difficult to remove from the substratum because basal cells of ascomata remain immersed in the substratum, yellow to brownish-yellow, dark-red at base, not changing colour in 3 % KOH or lactic acid, warty except at ostiolar region, ostiole papillate, composed of palisade-like, cylindrical to narrowly ellipsoidal cells. Ascomatal wall 50–65 µm thick of two regions; outer region comprising warts 50–55 µm thick, composed of globose to nearly angular, thick-walled cells, 10–30 × 5–16 µm, yellow, wall 1.5–2 µm thick; inner region 5–10 µm thick, composed of flattened, ellipsoidal cells, 12–18 × 3–5 µm, hyaline; warts globose to subglobose 25–40 × 15–30 µm, yellow. Asci clavate, long stipitate, 110–130 × 17–22 µm, 8-spored, multisieriate. Ascospores narrowly fusiform with rounded ends, lightly curved, guttulate, hyaline, smooth, (53–)60–86–(89) × 6.5–8–(9) µm, 3-septate, not constricted at the septa or constricted when overmature. Conidiophores consisting of a stipe bearing a penicillate arrangement of fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, 40–150 × 3–5 µm: stipe extensions septate, straight to flexuous, 120–200 µm long, 2.5–3 µm wide at the apical septum, terminating in an obpyriform to ellipsoid vesicle, (5–)7–8–(10) µm diameter. Conidigenous apparatus with primary branches aseptate or 1-septate, 15–20 × 4–5 µm; secondary branches aseptate, 8–15 × 4–5 µm; tertiary branches aseptate, 10–15 × 4–5 µm, each terminal branch producing 2–4 phialides; phialides doliform to reniform, hyaline, aseptate, 6–12 × 2.5–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Conidia cylindrical, rounded at both ends, straight, (45–)55–68–(73) × (4–)5–6–(7) µm (av. = 60 × 5.5 µm), (1–)3-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia unknown.

Culture characteristics: Colonies on MEA sienna to brick on the surface, and sienna in reverse; sienna on OA (surface); sienna to umber on PDA (surface), and umber in reverse; chlamydospores on MEA moderate, occurring throughout the medium, with sparse to moderate sporulation on aerial mycelium.

Specimens examined: IRELAND, Clare Island, Ilex aquifolium, Hawley, K 61269!, holotype of Cy. ilicicola, IMI 76542 isotype. NETHERLANDS, South-East Limburg, Vijlenerbos, Vijlen, Ilex aquifolium, Aug. 1970, H.A van der Aa, epitype CBS H-15110, ex-epitype culture CBS 749.70; Hilversum, on leaves of Ilex aquifolium, 11 Nov. 2008, W. Gams, CPC

Table 1. Collection details and GenBank accession numbers of isolates of Calonectria lauri included in this study.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Substrate</th>
<th>Country</th>
<th>Collector(s)</th>
<th>GenBank Accession No. (TUB, HIS, ITS)</th>
</tr>
</thead>
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<td>CPC 15683</td>
<td>Leaves of Ilex aquifolium</td>
<td>Netherlands</td>
<td>W. Gams</td>
<td>FR694682, FR694676, FR694679</td>
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<tr>
<td>CBS 126269</td>
<td>Leaves of I. aquifolium</td>
<td>France</td>
<td>A. Gardiennet</td>
<td>FR694683, FR694677, FR694680</td>
</tr>
<tr>
<td>CBS 553.69 = IMI 299390</td>
<td>Root of Buxus sempervirens</td>
<td>Belgium</td>
<td>—</td>
<td>FR694664, FR694678, —</td>
</tr>
<tr>
<td>CBS 749.70</td>
<td>I. aquifolium</td>
<td>Netherlands</td>
<td>H.A. van der Aa</td>
<td>FR694685, GQ267250, GQ280584</td>
</tr>
</tbody>
</table>

1CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of P.W. Crous, housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.

2TUB: partial beta-tubulin gene; HIS: partial histone H3 gene; ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA.
Fig. 1. Single most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the β-tubulin sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Cylindrocladiella lageniformis* (GenBank AY725652).
Calonectria lauri sp. nov.


