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Two novel species of *Calonectria* isolated from soil in a natural forest in China

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Abstract

Species of *Calonectria* include important pathogens of numerous agronomic and forestry crops worldwide, and they are commonly distributed in soils of tropical and subtropical regions of the world. Previous research results indicated that species diversity of *Calonectria* in China is relatively high. Most *Calonectria* spp. reported and described from China were obtained from diseased *Eucalyptus* tissues or soils in *Eucalyptus* plantations established in tropical and subtropical areas in southern China. Recently, a number of *Calonectria* isolates were isolated from soils in a natural forest in the temperate region of central China. These isolates were identified by DNA sequence comparisons for the translation elongation factor 1-alpha (*tef1*), histone H3 (*his3*), calmodulin (*cmdA*) and β-tubulin (*tub2*) gene regions, combined with morphological characteristics. Two novel species of *Calonectria* were identified and described, and are named here as *Calonectria lichi* and *Ca. montana*, which reside in the Prolate Group and Sphaero-Naviculate Group, respectively. This study revealed that more species of *Calonectria* may occur in natural forests in central China than previously suspected.

Key words

Cylindrocladium, pathogen, phylogeny, taxonomy

Introduction

Calonectria species include many notorious plant pathogens and are widely distributed in tropical and subtropical areas of the world (Crous 2002, Lombard et al. 2010d, Aiello et al. 2013, Vitale et al. 2013, Alfenas et al. 2015). These species can cause serious plant

epidemics on a wide range of plant hosts (Peerally 1991, Schoch et al. 2001, Crous 2002), and result in considerable economic losses to agriculture and forestry. Example include shoot blight on *Pinus* spp. in South African nurseries (Crous et al. 1991), root rot on *Myrtus communis* in Tunisia (Lombard et al. 2011), and leaf blight on *Buxus sempervirens* in Iran (Mirabolfathy et al. 2013). In addition, members of the genus *Calonectria* are responsible for red crown rot of *Glycine max* (soybean) in Japan (Yamamoto et al. 2017), fruit rot of *Nephelium lappaceum* (rambutan) in Puerto Rico (Serrato-Diaz et al. 2013) and root rot of *Arbutus unedo* (strawberry) in Italy (Vitale et al. 2009). As an important fast-growing tree species, *Eucalyptus* plays a significant role in the global pulpwood supply. Previous research showed that Calonectria leaf blight (CLB), associated with several species of *Calonectria*, is considered to be one of the most prominent *Eucalyptus* leaf diseases that has occurred in numerous countries such as Brazil (Alfenas et al. 2015, Lombard et al. 2016), China (Zhou et al. 2008, Chen et al. 2011), Colombia (Rodas et al. 2005), India (Sharma et al. 1984) and Vietnam (Old et al. 1999). Other fungal diseases of *Eucalyptus* spp. caused by *Calonectria* species include damping-off, shoot blight, and root rot, which have been observed in Brazil (Ferreira 1989) and South Africa (Crous et al. 1991), and these diseases have received considerable attention.

Calonectria spp. are soil-borne fungi, they can form microsclerotia in soil and infected plant roots, stem and leaves as primary inoculum. After diseased tissues decompose or the plants are harvested, microsclerotia are released into the soil, which allows them to survive for extended periods even up to 15 years or more (Sobers and Littrell 1974, Crous 2002). Species of *Calonectria* are also rapidly dispersed via aerial dissemination and water movement, which leads to the transmission of *Calonectria* disease (Vitale et al. 2013). Based on previous studies, at least 145 *Calonectria* species have been identified using molecular data and have been described worldwide (Crous 2002, Crous et al. 2004, 2006, 2012, 2013, 2015, Lombard et al. 2010a, b, c, 2011, 2015, 2016, Chen et al. 2011, Xu et al. 2012, Alfenas et al. 2013a, b, 2015, Gehesquière et al. 2015). Sixty species were isolated from soil samples collected in subtropical or tropical regions (Crous 2002, Crous et al. 2004, Lombard et al. 2010a, b, c, 2015, 2016, Chen et al. 2011, Xu et al. 2012, Alfenas et al. 2015).

In China, *Calonectria* has a relatively high species diversity, and to date, 28 *Calonectria* species have been identified and described. Based on previous studies, *Calonectria* species have been reported in nine provinces and one Special Administrative Region (SAR), which with the exception of LiaoNing and ShanDong Provinces belong to temperate regions (Luan et al. 2006, Li et al. 2010). Most *Calonectria* have been isolated from agronomic crops or forestry plantations in subtropical and tropical regions, including FuJian, GuangDong, GuangXi, GuiZhou, HaiNan, JiangXi and YunNan Provinces, as well as Hong Kong SAR (Crous et al. 2004, Lombard et al. 2010a, 2015, Chen et al. 2011, Gai et al. 2012, Xu et al. 2012, Pei et al. 2015).

China has large areas of plantation and natural forests. To date 27 *Calonectria* species have been isolated from *Eucalyptus* tissues with CLB/leaf rot symptoms or from soils originating from *Eucalyptus* plantations in tropical or subtropical areas in FuJian, GuangDong, GuangXi and HaiNan Provinces (Crous et al. 2004, Lombard et

al. 2010a, 2015, Chen et al. 2011). However, little information is known about the species diversity of *Calonectria* in natural forests. In this study, a number of soil samples were collected from a natural forest in the temperate region of central China, and baited with alfalfa seeds for *Calonectria*. The aim of the current study was to identify these isolates using a combination of phylogenetic analyses and morphological characteristics and to gain a preliminary understanding of the species diversity of *Calonectria* in natural forests in China.

Materials and methods

Fungal isolates

In April 2016, 17 soil samples were collected from a natural forestry area in central China. The collected soils were baited with surface-disinfested (30 s in 75% ethanol and washed several times with sterile water) *Medicago sativa* (alfalfa) seeds using the method described by Crous (2002). After one week, sporulating conidiophores were produced on infected alfalfa tissue. Using a dissection microscope AxioCam Stemi 2000C (Carl Zeiss, Germany), conidial masses were selected and scattered onto 2 % malt extract agar (MEA) (20 g malt extract powder and 20 g agar powder per liter of water: malt extract powder was obtained from Beijing Shuangxuan microbial culture medium products factory, Beijing, China; the agar powder was obtained from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) using sterile needles. After incubation at 25 °C for one day, germinated spores were individually transferred onto fresh MEA under the dissection microscope and were incubated at 25 °C for one week.

Single conidial cultures were deposited in the Culture Collection of the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong Province, China. Representative isolates were stored in the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. The specimens (pure fungal cultures) were deposited in the Collection of Central South Forestry Fungi of China (CSFF), GuangDong Province, China.

DNA extraction, PCR and sequence reactions

Single conidial cultures grew on MEA for one week at 25 °C, after which actively growing mycelium was scraped using a sterilized scalpel and transferred into 2 mL Eppendorf tubes. Total genomic DNA was extracted following the protocols "Extraction method 5: grinding and CTAB" described by Van Burik et al. (1998). The extracted DNA was dissolved in 30 μL TE buffer (1 M Tris-HCl and 0.5 M EDTA, pH 8.0), and a Nano-Drop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the concentration.

Based on previous research (Lombard et al. 2010d, Alfenas et al. 2015), partial gene regions including translation elongation factor 1-alpha (*tef1*), histone H3 (*his3*), calmodulin (*cmdA*) and β-tubulin (*tub2*), were used as successful DNA barcodes at species, being able to clearly distinguish between intra- and inter-specific divergence. The primer pairs EF1-728F/EF2, CYLH3F/CYLH3R, CAL-228F/CAL-2Rd and T1/CYLTUB1R were used to amplify the fragments of the respective *tef1*, *his3*, *cmdA* and *tub2* genes (Lombard et al. 2010d).

The PCR reaction mixture used to amplify the different loci consisted of TopTaq™ Master Mix 12.5 μL (Qiagen Inc., Hilden, Germany), forward primer 1 μL, 10 μM (Invitrogen, Shanghai, China), reverse primer 1 μL, 10 μM (Invitrogen, Shanghai, China), and RNase-Free H_2O 8.5 μL (Qiagen Inc., Hilden, Germany), and 2 μL (100 ng/μL) of the DNA samples was added as the template to each PCR reaction. The amplifications were performed in 25 μL reaction volumes on an MJ Mini Cycler (BIO-RAD, Hercules, CA, USA) under the conditions described by Groenewald et al. (2013). The amplification products were separated by 1.5% agarose gel electrophoresis and visualized with SYBR Safe DNA gel stain (Thermo Fisher Scientific Inc., USA).

Amplified fragments were sequenced in both directions using the same primer pairs used for amplification by the Beijing Genomics Institute, Guangzhou, China. Sequences were edited using MEGA v. 6.0.5 software (Tamura et al. 2013). All sequences of the isolates obtained in this study were submitted to GenBank (http://www.ncbi.nlm.nih.gov) (Table 1).

Phylogenetic analyses

The sequences generated from this study were added to other sequences of closely related *Calonectria* species downloaded from GenBank for phylogenetic analyses. All sequences used in this study were aligned using the online MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server) with the alignment strategy FFT-NS-i (Slow; interactive refinement method). The aligned sequences were manually edited using MEGA v. 6.0.5 and were deposited in TreeBASE (http://treebase.org).

Phylogenetic analyses were conducted on individual *tef1*, *his3*, *cmdA* and *tub2* sequence datasets and on the combined datasets for the four gene regions, depending on the sequence availability. Two methods, maximum parsimony (MP) and maximum likelihood (ML) were used for phylogenetic analyses.

MP analyses were performed using PAUP v. 4.0 b10 (Swofford 2003), gaps were treated as a fifth character, and characters were unordered and of equal weight with 1000 random addition replicates. A partition homogeneity test (PHT) was conducted to determine whether data for the four genes could be combined. The most parsimonious trees were acquired using the heuristic search option with stepwise addition, tree bisection, and reconstruction branch swapping. MAXTREES was set to 5,000, and zero-length branches were collapsed. A bootstrap analysis (50% majority rule, 1,000 replicates) was carried out to determine statistical support for internal nodes in trees.

Table 1. The species of *Calonectria* used in this study.

