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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.

Key words:
Hypocreales
Calonectria
Cylindrocladium
Ilex aquifolium
TUB
HIS
systematics

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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 *Nectricladiella* teleomorphs (Schoch *et al*. 2000). Other morphologically similar genera that have also since been separated from this complex include *Xenocylindrocladium* (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

Island (Ireland), and *Calonectria spathulata* on *Ilex paraguariensis* in Brazil (Crous 2002). Hawksworth & Sivanesan (1976) also reported a *Calonectria* species on *Ilex aquifolium* from Slapton, South Devon, England, which appears to be undescribed, with ascospores 3-septate, 14–22 ×3–4 μm. The collection obtained from France and treated in this study, is morphologically distinct from taxa presently reported from *Ilex*.

In recent years there have been several revisions focused on either *Calonectria* or its anamorph genus, *Cylindrocladium* (Rossman 1979, Peerally 1991, Crous & Wingfield 1994, Crous 2002). The first attempt to provide a molecular phylogeny of the genus was that of Schoch *et al*. (2001) based on β-tubulin DNA sequences. This gene region, however, proved insufficiently variable to reliably distinguish all species complexes in the genus (Kang *et al*. 2001a, b, Henricot & Culham 2002, Crous *et al*. 2004b, 2006a). Since then, a concerted effort has been made to generate a multi-gene phylogeny for taxa in the genus, and identify the best suited gene for species delimitation (Lombard *et al*. 2009, 2010a–d). Based on these findings, a combination of β-tubulin DNA sequence data, supplemented with either calmodulin or elongation factor 1-α, proved the most effective in distinguishing all known taxa.
The aim of the present study was to compare the new collections on *Ilex* from France to all species known in the genus, using morphology and DNA sequence analysis of their β-tubulin and histone gene regions in order to determine if it represented a novel taxon.

MATERIALS AND METHODS

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 nrRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S nrRNA gene, the second ITS region (ITS2) and the 5' end of the 28S nrRNA 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

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

Strain No.[1]	Substrate	Country	Collector(s)	GenBank Accession No. (TUB, HIS, ITS)[2]
CPC 15683	Leaves of *Ilex aquifolium*	Netherlands	W. Gams	FR694682, FR694676, FR694679
CBS 126269 = CPC 17978	Leaves of *I. aquifolium*	France	A. Gardiennet	FR694683, FR694677, FR694680
CBS 553.69 = IMI 299390	Root of *Buxus sempervirens*	Belgium	—	FR694684, FR694678, —
CBS 749.70	*I. aquifolium*	Netherlands	H.A. van der Aa	FR694685, GQ267250, GQ280584

[1]CBS: 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.

[2]TUB: partial beta-tubulin gene; HIS: partial histone H3 gene; ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA.

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: *Tetracytum lauri* Vanderw., Parasitica 1: 145. 1945. (as "*laurii*").

= *Candelospora ilicicola* Hawley, Proc. Roy. Irish Acad. 31: 11. 1912. [non *Calonectria ilicicola* Boedijn & Reitsma, 1950]

= *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 subtratum 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, warted 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, multiseriate. *Ascospores* narrowly fusiform with rounded ends, lightly curved, guttulate, hyaline, smooth, (53–)60–86(–89) × 6.5–8(–9) µm, 3-septate, not conctricted 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 diam. *Conidiogenous 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 doliiform 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

