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Cryphonectriaceae (Diaporthales), a new family including *Cryphonectria*, *Chrysoporthe*, *Endothia* and allied genera

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Abstract: Recent phylogenetic studies on the members of the Diaporthales have shown that the order includes a number of distinct phylogenetic groups. These groups represent the Gnomoniaceae, Melanconidaceae, Valsaceae, Diaporthaceae and Togniniaceae. New groups representing undescribed families also have emerged and they have been referred to as the *Schizoparme*, *Cryphonectria-Endothia* and *Harknessia* complexes. In this study we define the new family Cryphonectriaceae (Diaporthales) to accommodate genera in the *Cryphonectria-Endothia* complex. These genera can be distinguished from those in other families or undescribed groups of the Diaporthales by the formation of orange stromatic tissue at some stage of their life cycle and a purple reaction in KOH and a yellow reaction in lactic acid associated with pigments in the stromatic tissue or in culture.

Key words: *Amphilogia*, *Cryptodiaporthe corni*, LSU sequences, *Rostraureum*

INTRODUCTION

The Diaporthales represents a fungal order incorporating approximately 100 genera (Eriksson 2005a). Genera in this order occur on a wide diversity of plant substrates as either saprophytes or parasites (Barr 1978). The parasites include some of the most economically and ecologically important pathogens of trees and agricultural crops. Examples of such a pathogen are *Cryphonectria parasitica* (Murrill) M. E. Barr, which has devastated American chestnut (*Castanea dentata* Borkh.) populations in North America (Anagnostakis 1987, Heiniger and Rigling 1994), and *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., the causal agent of stem canker of soybeans (Kulik 1984).

Members of the Diaporthales are united morphologically by a *Diaporthe*-type centrum (Alexopoulos and Mims 1978, Barr 1978). Morphological characteristics include perithecia with long necks that are located in pseudostromata with no paraphyses and thick-walled asci that are either evanescent with short stalks or intact (Alexopoulos and Mims 1978, Hawksworth et al 1995). Features such as the presence or absence of stromatic tissue, stromatal tissue type, the position of the perithecia and perithecial beaks relative to the substrate, ascospore shape and ascospore septation have been used to differentiate families and genera in the Diaporthales (Barr 1978).

Six families currently are recognized in the Diaporthales (Eriksson 2005b). These include the Diaporthaceae Höhn. ex Wehm., Gnomoniaceae G. Winter, Melanconidaceae G. Winter, Valsaceae Tul. & C. Tul., Vialaeaceae P.F. Cannon and Togniniaceae Réblová, L. Mostert, W. Gams & Crous. This classification has largely emerged from recent DNA sequence comparisons of Castlebury et al (2002), who compared genera representing the families previously recognized in the Diaporthales. The Togniniaceae is a new family that was described by Réblová et al (2004). The family level status of the Vialaeaceae, established by Cannon (Cannon 1995), has not been confirmed with DNA sequence data (Castlebury et al 2002).

In addition to the described families groups not recognized previously also were noted by Castlebury et al (2002). One of these groups includes species of *Schizoparme* Shear and their *Coniella* Höhn. and *Pilidiella* Petr. & Syd. anamorphs, which have been referred to as members of the *Schizoparme* complex (Castlebury et al 2002). The second group included species of *Cryphonectria* (Sacc.) Sacc. and *Endothia* Fr.; this was referred to as the *Cryphonectria-Endothia* complex (Castlebury et al 2002). Species of *Harknessia* M.C. Cooke and allied genera *Dwiroopa* C.V. Subramanian & J. Muthumary and *Apoharknessia* Crous & S. Lee also formed a group within the Diaporthales, although not well supported phylogenetically with the available DNA sequence data

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(Castlebury et al 2002, Lee et al 2004). This group could not be described as a family because the status of *Wuestneia* Auersw. ex Fuckel as the teleomorph of this coelomycete genus still must be confirmed (Lee et al 2004). In addition to these undescribed complexes of species, several species, such as *Greeneria uvicola* (Berk. & M.A. Curtis) Punith., did not group in any of the families or undescribed complexes (Castlebury et al 2002). This suggests that additional groups might emerge in the Diaporthales as more species are described or included in phylogenetic comparisons.

Various taxonomic studies considering genus and species delimitation for species of *Cryphonectria* and *Endothia* have been conducted recently (Venter et al 2002; Gryzenhout et al 2004, 2005a, b; Myburg et al 2004a). These studies have included the recognition of at least four new genera containing species previously placed in *Cryphonectria*. Of these *Chrysoporthe* Gryzenh. & M. J. Wingf. was described to accommodate the important stem canker pathogen *Cryphonectria cubensis* (Bruner) Hodges and two additional species, *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf. and the anamorph species *Chrysoporthella hodgesiana* Gryzenh. & M.J. Wingf. (Gryzenhout et al 2004). *Rostraureum* Gryzenh. & M.J. Wingf. was described to include the fungus previously known as *Cryphonectria longirostris* (Earle) Micales & Stipes and also includes *Rostraureum tropicale* Gryzenh. & M.J. Wingf., a pathogen of *Terminalia ivorensis* A. Chev. trees in Ecuador (Gryzenhout et al 2005a). Another genus, represented by isolates from *Elaeocarpus* spp. in New Zealand and including *Cryphonectria gyrosa* (Berk. & Broome) Sacc., was identified in a study by Myburg et al (2004a) and subsequently was described as *Amphilogia* Gryzenh. & M.J. Wingf. (Gryzenhout et al 2005b). A genus closely related to *Cryphonectria* and *Endothia* and representing isolates from *Syzygium aromaticum* (L.) Murr. & Perry (clove) in Indonesia, was recognized in a study by Myburg et al (2003). This genus was not assigned a name because insufficient herbarium material, linked to isolates, is available.