Species	Isolate No.[†,‡]	Substrate	Sampling site	Collector	GenBank accession No.[§,|]				Reference
					tef1	*his3*	*cmdA*	*tub2*	
Calonectria acicola	**CBS 114813**	*Pinus radiata*	New Zealand	H. Pearson	GQ267292	DQ190693	GQ267360	DQ190591	Gadgil and Dick 2004
	CBS 114812	*P. radiata*	New Zealand	H. Pearson	GQ267291	DQ190692	GQ267359	DQ190590	Gadgil and Dick 2004
Ca. aconidialis	**CBS 136086**	Soil in *Eucalyptus* plantation	HaiNan, China	X. Mou & S.F. Chen	KJ462785	KJ463133	KJ463017	N/A[¶]	Lombard et al. 2015
Ca. arbusta	**CBS 136079**	Soil in *Eucalyptus* plantation	GuangXi, China	X. Zhou & G. Zhao	KJ462787	KJ463135	KJ463018	KJ462904	Lombard et al. 2015
Ca. asiatica	**CBS 114073**	Leaf litter	Thailand	N.L. Hywel-Jones	AY725705	AY725658	AY725741	AY725616	Crous et al. 2004
	CBS 112711	Leaf litter	Thailand	N.L. Hywel-Jones	AY725702	AY725655	AY725738	AY725613	Crous et al. 2004
Ca. australiensis	**CBS 112954**	*Ficus pleurocarpa*	Australia	C. Pearce & B. Paulu	GQ267293	DQ190699	GQ267363	DQ190596	Crous et al. 2006
Ca. brassicicola	**CBS 112841**	*Brassica* sp.	Indonesia	M.J. Wingfield	KX784689	N/A	KX784561	KX784619	Lombard et al. 2016
Ca. canadiana	**CBS 110817**	*Picea* sp.	Canada	S. Greifenhagen	GQ267297	AF348228	AY725743	AF348212	Lombard et al. 2010b
Ca. chinensis	**CBS 114827**	Soil	Hong Kong	E.C.Y. Liew	AY725710	AY725661	AY725747	AY725619	Lombard et al. 2010b
	CBS 112744	Soil	Hong Kong	E.C.Y. Liew	AY725709	AY725660	AY725746	AY725618	Lombard et al. 2010b
Ca. colhounii	**CBS 293.79**	*Camellia sinensis*	Indonesia	N/A	GQ267301	DQ190639	GQ267373	DQ190564	Lombard et al. 2010b
	CBS 114704	*Arachis pintoi*	Australia	D. Hutton	GQ267300	DQ190638	GQ267372	DQ190563	Lombard et al. 2010b
Ca. colombiensis	**CBS 112220**	*Eucalyptus grandis*	Colombia	M.J. Wingfield	AY725711	AY725662	AY725748	GQ267207	Lombard et al. 2010b
	CBS 112221	*E. grandis*	Colombia	M.J. Wingfield	AY725712	AY725663	AY725749	AY725620	Lombard et al. 2010b
Ca. crousiana	**CBS 127198**	*E. grandis*	FuJian, China	M.J. Wingfield	HQ285822	HQ285808	**MF527084**	HQ285794	Chen et al. 2011; This study
	CBS 127199	*E. grandis*	FuJian, China	M.J. Wingfield	HQ285823	HQ285809	**MF527085**	HQ285795	Chen et al. 2011; This study
Ca. curvispora	**CBS 116159**	Soil	Madagascar	P.W. Crous	GQ267302	AY725664	GQ267374	AF333394	Lombard et al. 2010b
Ca. eucalypti	**CBS 125275**	*E. grandis*	Sumatra Utara	M.J. Wingfield	GQ267338	GQ267267	GQ267430	GQ267218	Lombard et al. 2010b
	CBS 125276	*E. grandis*	Sumatra Utara	M.J. Wingfield	GQ267339	GQ267268	GQ267431	GQ267219	Lombard et al. 2010b
Ca. expansa	**CBS 136247**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Zhou & G. Zhao	KJ462798	KJ463146	KJ463029	KJ462914	Lombard et al. 2015
	CBS 136078	Soil in *Eucalyptus* plantation	Guangdong, China	X. Zhou & G. Zhao	KJ462797	KJ463145	KJ463028	KJ462913	Lombard et al. 2015
Ca. fujianensis	**CBS 127201**	*E. grandis*	FuJian, China	M.J. Wingfield	HQ285820	HQ285806	**MF527089**	HQ285792	Chen et al. 2011; This study
	CBS 127200	*E. grandis*	FuJian, China	M.J. Wingfield	HQ285819	HQ285805	**MF527088**	HQ285791	Chen et al. 2011; This study

Species	Isolate No.[†,‡]	Substrate	Sampling site	Collector	GenBank accession No.[§,|]				Reference
					tef1	*his3*	*cmdA*	*tub2*	
Ca. guangxiensis	**CBS 136092**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Mou & R. Chang	KJ462803	KJ463151	KJ463034	KJ462919	Lombard et al. 2015
	CBS 136094	Soil in *Eucalyptus* plantation	Guangxi, China	X. Mou & R. Chang	KJ462804	N/A	KJ463035	KJ462920	Lombard et al. 2015
Ca. hainanensis	**CBS 136248**	Soil in *Eucalyptus* plantation	Hainan, China	X. Mou & S.F. Chen	KJ462805	KJ463152	KJ463036	N/A	Lombard et al. 2015
Ca. hongkongensis	**CBS 114828**	Soil	Hong Kong	E.C.Y. Liew	AY725717	AY725667	AY725755	AY725622	Lombard et al. 2010b
	CBS 114711	Soil	Hong Kong	M.J. Wingfield	AY725716	AY725666	AY725754	AY725621	Lombard et al. 2010b
Ca. ilicicola	**CBS 190.50**	*Solanum tuberosum*	Indonesia	K.B. Boedijn & J. Reitsma	AY725726	AY725676	AY725764	AY725631	Lombard et al. 2010b
	CBS 112215	*A. hypogaea*	U.S.A.	Beute	AY725726	AY725684	AY725765	AY725639	Crous et al. 2004
Ca. indonesiae	**CBS 112823**	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY725718	AY725668	AY725756	AY725623	Lombard et al. 2010b
	CBS 112840	*S. aromaticum*	Indonesia	M.J. Wingfield	AY725720	AY725670	AY725758	AY725625	Lombard et al. 2010b
C. indonesiana	**CBS 112936**	Soil	Indonesia	M.J. Wingfield	KX784701	N/A	KX784573	KX784631	Lombard et al. 2016
Ca. indusiata	CBS 144.36	N/A	N/A	N/A	GQ267332	GQ267262	GQ267453	GQ267239	Lombard et al. 2010b
	CBS 114684	*Rhododendron* sp.	U.S.A.	N.E. El-Gholl	GQ267333	DQ190653	GQ267454	AF232862	Lombard et al. 2010b
Ca. kyotensis	CBS 170.77	*Idesia polycarpa*	New Zealand	N/A	GQ267308	GQ267249	GQ267380	GQ267209	Lombard et al. 2010b
	CBS 413.67	*Paphiopedilum callosum*	Celle, Germany	W. Gerlach	GQ267307	GQ267248	GQ267379	GQ267208	Lombard et al. 2010b
Ca. lateralis	**CBS 136629**	Soil in *Eucalyptus* plantation	Guangxi,China	X. Zhou & G. Zhao	KJ462840	KJ463186	KJ463070	KJ462955	Lombard et al. 2015
Ca. lichi	**CERC 8866**	Soil	Central China	S.F. Chen	**MF527039**	**MF527055**	**MF527071**	**MF527097**	This study
	CERC 8841	Soil	Central China	S.F. Chen	**MF527036**	**MF527052**	**MF527068**	**MF527094**	This study
	CERC 8848	Soil	Central China	S.F. Chen	**MF527037**	**MF527053**	**MF527069**	**MF527095**	This study
	CERC 8850	Soil	Central China	S.F. Chen	**MF527038**	**MF527054**	**MF527070**	**MF527096**	This study
	CERC 8871	Soil	Central China	S.F. Chen	**MF527040**	**MF527056**	**MF527072**	**MF527098**	This study
	CERC 8890	Soil	Central China	S.F. Chen	**MF527041**	**MF527057**	**MF527073**	**MF527099**	This study
	CERC 8900	Soil	Central China	S.F. Chen	**MF527042**	**MF527058**	**MF527074**	**MF527100**	This study
	CERC 8906	Soil	Central China	S.F. Chen	**MF527043**	**MF527059**	**MF527075**	**MF527101**	This study
	CERC 8928	Soil	Central China	S.F. Chen	**MF527044**	**MF527060**	**MF527076**	**MF527102**	This study
Ca. macroconidialis	**CBS 114880**	*E. grandis*	South Africa	P.W. Crous	GQ267313	DQ190655	GQ267393	AF232855	Lombard et al. 2010b
Ca. magnispora	**CBS 136249**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Mou & R. Chang	KJ462841	KJ463187	KJ463071	KJ462956	Lombard et al. 2015
Ca. malesiana	**CBS 112752**	Soil	Indonesia	M.J. Wingfield	AY725722	AY725672	AY725760	AY725627	Lombard et al. 2010b
	CBS 112710	Debris	Thailand	N.L. Hywel-Jones	AY725721	AY725671	AY725759	AY725626	Lombard et al. 2010b