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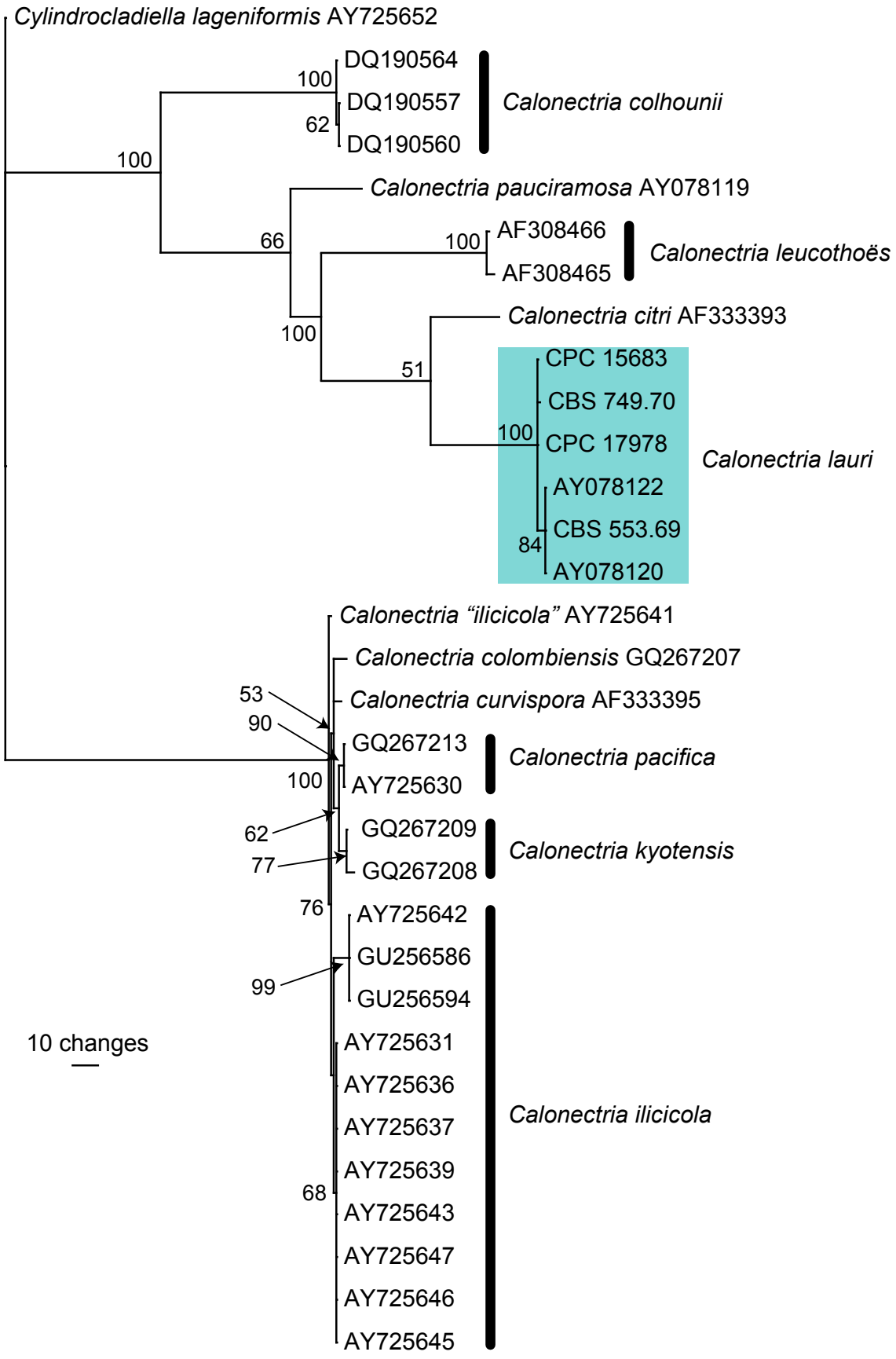

Fig. 1. Single most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the β-tubulin sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Cylindrocladiella lageniformis* (GenBank AY725652).