A collection of isolates representing species of *Cryphonectria* and *Endothia*, as well as those of the newly described genera, has provided the opportunity to substantially expand the LSU DNA sequence dataset for the *Cryphonectria-Endothia* complex defined by Castlebury et al (2002). The expanded LSU sequence dataset ultimately was used to characterize and describe a family for species and genera in the *Cryphonectria-Endothia* complex of genera. LSU sequences also were supplemented with more variable sequences of the ribosomal ITS region and β-tubulin genes, to show infrafamilial relationships.

MATERIALS AND METHODS

Isolates studied.—Representative isolates for species of *Cryphonectria*, *Endothia*, *Chrysoporthe*, *Amphilogia* and *Rostraureum*, used in previous studies (Myburg et al 2002, 2003; Venter et al 2002; Gryzenhout et al 2004; Myburg et al 2004a, b; Gryzenhout et al 2005a), were included in sequence data analyses (TABLE I). Isolates (CMW 10779–10781) that represented the undescribed genus from *S. aromaticum* in Indonesia (Myburg et al 2003) also were included (TABLE I).

Isolates of some of the species studied by Castlebury et al (2002) and Zhang and Blackwell (2001) were included. These isolates included *Cryptodiaporthe corni* (Wehm.) Petr. (AR 2814), and isolates of *C. macrospora* (Tak. Kobay. & Kaz. Itô) M.E. Barr (AR 3444) and *C. nitschkei* (G.H. Otth) M.E. Barr (AR 3433) from Siberia, Russia. These were kindly provided for additional analyses by Drs A.Y. Rossman and L.A. Castlebury (Systematic Botany and Mycology Laboratory, USDA-ARS, Beltsville, Maryland). *Cryptodiaporthe corni* was of interest because it grouped separately from other *Cryptodiaporthe* Petr. species in the *Gnomoniaceae* clade, including the type species *Cryptodiaporthe aesculi* (Fuckel) Petr. (Castlebury et al 2002).

The isolate referred to as *E. eugeniae* (Nutman & F.M. Roberts) J. Reid & C. Booth (CBS 534.82), sequenced by Zhang and Blackwell (2001) and used by Castlebury et al (2002), was acquired from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. It was necessary to include this isolate because it did not group with the isolate of *Chr. cubensis*, even though it represents a previous synonym of *C. cubensis* (Hodges et al 1986, Micales et al 1987, Myburg et al 2003). The isolate of *C. havanensis* (Bruner) M.E. Barr (CBS 505.63) used in the study of Castlebury et al (2002) has been shown (as E40 or CMW 10453) to represent *Chr. cubensis* (Hodges et al 1986, Micales et al 1987, Myburg et al 2004a).

Isolates in this study (TABLE I) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A representative subset of these isolates not in other internationally recognized culture collections is stored in the culture collection of the Centraalbureau voor Schimmelcutures, Utrecht, Netherlands (TABLE I). Background information pertaining to other isolates included in the phylogenetic analyses can be found in the studies of Zhang and Blackwell (2001) and Castlebury et al (2002).

PCR amplification and sequencing.—Isolates were grown in malt extract broth (20 g/L Biolab malt extract). DNA was extracted from the mycelium following the method used by Myburg et al (1999). To characterize the isolates of *Cryptodiaporthe corni* (AR 2814), *C. macrospora* (AR 3444), *C. nitschkei* (AR 3433) and *E. eugeniae* (CBS 534.82), the ITS1, 5.8S and ITS2 regions of the rRNA operon as well as two regions within the β-tubulin gene were amplified with previously described methods (Myburg et al 1999, 2002).

DNA from isolates representing key species of *Cryphonectria*, *Endothia*, *Chrysoporthe*, *Rostraureum*, *Amphilogia* and

the undescribed fungi from Indonesia (TABLE I) was used to amplify a region of the LSU rDNA gene. Primers pairs ITS3 (White et al 1990) and LR3 (Vilgalys and Hester 1990) were used. The reaction mix used the same reagents and concentrations as those used for the ITS and β-tubulin reactions. PCR conditions were 95 C for 3 min (denature), 30 cycles of 95 C for 30 s (denature), 56 C for 45 s (anneal), 72 C for 1 min (elongation) and a final elongation step of 72 C for 4 min. Amplification products were purified with a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and used directly as templates in subsequent sequencing reactions.