Species	Isolate No.[†,‡]	Substrate	Sampling site	Collector	GenBank accession No.[§,|]				Reference
					tef1	*his3*	*cmdA*	*tub2*	
Ca. microconidialis	**CBS 136638**	*E. urophylla* × *E. grandis* clone seedling leaf	Guangdong, China	G. Zhao	KJ462845	KJ463191	KJ463075	KJ462960	Lombard et al. 2015
	CBS 136633	*E. urophylla* × *E. grandis* clone seedling leaf	Guangdong, China	G. Zhao	KJ462842	KJ463188	KJ463072	KJ462957	Lombard et al. 2015
Ca. montana	**CERC 8952**	Soil	Central China	S.F. Chen	**MF527049**	**MF527065**	**MF527081**	**MF527107**	This study
	CERC 8930	Soil	Central China	S.F. Chen	**MF527045**	**MF527061**	**MF527077**	**MF527103**	This study
	CERC 8932	Soil	Central China	S.F. Chen	**MF527046**	**MF527062**	**MF527078**	**MF527104**	This study
	CERC 8936	Soil	Central China	S.F. Chen	**MF527047**	**MF527063**	**MF527079**	**MF527105**	This study
	CERC 8938	Soil	Central China	S.F. Chen	**MF527048**	**MF527064**	**MF527080**	**MF527106**	This study
	CERC 8957	Soil	Central China	S.F. Chen	**MF527050**	**MF527066**	**MF527082**	**MF527108**	This study
	CERC 8966	Soil	Central China	S.F. Chen	**MF527051**	**MF527067**	**MF527083**	**MF527109**	This study
Ca. monticola	**CPC 28835**	Soil	Thailand	P.W. Crous	KT964773	N/A	KT964771	KT964769	Crous et al. 2015
	CPC 28836	Soil	Thailand	P.W. Crous	KT964774	N/A	KT964772	KT964770	Crous et al. 2015
Ca. multiseptata	**CBS 112682**	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	FJ918535	DQ190659	GQ267397	DQ190573	Lombard et al. 2010b
Ca. nymphaeae	**CBS 131802**	*Nymphaea tetragona*	Guiyang, Guizhou	S.Y. Qin	KC555273	N/A	N/A	JN984864	Xu et al. 2012
	HGUP 100004	*N. tetragona*	Guiyang, Guizhou	Y. Wang	KC555274	N/A	N/A	JN984865	Xu et al. 2012
Ca. pacifica	**CBS 109063**	*Araucaria heterophylla*	Hawaii, USA	M. Aragaki	AY725724	GQ267255	AY725762	GQ267213	Lombard et al. 2010b
	CBS 114038	*Ipomoea aquatica*	New Zealand	C.F. Hill	GQ267320	AY725675	GQ267402	AY725630	Lombard et al. 2010b
Ca. paracolhounii	**CBS 114679**	N/A	USA	A.Y. Rossman	KX784714	N/A	KX784582	KX784644	Lombard et al. 2016
	CBS 114705	*Annona reticulata*	Australia	D. Hutton	KX784715	N/A	N/A	KX784645	Lombard et al. 2016
Ca. parakyotensis	**CBS 136085**	Soil in *Eucalyptus* plantation	Guangdong, China	X. Mou & R. Chang	KJ462851	KJ463197	KJ463081	N/A	Lombard et al. 2015
	CBS 136095	Soil in *Eucalyptus* plantation	Guangxi, China	X. Mou & R. Chang	KJ462852	KJ463198	KJ463082	N/A	Lombard et al. 2015
Ca. parva	**CBS 110798**	*Eucalyptus grandis* roots	South Africa	P.W. Crous	KX784716	N/A	KX784583	KX784646	Lombard et al. 2016
Ca. pauciramosa	**CMW 5683**	*E. grandis*	South Africa	P.W. Crous	FJ918565	FJ918531	GQ267405	FJ918514	Lombard et al. 2010b
	CMW 30823	*E. grandis*	South Africa	P.W. Crous	FJ918566	FJ918532	GQ267404	FJ918515	Lombard et al. 2010b
Ca. penicilloides	**CBS 174.55**	*Prunus* sp.	Japan	Tubaki	GQ267322	GQ267257	GQ267406	AF333414	Lombard et al. 2010b
Ca. pluriramosa	**CBS 136976**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Zhou & G. Zhao	KJ462882	KJ463228	KJ463112	KJ462995	Lombard et al. 2015
Ca. pseudokyotensis	**CBS 137332**	Soil in *Eucalyptus* plantation	Guangxi,China	X. Zhou & G. Zhao	KJ462881	KJ463227	KJ463111	KJ462994	Lombard et al. 2015

Species	Isolate No.[†,‡]	Substrate	Sampling site	Collector	GenBank accession No.[§,|]				Reference
					tef1	*his3*	*cmdA*	*tub2*	
Ca. pseudocolhounii	**CBS 127195**	*E. dunnii*	FuJian, China	M.J. Wingfield	HQ285816	HQ285802	**MF527091**	HQ285788	Chen et al. 2011; This study
	CBS 127196	*E. dunnii*	FuJian, China	M.J. Wingfield	HQ285817	HQ285803	**MF527092**	HQ285789	Chen et al. 2011; This study
Ca. pseudoreteaudii	**CBS 123694**	*E. urophylla* × *E. grandis* cutting	Guangdong, China	M.J. Wingfield	FJ918541	FJ918519	GQ267411	FJ918504	Lombard et al. 2010b
	CBS 123696	*E. urophylla* × *E. grandis* cutting	Guangdong, China	M.J. Wingfield	FJ918542	FJ918520	GQ267410	FJ918505	Lombard et al. 2010b
Ca. queenslandica	**CBS 112146**	*E. urophylla*	Australia	B. Brown	FJ918543	FJ918521	GQ267415	AF389835	Lombard et al. 2010b
	CBS 112155	*E. pellita*	Australia	K.M. Old	FJ918544	DQ190667	GQ267416	AF389834	Lombard et al. 2010b
Ca. reteaudii	**CBS 112144**	*E. camaldulensis*	Vietnam	M.J. Dudzinski	FJ918537	DQ190661	GQ267417	AF389833	Lombard et al. 2010b
	CBS 112143	*E. camaldulensis*	Vietnam	M.J. Dudzinski	FJ918536	DQ190660	GQ267418	GQ240642	Lombard et al. 2010b
Ca. sphaeropendunculata	**CBS 136081**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Zhou & G. Zhao	KJ462890	KJ463236	KJ463120	KJ463003	Lombard et al. 2015
Ca. sumatrensis	**CBS 112829**	Soil	Indonesia	M.J. Wingfield	AY725733	AY725696	AY725771	AY725649	Lombard et al. 2010b
	CBS 112934	Soil	Indonesia	M.J. Wingfield	AY725735	AY725698	AY725773	AY725651	Lombard et al. 2010b
Ca. syzygiicola	**CBS 112831**	Soil	Indonesia	M.J. Wingfield	KX784736	N/A	N/A	KX784663	Lombard et al. 2016
Ca. terrae-reginae	**CBS 112151**	*E. urophylla*	Australia	C. Hanwood	FJ918545	FJ918522	GQ267451	FJ918506	Lombard et al. 2010b
	CBS 112634	*Xanthorrhoea australis*	Australia	T. Baigent	FJ918546	DQ190668	GQ267452	FJ918507	Lombard et al. 2010b
Ca. turangicola	**CBS 136077**	Soil in *Eucalyptus* plantation	Guangxi, China	X. Zhou & G. Zhao	KJ462900	KJ463246	N/A	KJ463013	Lombard et al. 2015
	CBS 136093	Soil in *Eucalyptus* plantation	Guangxi, China	X. Mou & R. Chang	KJ462901	KJ463247	KJ463130	KJ463014	Lombard et al. 2015

[†] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CERC: China Eucalypt Research Centre, Zhanjiang, GuangDong Province, China; CMW: culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC: Pedro Crous working collection housed at CBS; HGUP: Plant Pathology Herbarium of Gui Zhou University, GuiYang 550025, China.

[‡] Isolates represent ex-type and indicated in bold.

[§] *tef1*: translation elongation factor 1-alpha; *his3*: histone H3; *cmdA*: calmodulin; *tub2*: β-tubulin.

[|] GenBank accession no. indicated in bold were generated in this study

[¶] "N/A" represents information that are not available.

The tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were used to assess phylogenetic trees (Hillis and Huelsenbeck 1992).

ML analyses were performed using PHYML v. 3.0 (Guindon and Gascuel 2003), and the best evolutionary model was obtained using JMODELTEST v. 2.1.5 (Posada 2008). In PHYML, the maximum number of retained trees was set to 1,000, and nodal support was determined by non-parametric bootstrapping with 1,000 replicates.

Based on the morphological characteristics, datasets were separated into two groups: the Prolate Group and the Sphaero-Naviculate Group (Lombard et al. 2010b), and therefore phylogenetic analyses were performed with two separate sequence datasets. *Calonectria hongkongensis* (CBS 114711 and CBS 114828) and *Ca. pauciramosa* (CMW 5683 and CMW 30823) represented the outgroup taxa for the Prolate Group and Sphaero-Naviculate Group, respectively. The phylogenetic trees were viewed using MEGA v. 6.0.5 for both MP and ML analyses.

Sexual compatibility

Based on multi-gene phylogenetic analyses, isolates of each identified *Calonectria* species were crossed with each other in all possible combinations. Crosses were performed on minimal salt agar (MSA; Guerber and Correll 2001) on the surface of the medium using three sterile toothpicks. Isolates crossed with themselves were regarded as controls. These crosses were used to determine whether the identified species had a heterothallic or a homothallic mating system. The cultures were incubated at 25 °C for six weeks. When isolate combinations produced extruding viable ascospores, crosses were considered successful.

Morphology

To determine the morphological characteristics of the asexual morphs, representative isolates identified by DNA sequence comparisons were selected. Agar plugs from the periphery of actively growing single conidial cultures were transferred onto synthetic nutrient-poor agar (SNA; Nirenburg 1981) and incubated at 25 °C for one week (there were five replicates per isolate). Asexual structures that emerged on the surface of the SNA medium were mounted in one drop of 80% lactic acid on glass slides and examined under an Axio Imager A1 microscope (Carl Zeiss Ltd., Munchen, Germany) and an AxioCam ERc 5S digital camera with Zeiss Axio Vision Rel. 4.8 software (Carl Zeiss Ltd., Munchen, Germany). Sexual morphs were studied by transferring perithecia obtained from the sexual compatibility tests into a tissue-freezing medium (Leica Biosystems, Nussloch, Germany) and were hand-sectioned using an HM550 Cryostat Microtome (Microm International GmbH, Thermo Fisher Scientific, Wall-

dorf, Germany) at –20 °C. The 10-μm sections were mounted in 80% lactic acid and 3% KOH.

Fifty measurements were made for each morphological structure of the isolates selected as the holotype specimen, 30 measurements were made for the isolates selected as the paratype specimen. Minimum, maximum and average (mean) values were determined and presented as follows: (minimum–) (average – standard deviation) – (average + standard deviation) (–maximum).

The optimal growth temperature of the *Calonectria* species was determined by transferring the representative isolates to fresh 9 mm MEA Petri dishes, which were incubated under temperatures ranging from 5 to 35 °C at 5 °C intervals in the dark (there were five replicates per isolate). Colony colors were determined by inoculating the isolates on fresh MEA at 25 °C in the dark, after seven days incubation, a comparison was performed using the colour charts of Rayner (1970).