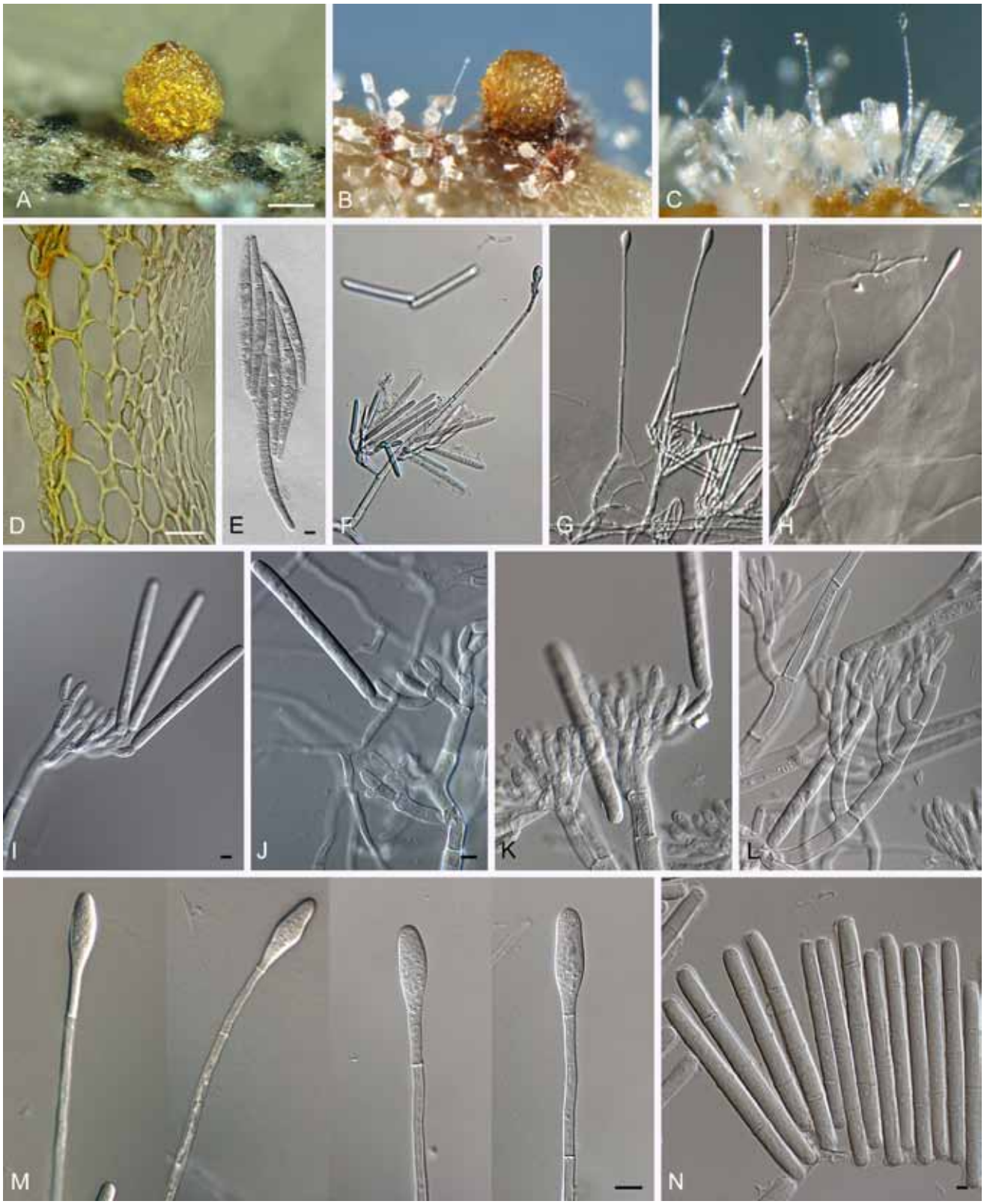

Fig. 2. *Calonectria lauri* and its *Cylindrocladium* anamorph. **A, B.** Yellowish perithecia *in vivo* (A), and *in vitro* (B). **C.** *Cylindrocladium* anamorph. **D.** Vertical section through perithecium, showing wall anatomy. **E.** Ascospores. **F–H.** Conidiophores. **I–L.** Conidiogenous apparatus with phialides. **M.** Ellipsoid to obpyriform vesicles. **N.** Three-septate conidia. Scale bars: A, B = 200 µm, C, E–H, M = 8 µm, D, J–L, N = 10 µm, I = 5.5 µm.

15683 = CBS 128031, CPC 15684, CPC 15685. FRANCE, Pressigny (52), on leaves of *Ilex aquifolium*, 05 Dec. 2009, *A. Gardiennet*, AG09308, CBS H-20476, culture CPC 17978 = CBS 126269; Forêt de Chizé, Villiers en Bois (79) on leaves of *Ilex aquifolium*, 19 Sept. 2006, *C. Lechat*, CLL696. BELGIUM, Gent, on roots of *Buxus sempervirens*, July 1969, *A. Roos*, IMI 299390 = CBS 553.69.

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* teleomorph 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 discolour 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:

Calonectria canadiana L. Lombard, M.J. Wingf. & Crous, ***nom. nov.*** MycoBank MB517424.

Basionym: *Cylindrocladium canadense* J.C. Kang, Crous & C.L. Schoch, Syst. Appl. Microbiol. 24: 210. 2001.

= *Calonectria canadensis* (J.C. Kang, Crous & C.L. Schoch) L. Lombard, M.J. Wingf. & Crous, Stud. Mycol. 66: 56. 2010, non *Calonectria canadensis* (Ellis & Everh.) Berl. & Voglino, Addendum to Syll. Fung. 4: 212. 1886.

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