**Notes:** The name *Calonectria ilicicola* is already occupied, and thus the next available epithet for this species in *Calonectria* is that of *Tetracytum lauri*. *Calonectria lauri* is phylogenetically closely related to *Ca. citri* (known on *Citrus* from Florida). Morphologically the two species can be separated in that *Ca. citri* has ellipsoid to pyriform or obovoid vesicles, and 3-septate conidia that are slightly shorter and narrower, (25–)53–60(–65) × 3–4(–5) μm (Crous 2002).

**DISCUSSION**

The genus *Calonectria* is based upon *Calonectria pyrochroa* (on *Platanus* leaf litter, France, lectotype BPI), which Rossman (1979) found to be indistinguishable from *Ca. daldiniana* (on *Magnolia grandiflora* leaf litter, Italy, holotype RO). A separate collection from decaying leaves of *Pittosporum undulatum* collected in Madeira (CUP-MM 2407) produced a *Cylindrocladium* anamorph with clavate vesicles, which later led Rossman (1983) to conclude that the oldest anamorph epithet that could be linked to *Ca. pyrochroa* was *C. ilicicola*.

Brayford & Chapman (1987) reported a wilting disease of *Laurus nobilis* in nurseries on the Isles of Scilly, and later on *Arbutus andrachnoides* and *Gaultheria shallon* in West Devon, U.K. The causal organism was identified as *C. ilicicola*, but incorrectly linked to the teleomorph name, *Ca. ilicicola*. Based on a molecular comparison of ex-type strains, Crous et al. (1993) showed *Ca. ilicicola* was the teleomorph of *C. parasiticum*, a major pathogen associated with *Cylindrocladium* black rot of peanut. In a later study, Crous & Wingfield (1994) accepted the relationship between *Ca. pyrochroa* and *C. ilicicola*, as there were no cultures available at the time to refute this proposed link (Crous 2002). Following a revision of *Cylindrocladium* strains in the CBS culture collection, Crous et al. (2006a) discovered a strain linked to a specimen that closely matched the type of *C. ilicicola*, and subsequently designated CBS 749.70 (on *Ilex aquifolium*, the Netherlands) as ex-epitype strain for *C. ilicicola*. Sequence data derived from the ex-epitype strain, and morphology, proved to be identical to that of the new collection obtained from France (Figs 1–2), confirming it to be *C. ilicicola*.

However, isolate CBS 126269 produced a *Calonectria* telemorph in culture, which is clearly distinct from *Ca. pyrochroa*. The latter species (and its synonyms) have scarlet-red perithecia, which turn purple in 2 % KOH (Rossman 1979). The present collection (on the host and on CLA in culture), forms yellow to brownish yellow perithecia that do not discolor in KOH (except at the perithecial base). The teleomorph of *C. ilicicola* could therefore not be *Ca. pyrochroa* as currently accepted (Lombard et al. 2010c). Because the name *Ca. ilicicola* is already occupied by the pathogen causing *Cylindrocladium* black rot of peanut (*Crous et al. 1993*), a new name, *Ca. lauri*, is proposed for this species, which appears to occur commonly on *Laurus*, *Ilex*, as well as several other hosts in Europe (Brayford & Chapman 1987). Presently no cultures are available of *Ca. pyrochroa*, and further collections will have to be made from *Platanus* leaf litter in France to help clarify the morphology of its *Cylindrocladium* anamorph.

**APPENDIX**

In the recent treatment of the genus *Calonectria*, Lombard et al. (2010c) allocated the name *Cylindrocladium canadense* to *Calonectria* as *Ca. canadensis* (J.C. Kang, Crous & C.L. Schoch) L. Lombard, M.J. Wingf. & Crous, but overlooked the older existing name, *Ca. canadensis* (Ellis & Everh.) Berl. & Voglino. A new combination is required to resolve this homonym as follows:


**ACKNOWLEDGEMENTS**

The authors thank the technical staff, A. van Iperen (cultures), M. Vermaas (photo plates), and M. Starink-Willems (DNA isolation, amplification and sequencing) for their invaluable assistance. Drew Minnis (USDA, Beltsville, U.S.A.) is also thanked for bringing the homonym associated with epithet "canadensis" to our attention. Finally, we thank Alain Gardiennet for the supply of specimens.
REFERENCES