Results

Fungal isolates

A total of 40 isolates with the typical morphological of *Calonectria* species were obtained from the infected alfalfa tissue cultivated in the soil samples. Based on preliminary phylogenetic analysis of the *tef1* gene region (data not shown), 16 isolates from all soil samples were selected for further study (Table 1).

Phylogenetic analyses

Sequences for the 78 ex-type and other strains of 48 *Calonectria* species closely related to isolates obtained in this study were downloaded from GenBank (Table 1). For the 16 isolates collected in this study, nine resided in the Prolate Group, and seven were clustered in the Sphaero-Naviculate Group. Phylogenetic analyses of individual *tef1*, *his3*, *cmdA* and *tub2* and the combined sequence datasets were conducted using both MP and ML method. For both the Prolate and Sphaero-Naviculate Groups, although the related position of some *Calonectira* species were slightly different between the MP and ML trees, the overall topologies were similar, and the ML trees were exhibited.

For the Prolate and Sphaero-Naviculate Groups, the PHT comparing the combined *tef1*, *his3*, *cmdA* and *tub2* gene datasets generated P values of 0.141 and 0.333, respectively, which indicated that no significant difference existed between these datasets. These datasets were consequently combined and subjected to phylogenetic analyses. For each of the two groups, the sequence alignments of *tef1*, *his3*, *cmdA*, *tub2* and the combination of the four genes were deposited in TreeBASE (TreeBASE No. 21357). The number of parsimony informative characters, the statistical values for the

Table 2. Statistics resulting from phylogenetic analyses.

Dataset	Phylogenetic group	No. of taxa	No. of bp[†]	Maximum parsimony						
				PIC[‡]	No. of trees	Tree length	CI[§]	RI[|]	RC[¶]	HI[#]
tef1	Prolate	45	515	210	8	448	0.7054	0.8847	0.6240	0.2946
his3	Prolate	38	449	140	6	340	0.6941	0.9176	0.6369	0.3059
cmdA	Prolate	42	476	152	792	245	0.7591	0.9295	0.7056	0.2408
tub2	Prolate	45	579	204	18	350	0.8085	0.9395	0.7597	0.1914
tef1/*his3*/*cmdA*/*tub2*	Prolate	45	2019	706	1	1484	0.6880	0.8940	0.6150	0.3120
tef1	Sphaero-Naviculate	51	522	159	33	330	0.7030	0.9056	0.6367	0.2969
his3	Sphaero-Naviculate	47	455	138	11	386	0.6632	0.9110	0.6042	0.3367
cmdA	Sphaero-Naviculate	49	473	138	48	228	0.7763	0.9406	0.7302	0.2236
tub2	Sphaero-Naviculate	47	534	174	4	401	0.7107	0.9216	0.6550	0.2892
tef1/*his3*/*cmdA*/*tub2*	Sphaero-Naviculate	51	1984	609	1350	1535	0.6190	0.8790	0.6047	0.3810

Dataset	Phylogenetic group	Maximum likelihood							
		Subst. model[††]	NST[‡‡]	Rate matrix					Rates
tef1	Prolate	TIM2+G	6	1.6588	2.3553	1.6588	1.0000	4.4652	Gamma
his3	Prolate	GTR+G	6	1.8190	7.5654	4.6281	1.4320	15.6259	Gamma
cmdA	Prolate	HKY+G	2						Gamma
tub2	Prolate	TPM3uf+G	6	1.5151	4.2112	1.0000	1.5151	4.2112	Gamma
tef1/*his3*/*cmdA*/*tub2*	Prolate	TIM2+I+G	6	1.3725	3.6221	1.3725	1.0000	5.1226	Gamma
tef1	Sphaero-Naviculate	GTR+G	6	2.3612	2.5155	0.6227	0.7074	5.0226	Gamma
his3	Sphaero-Naviculate	HKY+I+G	2						Gamma
cmdA	Sphaero-Naviculate	TrN+G	6	1.0000	3.8308	1.0000	1.0000	6.4755	Gamma
tub2	Sphaero-Naviculate	TPM3uf+G	6	1.5714	4.6055	1.0000	1.5714	4.6055	Gamma
tef1/*his3*/*cmdA*/*tub2*	Sphaero-Naviculate	GTR+I+G	6	1.6318	3.8130	1.0888	1.1609	5.2579	Gamma

[†] bp = base pairs.

[‡] PIC = number of parsimony informative characters.

[§] CI = consistency index.

[|] RI = retention index.

[¶] RC = rescaled consistency index.

[#] HI = homoplasy index.

[††] Subst. model = best fit substitution model.

[‡‡] NST = number of substitution rate categories.

phylogenetic trees of the MP analyses, and the parameters for the best-fit substitution models of ML analyses are shown in Table 2.

Phylogenetic analyses of each of the individual and combined sequence datasets indicated that in the Prolate Group, the nine isolates resided in the *Ca. colhounii* species complex and were closely related to *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis*, *Ca. nymphaeae*, *Ca. paracolhounii* and *Ca. pseudocolhounii*. In the *his3* and *cmdA* phylogenetic trees, the nine isolates and *Ca. fujianensis* were clustered in the same clade (Suppl. materials 2, 3), while in the trees based on the *tef1* and *tub2* sequences, the nine isolates formed an independent clade (Supplementary Figures 1, 4). Based on the phylogenetic analyses of the combined sequences of the four genes, the nine isolates formed a new, strongly defined phylogenetic clade that was distinct from other *Calonectria* species and was supported by high bootstrap values (ML = 94%, MP = 93%) (Figure 1). Fixed unique single nucleotide polymorphisms (SNPs) were identified in the new phylogenetic clades of the nine isolates and their phylogenetically closed *Calonectria* species (Table 3). The total number of SNP differences between the new clade and the other closely related species varied between 10–34 for all four gene regions combined (Table 4). The results of these phylogenetic and SNP analyses indicate that the nine isolates in the Prolate Group represent a distinct, undescribed species.

Phylogenetic analyses of each of the individual and combined datasets indicated that in the Sphaero-Naviculate Group, the seven isolates were clustered in the *Ca. kyotensis* species complex and were closely related to *Ca. canadiana*. In the *tef1* phylogenetic trees, the seven isolates were grouped in the same clade with *Ca. canadiana* (Suppl. material 5). In the phylogenetic trees based on the *his3*, *cmdA* and *tub2* sequences, the seven isolates formed an independent clade distinct from *Ca. canadiana* and other species in the *Ca. kyotensis* species complex (Suppl. materials 6, 7 and 8). Based on the combined sequences of the four genes, the seven isolates formed a strongly defined phylogenetic clade that was distinct from *Ca. canadiana* and was supported by high bootstrap values (ML = 100%, MP = 100%) (Figure 2). The seven isolates obtained in this study were distinguished from *Ca. canadiana* using SNP analyses for each of the *tef1*, *his3*, *cmdA* and *tub2* gene region sequences (Tables 5). The total number of SNP differences between the seven isolates and *Ca. canadiana* for all four genes was 51 (Table 6). The results indicate that the seven isolates in the Sphaero-Navivulate Group represent a novel species.

Sexual compatibility

After a six-week mating test on MSA, all 16 isolates and the crosses of isolates of each identified species failed to yield sexual structures, indicating that they were either self-sterile (heterothallic) or had retained the ability to recombine to produce fertile progeny.

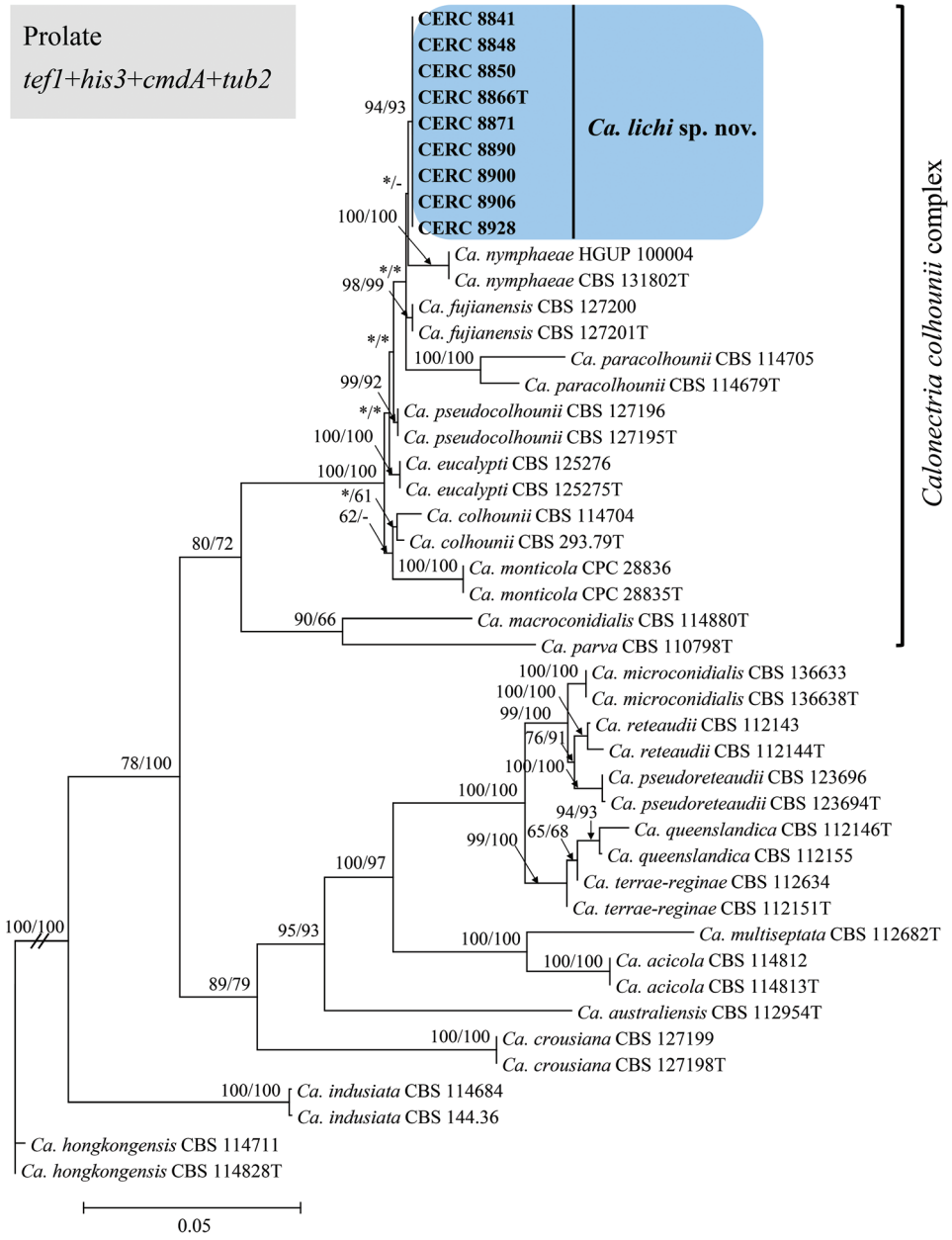

Figure 1. Phylogenetic tree of *Calonectria* species in the Prolate group based on maximum likelihood (ML) analysis of combined DNA dataset of *tef1*, *his3*, *cmdA* and *tub2* gene sequences. ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in blue. The tree was rooted to *Ca. hongkongensis* (CBS 114711 and CBS 114828).

Table 3. Single nucleotide polymorphism comparisons in four gene regions between *Calonectria lichi* and the phylogenetically closest related species.

Species	Isolate no.	*tef1*†																	
		28‡	81	89	90	91	92	93	100	120	121	124	184	185	186	243	418	425	432
Ca. lichi	**CERC 8866**§	A	**A**|	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8841	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8848	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8850	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8871	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8890	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8900	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8906	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
	CERC 8928	A	**A**	–	–	–	–	–	C	T	T	A	–	–	–	A	C	–	A
Ca. colhounii	**CBS 293.79**	**C**	T	**A**	**C**	**A**	**A**	**C**	C	**–**	–	A	–	–	–	**G**	C	–	A
	CBS 114704	**C**	T	**A**	**C**	**A**	**A**	**C**	C	**–**	–	A	–	–	–	**G**	C	–	A
Ca. eucalypti	**CBS 125275**	A	T	–	–	–	–	–	C	T	–	A	–	–	–	A	C	–	A
	CBS 125276	A	T	–	–	–	–	–	C	T	–	A	–	–	–	A	C	–	A
Ca. fujianensis	**CBS 127201**	A	T	–	–	–	–	–	C	T	–	G	**A**	**A**	**A**	A	C	–	A
	CBS 127200	A	T	–	–	–	–	–	C	T	–	G	**A**	**A**	**A**	A	C	–	A
Ca. nymphaeae	**CBS 131802**	A	T	–	–	–	–	–	C	T	–	G	–	–	–	A	C	–	A
	HGUP 100004	A	T	–	–	–	–	–	C	T	–	G	–	–	–	A	C	–	A
Ca. paracolhounii	**CBS 114679**	A	T	–	–	–	–	–	**T**	T	T	A	–	–	–	A	**G**	**C**	**C**
Ca. pseudocolhounii	**CBS 127195**	A	T	–	–	–	–	–	C	T	–	A	–	–	–	A	C	–	A
	CBS 127196	A	T	–	–	–	–	–	C	T	–	A	–	–	–	A	C	–	A

Table 3. Continue.

Species	Isolate no.	*tef1*																
		433	435	436	437	438	441	443	444	446	447	448	450	452	453	457	473	483
Ca. lichi	**CERC 8866**[§]	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8841	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8848	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8850	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8871	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8890	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8900	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8906	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CERC 8928	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
Ca. colhounii	**CBS 293.79**	T	T	C	C	C	**C**	T	A	C	T	A	C	C	T	C	G	C
	CBS 114704	T	T	C	C	C	**C**	T	A	C	T	A	C	C	T	C	G	C
Ca. eucalypti	**CBS 125275**	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	C
	CBS 125276	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	C
Ca. fujianensis	**CBS 127201**	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CBS 127200	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
Ca. nymphaeae	**CBS 131802**	T	T	C	T	C	T	T	A	C	T	A	C	–	T	T	–	N/A[¶]
	HGUP 100004	T	T	C	T	C	T	T	A	C	T	A	C	–	T	T	–	N/A
Ca. paracolhounii	**CBS 114679**	**A**	**G**	**–**	**–**	**T**	T	**C**	**T**	**G**	**G**	**G**	**T**	G	**G**	N/A	N/A	N/A
Ca. pseudocolhounii	**CBS 127195**	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–
	CBS 127196	T	T	C	T	C	T	T	A	C	T	A	C	T	T	T	G	–

Table 3. Continue.

Species	Isolate no.	*His3*							*cmdA*														
		45	234	272	293	344	353	368	169	204	205	210	238	244	266	293	325	334	411	429	432	474
Ca. lichi	**CERC 8866**[§]	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8841	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8848	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8850	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8871	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8890	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8900	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8906	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CERC 8928	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
Ca. colhounii	**CBS 293.79**	A	T	A	T	C	T	C	G	A	C	C	G	G	G	A	G	G	C	T	C	C
	CBS 114704	A	T	A	T	C	T	C	G	A	C	C	G	G	G	A	G	G	C	T	C	C
Ca. eucalypti	**CBS 125275**	–	T	T	T	T	T	C	G	A	C	C	G	G	G	A	G	G	C	C	T	C
	CBS 125276	–	T	T	T	T	T	C	G	A	C	C	G	G	G	A	G	G	C	C	T	C
Ca. fujianensis	**CBS 127201**	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
	CBS 127200	A	T	A	C	C	C	A	G	A	C	C	G	G	G	A	G	G	C	C	C	T
Ca. nymphaeae	**CBS 131802**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	HGUP 100004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ca. paracolhounii	**CBS 114679**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	T	T	G	A	C	C	A	T	C	A	G	C	C	N/A
Ca. pseudocolhounii	**CBS 127195**	A	C	A	T	C	T	C	G	A	C	C	G	G	G	A	G	G	C	C	T	T
	CBS 127196	A	C	A	T	C	T	C	G	A	C	C	G	G	G	A	G	G	C	C	T	T

Table 3. Continue.

Species	Isolate no.	*tub2*																	
		24	28	33	68	98	103	427	442	446	455	534	535	536	537	541	547	550	551
Ca. lichi	**CERC 8866**[§]	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8841	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8848	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8850	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8871	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8890	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8900	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8906	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
	CERC 8928	C	G	C	C	A	C	C	C	T	A	G	T	G	C	T	C	T	C
Ca. colhounii	**CBS 293.79**	N/A	N/A	N/A	C	C	C	C	C	T	A	G	T	G	C	T	C	T	C
	CBS 114704	N/A	N/A	N/A	C	C	C	C	C	T	A	G	T	G	C	T	C	T	C
Ca. eucalypti	**CBS 125275**	T	A	T	C	C	C	C	C	**C**	**G**	G	T	G	C	T	C	T	C
	CBS 125276	T	A	T	C	C	C	C	C	**C**	**G**	G	T	G	C	T	C	T	C
Ca. fujianensis	**CBS 127201**	C	A	C	C	C	**T**	C	C	T	A	G	T	G	C	T	C	T	C
	CBS 127200	C	A	C	C	C	**T**	C	C	T	A	G	T	G	C	T	C	T	C
Ca. nymphaeae	**CBS 131802**	C	A	C	C	A	C	C	C	T	A	–	–	–	–	–	**T**	**C**	**G**
	HGUP 100004	C	A	C	C	A	C	C	C	T	A	–	–	–	–	–	**T**	**C**	**G**
Ca. paracolhounii	**CBS 114679**	N/A	N/A	N/A	**A**	C	C	C	**G**	T	A	G	T	G	C	T	C	T	C
Ca. pseudocolhounii	**CBS 127195**	T	A	T	C	C	C	**T**	C	T	A	G	T	G	C	T	C	T	C
	CBS 127196	T	A	T	C	C	C	**T**	C	T	A	G	T	G	C	T	C	T	C

[†] Polymorphic nucleotides occurring only in all of the isolates are shown, not alleles that partially occur in individuals per phylogenetic group.

[‡] Numerical positions of the nucleotides in the DNA sequence alignments are indicated.

[§] Ex-type isolates are indicated in bold.

[|] Fixed polymorphisms for each group are shaded and in bold, those fixed but shared between two or more groups are only shaded.

[¶] "N/A" represents sequences that are not available.

Table 4. Number of unique alleles found in *Calonectria lichi* and the phylogenetically closest related species in total and in the four gene regions.

	Ca. colhounii	***Ca. eucalypti***	***Ca. fujianensis***	***Ca. nymphaeae***	***Ca. paracolhounii***	***Ca. pseudocolhounii***
Ca. lichi	22(16/3/2/1)[†]	19(4/6/2/7)	10(6/0/0/4)	14(5/NA[‡]/NA/9)	34(19/NA/11/4)	13(3/4/1/5)
Ca. colhounii		19(12/3/2/2)	24(18/3/2/1)	24(15/NA/NA/9)	42(28/NA/12/2)	18(13/1/3/1)
Ca. eucalypti			22(6/6/2/8)	18(4/NA/NA/14)	45(26/NA/12/7)	11(1/4/1/5)
Ca. fujianensis				16(5/NA/NA/11)	37(23/NA/11/3)	15(5/4/1/5)
Ca. nymphaeae					32(20/NA/NA/12)	16(4/NA/NA/12)
Ca. paracolhounii						36(20/NA/12/4)

[†] The order of the four genes: total (*tef1*, *his3*, *cmdA* and *tub2*).

[‡] "NA" represents sequences that are not available.

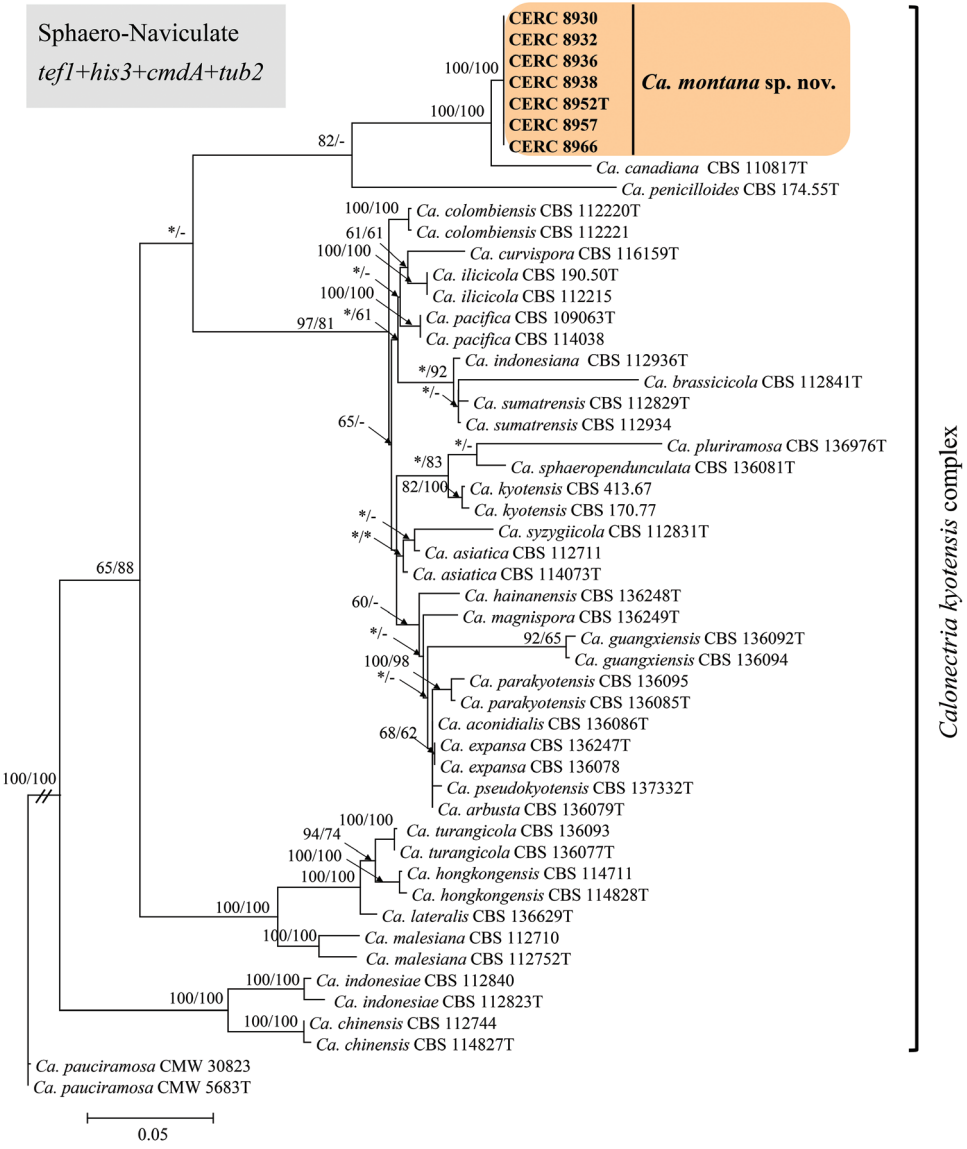

Figure 2. Phylogenetic tree of *Calonectria* species in the Sphaero-Naviculate group based on maximum likelihood (ML) analysis of combined DNA dataset of *tef1*, *his3*, *cmdA* and *tub2* gene sequences. ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in orange. The tree was rooted to *Ca. pauciramosa* (CMW 5683 and CMW 30823).

Table 5. Single nucleotide polymorphism comparisons in four gene regions between *Calonectria montana* and *Ca. canadiana.*

Species	Isolate no.	*tef1*[†]		*his3*																		
		50[‡]	497	28	29	34	47	49	50	58	64	92	110	123	138	157	159	177	180	188	196
Ca. montana	**CERC 8952**[§]	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8930	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8932	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8936	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8938	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8957	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
	CERC 8966	C	–	C	C	C	–	G	C	G	C	C	C	C	C	–	C	G	A	T	G
Ca. canadiana	**CBS 110817**	–	T	G	G	T	A	T	G	A	T	T	G	T	A	A	A	C	T	A	T

Species	Isolate no.	*his3*																				
		199	202	205	212	213	220	227	229	257	300	321	336	339	372	378	397	400	403	418	421
Ca. montana	**CERC 8952**	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8930	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8932	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8936	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8938	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8957	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
	CERC 8966	C	C	C	C	T	C	C	C	C	C	C	C	T	C	–	C	C	C	T	C
Ca. canadiana	**CBS 110817**	T	A	T	G	G	A	G	T	G	G	A	A	C	G	G	T	T	–	G	A

Species	Isolate no.	*cmdA*	*tub2*									
		470	3	4	7	10	174	181	336	403	439	502
Ca. montana	**CERC 8952**	T	G	T	C	T	C	T	C	C	T	C
	CERC 8930	T	G	T	C	T	C	T	C	C	T	C
	CERC 8932	T	G	T	C	T	C	T	C	C	T	C
	CERC 8936	T	G	T	C	T	C	T	C	C	T	C
	CERC 8938	T	G	T	C	T	C	T	C	C	T	C
	CERC 8957	T	G	T	C	T	C	T	C	C	T	C
	CERC 8966	T	G	T	C	T	C	T	C	C	T	C
Ca. canadiana	**CBS 110817**	C	A	C	G	C	T	C	T	A	C	T

[†] Polymorphic nucleotides occurring only in all of the isolates are shown, not alleles that partially occur in individuals per phylogenetic group.

[‡] Numerical positions of the nucleotides in the DNA sequence alignments are indicated.

[§] Ex-type isolates are indicated in bold.

Table 6. Number of unique alleles found in *Calonectria montana* and *Ca. canadiana* in total and in the four gene regions.

	Ca. canadiana
Ca. montana	51(2/38/1/10)†

† The order of the four genes: total (*tef1*, *his3*, *cmdA* and *tub2*).

Taxonomy

Based on DNA sequence comparisons, the 16 isolates collected in this study presented two strongly defined phylogenetic clades in both the Prolate Group and the Sphaero-Naviculate Group. Morphological differences were observed between each phylogenetic clade and its phylogenetically closed species, especially with respect to the size of the macroconidia (Table 7). Based on the phylogenetic analyses, as well as morphological characteristics, the fungi isolated from the soil in this study represent two novel species of *Calonectria*, they are described as follows:

Calonectria lichi **Q.L. Liu & S.F. Chen, sp. nov.**
MycoBank MB821348
Figure 3

Etymology. *lichi*, which is *Calonectria* in Chinese.

Diagnosis. *Calonectria lichi* differs from the phylogenetically closely related species *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis*, *Ca. nymphaeae*, *Ca. paracolhounii* and *Ca. pseudocolhounii* with respect to the macroconidia dimensions.

Type. CHINA. From soil under a natural forest in central China, 07 April 2016, ShuaiFei Chen, CSFF 2019 – holotype, CERC 8866 = CGMCC 3.18733 – ex-type culture.

Description. Sexual morph unknown. Macroconidiophores consisting of a stipe, a suite of penicillate arranged fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, (39.5–)78.5–160.5(–206.5) × (4.5–)5.5–7.5(–8.5) µm; stipe extension septate, straight to flexuous, (124–)139.5–187.5(–218) µm long, 2.5–5 µm wide at the apical septum, terminating in a clavate vesicle, (3.5–)4–5(–5.5) µm diam, lateral stipe extensions (90° to main axis) absent. Conidiogenous apparatus (44–)56–92(–108.5) µm long, (35–)52–82.5(–94) µm wide; primary branches aseptate to 1–septate, (12–)16.5–33.5(–46.5) × (4–)4.5–6.5(–9) µm; secondary branches aseptate, (7–)9.5–16(–21) × (3–)3.5–5(–6) µm; tertiary branches aseptate, (7.5–)9–12.5(–14.5) × (3–)3.5–4.5(–6) µm; additional branches (–5), aseptate, (5.5–)8.5–12.5(–14) × (2.5–)3.5–4.5(–5.5) µm; each terminal branch producing 2–4 phialides; phialides doliiform to reniform, hyaline, aseptate, (6–)8–12(–14.5) × (2.5–)3–4(–5) µm, apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (53–)60.5–70.5(–79) × (5–)5.5–6.5(–7) µm (av. = 65.7 × 6 µm), 3–septate, lacking a visible abscission scar,

Table 7. Morphological comparisons of *Calonectria lichi*, *Ca. montana* and their phylogenetically closely related species.

Speices	Macroconidia (L × W)[†,‡]	Macroconidia average (L × W)[†,‡]	Macroconidia septation	Vesicle (Min. – Max.)[†,§]	Vesicle shape	Reference
Ca. lichi[|]	(53–)60.5–70.5(–79) × (5–)5.5–6.5(–7)[¶]	65.7 × 6	3	(3.5–)4–5(–5.5)	clavate	This study
Ca. colhounii	(45–)60–70(–80) × (4–)5(–6)	65 × 5	(1–)3	3–4	clavate	Crous 2002
Ca. eucalypti	(66–)69–75(–80) × (5–)6	72 × 6	3	4–6	broadly clavate	Lombard et al. 2010b
Ca. fujianensis	(48–)50–55(–60) × (2.5–)3.5–4.5(–5)	52.5 × 4	(1–)3	(3–)3.5–4.5(–5)	clavate	Chen et al. 2011
Ca. nymphaeae	55–63 × 5.3–6.3	61 × 5.9	3–4	3–5	clavate	Xu et al. 2012
Ca. paracolhounii	(37–)39–43(–45) × 4–5	41 × 5	3	3–5	narrowly clavate	Lombard et al. 2016
Ca. pseudocolhounii	(49–)55–65(–74) × (3.5–)4–5(–5.5)	60 × 4.5	(1–)3	(3.5–)4–5(–6)	clavate	Chen et al. 2011
Ca. montana	(37.5–)40.5–45.5(–51.5) × 4–5(–5.5)	43.2 × 4.6	1	(4–)7–11(–12.5)	sphaeropedunculate	This study
Ca. canadiana	(38–)48–55(–65) × 4(–5)	50 × 4	1	6–10	pyriform to sphaeropedunculate	Kang et al. 2001; Lechat et al. 2010

[†] All measurements are in µm.

[‡] L × W = length × width.

[§] Min.–Max. = minimum–maximum.

[|] Species indicated in bold are described in this study.

[¶] Measurements are presented in the format [(minimum–) (average – standard deviation) – (average + standard deviation) (–maximum)].

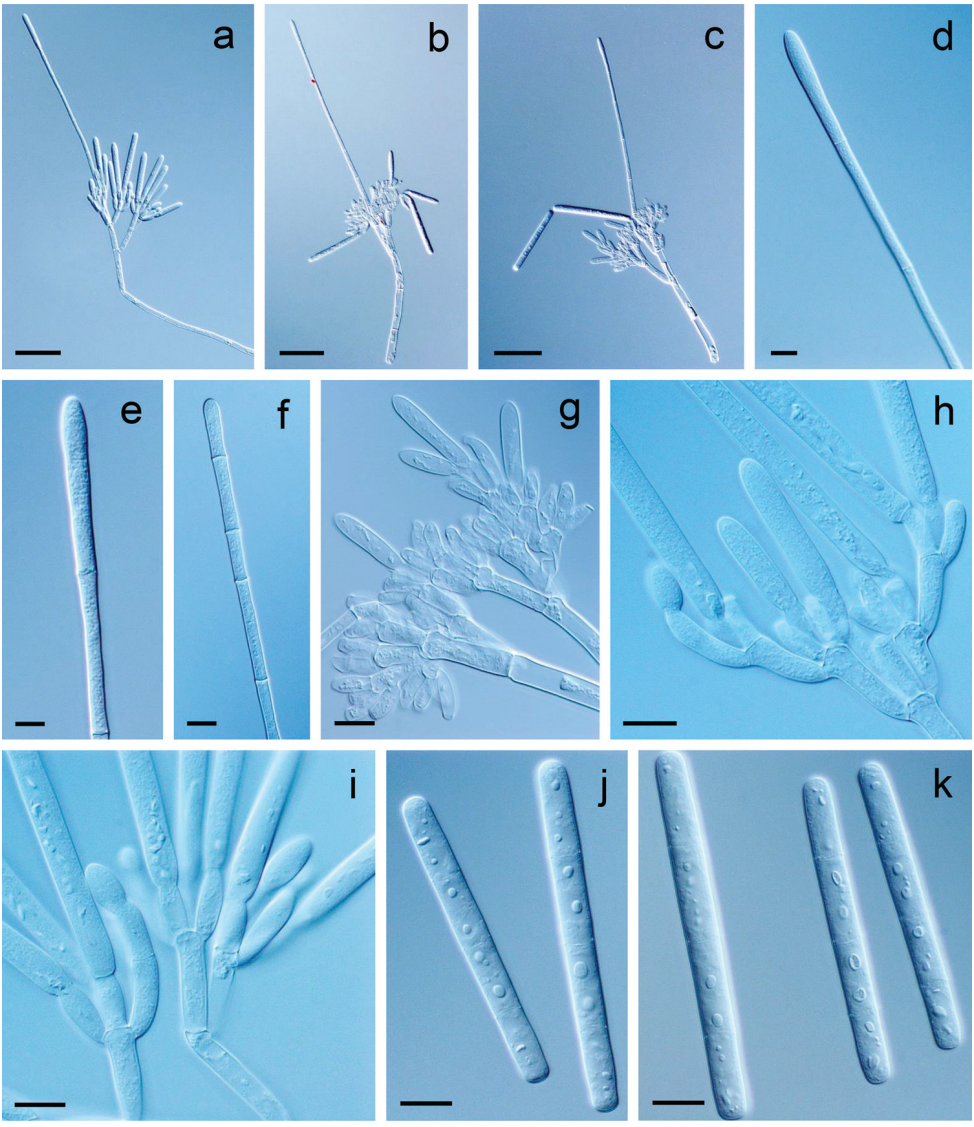

Figure 3. *Calonectria lichi*. **a–c** Macroconidiophore **d–f** Clavate vesicles **g–i** Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides **j–k** Macroconidia Scale bars: **a–c** = 50 µm; **d–f** = 5 µm; **g–k** = 10 µm.

held in parallel cylindrical clusters by colorless slime. Megaconidia and microconidia not observed.

Culture characteristics. Colonies forming abundant white aerial mycelium on MEA at 25 °C after seven days, with feathery, irregular margins at the edges, moderate sporulation. Surface with white to buff outer margins, and salmon (13'd) inner region, becoming ochreous (44) towards the center, reverse sienna (8) to umber (9) with abundant chlamydospores throughout the medium, forming microsclerotia. Optimal

growth temperature at 25 °C, no growth at 5 °C and 35 °C, after seven days, colonies at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C reached 21.9 mm, 30.8 mm, 41.5 mm, 54.4 mm and 37.2 mm, respectively.

Substratum. Soil in a natural forest.

Distribution. Central China.

Other specimens examined. CHINA. From soil in a natural forest in central China, 07 April 2016, ShuaiFei Chen, CSFF 2020, culture CERC 8850 = CGMCC 3.18732; CHINA. From soil under a natural forest in central China, 07 April 2016, ShuaiFei Chen, CSFF 2021, culture CERC 8890 = CGMCC 3.18734; CHINA. From soil in a natural forest in central China, 07 April 2016, ShuaiFei Chen, culture CERC 8841, CERC 8848, CERC 8871, CERC 8900, CERC 8906 and CERC 8928.

Notes. *Calonectria lichi* is a new species in the *Ca. colhounii* complex and is closely related to *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis*, *Ca. nymphaeae*, *Ca. paracolhounii* and *Ca. pseudocolhounii* (Crous 2002, Lombard et al. 2010b, 2016, Chen et al. 2011, Xu et al. 2012, Crous et al. 2015). The macroconidia of *Ca. lichi* (av. 65.7 × 6.0 µm) are longer and wider than those of *Ca. colhounii* (av. 65 × 5 µm), *Ca. fujianensis* (av. 52.5 × 4 µm), *Ca. nymphaeae* (av. 61 × 5.9 µm), *Ca. paracolhounii* (av. 41 × 5 µm) and *Ca. pseudocolhounii* (av. 60 ×4.5 µm), but narrower than those of *Ca. eucalypti* (av. 72 × 6µm).

Calonectria montana Q.L. Liu & S.F. Chen, sp. nov.

MycoBank MB821349

Figure 4

Etymology. *montis*, meaning mountain in Latin, referring to the location where this fungus was collected.

Diagnosis. *Calonectria montana* can be distinguished from the phylogenetically closely related species *Ca. canadiana* by the size of macroconidia.

Type. CHINA. From soil under a natural forest in central China, 07 April 2016, ShuaiFei Chen, holotype CSFF 2022, ex-type culture CERC 8952 = CGMCC 3.18735.

Description. Sexual morph unknown. Macroconidiophores consisting of a stipe, a suite of penicillate arranged fertile branches, a stipe extension, and a terminal vesicle; stipe septate, hyaline, smooth, (30–)52–91(–123.5) × (4–)5.5–8(–9.5) µm; stipe extension septate, straight to flexuous (76.5–)107–168(–211.5) µm long, (2.5–)3–4.5(–5.5) µm wide at the apical septum, terminating in a pyriform to sphaeropedunculate vesicle, (4–)7–11(–12.5) µm diam, lateral stipe extensions (90° to main axis) absent. Conidiogenous apparatus (40–)49–87.5(–102.5) µm long, (44–)62–91(–104) µm wide; primary branches aseptate to 1–septate, (14.5–)19.5–34(–55.5) × (4–)4.5–6(–7) µm; secondary branches aseptate, (11–)13.5–23(–33) × (3–)4–5(–6) µm; tertiary branches aseptate, (9–)11–15(–16.5) × (3.5–)3.5–4.5(–5) µm; each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, (8–)10.5–

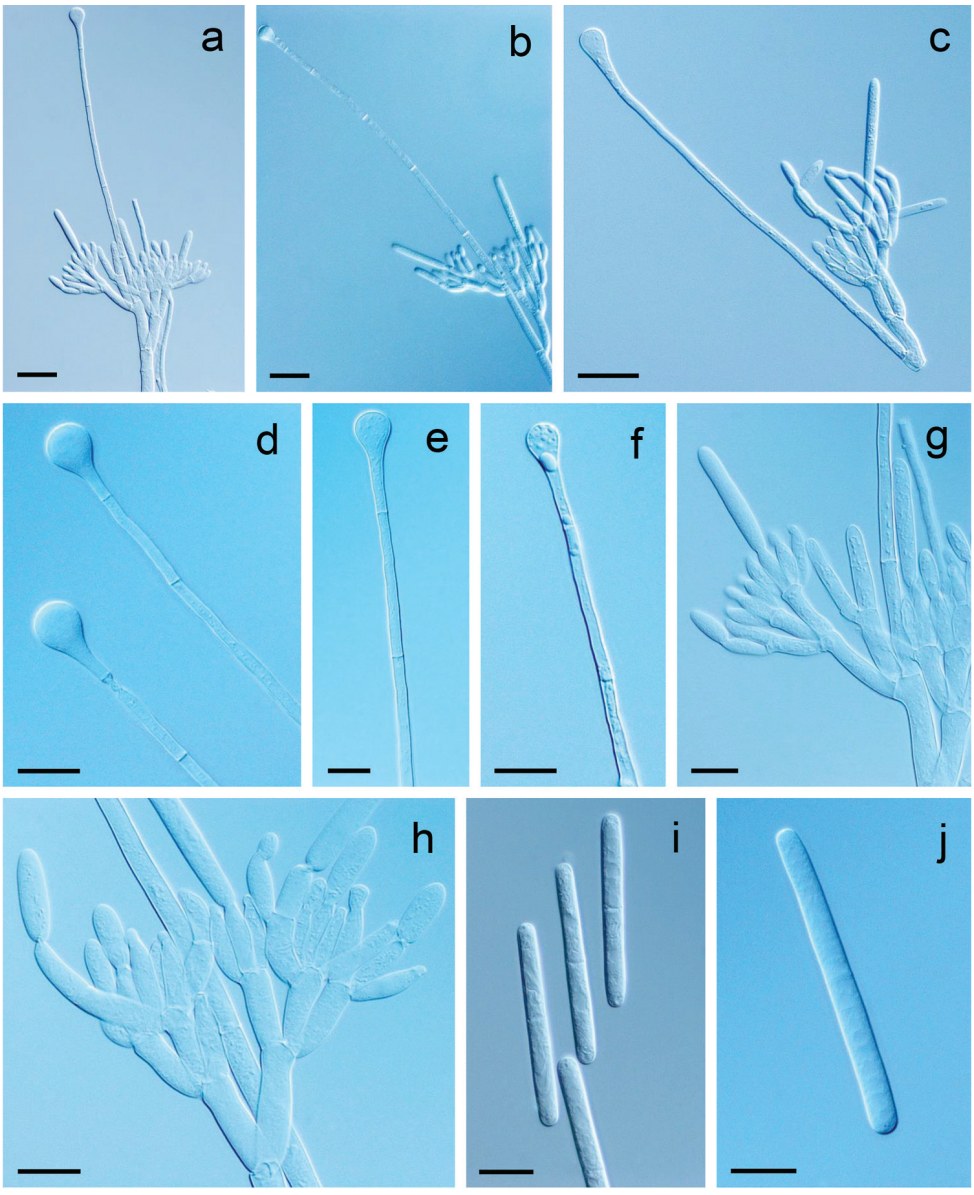

Figure 4. *Calonectria montana*. **a–c** Macroconidiophores **d–f** Sphaeropedunculate vesicles **g–h** Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides **i–j** Macroconidia Scale bars: **a–c** = 20 µm; **d–j** = 10 µm.

12.5(–15.5) × (2.5–)3.5–4.5(–5) µm, apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (37.5–)40.5–45.5(–51.5) × 4–5(–5.5) µm (av. = 43.2 × 4.6 µm), 1–septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colorless slime. Megaconidia and microconidia not observed.

Culture characteristics. Colonies forming abundant buff and wooly aerial mycelium on MEA at 25 °C after seven days, with feathery, irregular margins at the edges, sporulation moderate and more concentrated in the colony centre. Surface with buff to sienna (8) outer margins, reverse sienna (8) to umber (9), and chesnut (9'm) inner region, abundant chlamydospores throughout the medium, forming microsclerotia. Optimal growth temperature at 30 °C, no growth at 5 °C and 35 °C, after seven days, colonies at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C reached 22.9 mm, 31.5 mm, 51.1 mm, 61.9 mm and 77.2 mm, respectively, this is a high-temperature species.

Substratum. Soil under the natural forest.

Distribution. Central China.

Other specimens examined. CHINA. From soil in a natural forest in central China, 07 April 2016, ShuaiFei Chen, CSFF 2023, culture CERC 8957 = CGMCC 3.18736; From soil in a natural forest in central China, 07 April 2016, ShuaiFei Chen, CSFF 2024, culture CERC 8966 = CGMCC 3.18737; From soil in a natural forest in central China, 07 April 2016, ShuaiFei Chen, culture CERC 8930, CERC 8932, CERC 8936 and CERC 8938.

Notes. *Calonectria montana* is a new addition to the *Ca. kyotensis* complex and is phylogenetically closely related to *Ca. canadiana* (Crous 2002, Crous et al. 2004, Lombard et al. 2015, 2016). The macroconidia of *Ca. montana* (av. 43.2 × 4.6 μm) are shorter and wider than those of *Ca. canadiana* (av. 50 × 4 μm).

Discussion

This study identified two novel species of *Calonectria* from soil in a natural forest in the temperate region of central China. The identification of the fungi was supported by DNA sequence comparisons and morphological features. The two species were named *Calonectria lichi* and *Ca. montana*.

Calonectria lichi is a new addition to the *Ca. colhounii* complex that belongs to the Prolate Group. Based on phylogenetic analyses of four gene sequences, *Ca. lichi* formed a distinct and well-supported phylogenetic clade closely related to *Ca. fujianensis*, *Ca. nymphaeae* and *Ca. paracolhounii*, but it can be distinguished from these species by its larger macroconidia. To date, 10 species in the *Ca. colhounii* complex have been identified and described. Other than *Ca. lichi* described in this study, the other species include *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis*, *Ca. macroconidialis*, *Ca. monticola*, *Ca. nymphaeae*, *Ca. paracolhounii*, *Ca. parva* and *Ca. pseudocolhounii* (Crous 2002, Lombard et al. 2010b, 2016, Chen et al. 2011, Xu et al. 2012, Crous et al. 2015). Of these species, *Ca. colhounii*, *Ca. eucalypti*, *Ca. fujianensis*, *Ca. nymphaeae* and *Ca. pseudocolhounii* have been shown to be homothallic and always produce bright yellow perithecia (Crous 2002, Lombard et al. 2010b, Chen et al. 2011, Xu et al. 2012). In China, four species in the *Ca. colhounii* complex have been reported: except for *Ca. lichi*, which was isolated from a natural forest in the temperate zone in central China, the other species, including *Ca. fujianensis*, *Ca. pseudocolhounii* and *Ca. nym-*

phaeae, were previously isolated from tropical or subtropical regions in southern China (Chen et al. 2011, Xu et al. 2012).

Calonectria montana adds a new species to the *Ca. kyotensis* complex that belongs to the Sphaero-Naviculate Group. Phylogenetic analyses showed that *Ca. montana*, which formed an independent clade with a high bootstrap value, is closely related to *Ca. canadiana.* Morphological differences were observed between *Ca. montana* and *Ca. canadiana*, especially with respect to the size of the macroconidia and the shape of the vesicles (Kang et al. 2001, Crous 2002). Species in the *Ca. kyotensis* complex are characterized by having sphaeropedunculate vesicles with lateral stipe extensions on a conidiogenous apparatus (Crous et al. 2004, Lombard et al. 2010b, 2015, 2016). No lateral stipe extensions were produced by *Ca. montana*, indicating that this species is different from other species in the *Ca. kyotensis* complex. In this study, *Ca. montana* was isolated from soil in central China, 14 species residing in the *Ca. kyotensis* complex were previously reported in China, and all of them were isolated from soil in southern China (Crous et al. 2004, Lombard et al. 2015). The results from this study suggest that more species in *Ca. kyotensis* complex have yet to be discovered from China.

Species of *Calonectria* are important plant pathogens that can cause devastating diseases on various plant hosts worldwide, especially on horticultural, agronomic and forestry crops (Polizzi et al. 2001, 2009, Crous 2002, Saracchi et al. 2008, Chen et al. 2011, Pan et al. 2012). In China, *Calonectria* species have been reported as pathogens of various important agronomic and forestry crops. In agriculture, the *Fabaceae* and *Arecaceae* plant families are susceptible to infection by *Calonectria* species, including *Ca. ilicicola*, which causes black rot (CBR) of *Arachis hypogaea* (peanut) and *Medicago sativa* (Gai et al. 2012, Pan et al. 2012, Pei et al. 2015), *Ca. ilicicola* causes red crown rot of *Glycine max* (soybean) (Guan et al. 2010), and *Ca. colhounii* and *Ca. pteridis* cause leaf spot on *Phoenix canariensis* and *Serenoa repens*, respectively (Luo et al. 2009, Yang et al. 2014). In forestry, leaf blight caused by *Calonectria* species is considered as one of the most serious threats to *Eucalyptus* plantations and nurseries in southern China (Zhou et al. 2008, Lombard et al. 2010a, Chen et al. 2011). The leaf inoculations showed that all tested *Calonectria* species were pathogenic to the tested *Eucalyptus* clones, including the clones that are widely planted in southern China (Chen et al. 2011, Li et al. 2014a, b). These research results suggest that species of *Calonectria* need to be monitored carefully, both in agronomic crops and forests.

Accurate diagnosis of plant diseases and identification of their casual agents provide the foundation for developing effective disease management strategies (Booth et al. 2000, Crous 2002, Old et al. 2003, Vitale et al. 2013, Wingfield et al. 2015). Based on previous research results, the majority of *Calonectria* species identified and described in China were isolated from diseased plant tissues or soil under forestry plantations in subtropical and tropical regions (Crous et al. 2004, Lombard et al. 2010a, 2015, Chen et al. 2011). In this study, two novel *Calonectria* species were described, and they were isolated from soil in a natural forest in the temperate zone. The results from this study suggest that more extensive surveys need to be conducted to collect *Calonectria* in more geographic regions with different climate zones, which will help to clarify the species diversity of *Calonectria* in China.

Acknowledgments

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Supplementary material 1

Phylogenetic tree of *Calonectria* species in the Prolate group based on maximum likelihood (ML) analysis of *tef1* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in blue. The tree was rooted to *Ca. hongkongensis* (CBS 114711 and CBS 114828).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl1

Supplementary material 2

Phylogenetic tree of *Calonectria* species in the Prolate group based on maximum likelihood (ML) analysis of *his3* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in blue. The tree was rooted to *Ca. hongkongensis* (CBS 114711 and CBS 114828).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl2

Supplementary material 3

Phylogenetic tree of *Calonectria* species in the Prolate group based on maximum likelihood (ML) analysis of *cmdA* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in blue. The tree was rooted to *Ca. hongkongensis* (CBS 114711 and CBS 114828).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl3

Supplementary material 4

Phylogenetic tree of *Calonectria* species in the Prolate group based on maximum likelihood (ML) analysis of *tub2* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in blue. The tree was rooted to *Ca. hongkongensis* (CBS 114711 and CBS 114828).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl4

Supplementary material 5

Phylogenetic tree of *Calonectria* species in the Sphaero-Naviculate group based on maximum likelihood (ML) analysis of *tef1* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in orange. The tree was rooted to *Ca. pauciramosa* (CMW 5683 and CMW 30823).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl5

Supplementary material 6

Phylogenetic tree of *Calonectria* species in the Sphaero-Naviculate group based on maximum likelihood (ML) analysis of *his3* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in orange. The tree was rooted to *Ca. pauciramosa* (CMW 5683 and CMW 30823).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl6

Supplementary material 7

Phylogenetic tree of *Calonectria* species in the Sphaero-Naviculate group based on maximum likelihood (ML) analysis of *cmdA* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in orange. The tree was rooted to *Ca. pauciramosa* (CMW 5683 and CMW 30823).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl7

Supplementary material 8

Phylogenetic tree of *Calonectria* species in the Sphaero-Naviculate group based on maximum likelihood (ML) analysis of *tub2* gene sequences

Authors: QianLi Liu, ShuaiFei Chen

Data type: molecular data

Explanation note: ML and MP (maximum parsimony) bootstrap values (ML/MP) are shown above branches, with bootstrap values below 60 % marked with an *, and absent analysis values are marked with -. Isolates representing ex-type material are marked with "T", isolates highlighted in bold were sequenced in this study and novel species were covered in orange. The tree was rooted to *Ca. pauciramosa* (CMW 5683 and CMW 30823).

Link: https://doi.org/10.3897/mycokeys.26.14688.suppl8