Sequencing reactions were as specified by the manufacturers of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, UK). Nucleotide sequence data were generated with an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, UK). The primer pairs used in the respective sequencing reactions were: ITS1 and ITS4 (amplifying the ITS region), Bt1a and Bt1b (amplifying β-tubulin region 1), Bt2a and Bt2b (amplifying β-tubulin region 2), LS1 and LR3 (amplifying LSU rDNA).

Raw sequence data generated for the respective gene regions were edited with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems Inc., Foster City, California) software, exported to PAUP* (Phylogenetic Analysis Using Parsimony [*and other methods]) version 4.0b8 (Swofford 1998) and aligned to available sequence datasets. Subsequent phylogenetic analyses were executed with PAUP*.

Analyses of LSU rDNA sequences.—The subset of isolates used to generate large subunit ribosomal RNA sequence data for this study included 12 taxa (TABLE I). These sequences were aligned with the 650 bp dataset of Castlebury et al (2002) obtained from TreeBase (study accession number S815). This dataset was shown to be sufficient to define the various lineages within the Diaporthales, although higher bootstrap values were obtained with a larger dataset (Castlebury et al 2002). Only key taxa representing each lineage and the type species of genera were retained in the dataset. LSU sequences for additional species from other studies also were added to this database. These included species in the *Schizoparme*-complex derived from van Niekerk et al (2004); species of *Harknessia*, *Apoharknessia* and *Wuestneia* derived from Lee et al (2004); representatives of the *Togniniaceae* (Réblová et al 2004) and a second *Crypto. corni* isolate sourced from Zhang and Blackwell (2001).

Phylogenetic trees were generated by parsimony and distance analyses. LSU sequence data were deposited in GenBank (TABLE I) and the datamatrix in TreeBase (S1487, M2671). Gaps were treated as characters in the parsimony analyses using the NEWSTATE option in PAUP* and missing in the distance analyses. Parsimony was inferred from TBR swapping algorithms with MULTREES inactive and trees randomly added (100 reps). Uninformative characters were excluded and remaining characters were reweighted according to their consistency indices (CI) to reduce the number of trees. A 50% consensus bootstrap analysis was performed with the heuristic search modified by using no branch swapping with the MULTREES option turned off and only 10 random repeats (Castlebury et al 2002). This was done because bootstrap analysis was inordinately extensive using the parameters defined to generate the trees and could not run to completion (Castlebury et al 2002). For the distance analyses a neighbor joining tree was generated with the Tamura-Nei (TrNef+I+G) model (Tamura and Nei 1993) with invariable sites (I), gamma distribution (G) and equal base frequencies was used (I = 0.5726; G = 0.7028; rate matrix 1.0000, 4.2834, 1.0000, 1.0000, 8.9689, 1.0000). These parameters were determined with Modeltest version 3.5 (Posada and Crandall 1998).

The probabilities of branches occurring were tested with Bayesian inference employing the Markov chain Monte Carlo (MCMC) algorithm (Larget and Simon 1999). The program MrBayes version 3.1.1 (Huelsenbeck and Ronquist 2001) was used with these parameters: number of generations = 1 000 000, sample frequency = 100, number of chains = four (one cold, three hot) and a burn-in of 1000. Four independent analyses were run, with one having 3 000 000 generations. The likelihood model and settings were the same as for the distance methods, as determined by Modeltest.

Analyses of ITS rDNA and β-tubulin sequences.—The ribosomal DNA (ITS1, 5.8S, ITS2) and β-tubulin sequence data generated in this study were added to already published sequences of other species (TABLE I) using the TreeBase sequence matrix (study accession number S1128, matrix accession number M1935) from Myburg et al (2004a). Two isolates of *Diaporthe amibigua* Nitschke were used as outgroup because they are more distantly related members of the same order. The datasets for the two regions of the genome sequenced were subjected to a partition homogeneity test (Farris et al 1994) to ascertain whether they could be combined in a single sequence dataset in the phylogenetic analyses. Phylogenetic analyses were done with both parsimony and distance methods. All the sequence characters were unordered. Gaps were treated as characters with the NEWSTATE option in parsimony analyses and as missing in distance analyses. Parsimony was inferred from heuristic searches, with tree-bisection-reconnection (TBR) and MULTREES options (saving all optimal trees) effective and trees added randomly (100 repetitions). Uninformative characters were excluded, and remaining characters were reweighted according to their individual CI to reduce the number of trees.

The distance analysis was done with the neighbour joining method and the general time reversal model (GTR +I+G) (Rodríguez et al 1990), with G = 1.3390, I = 0.4877, base frequency 0.1929, 0.3348, 0.2315, 0.2409 and rate matrix 0.9638, 2.7348, 1.2919, 1.5236, 3.3995, 1.00. This model was chosen based on likelihood ratio tests performed by Modeltest version 3.5 (Posada and Crandall 1998). The confidence levels of the tree branch nodes were determined by a 1000-replicate bootstrap analysis showing values greater than 70%. Bayesian analyses were made with the same

TABLE I. Isolates used for the DNA sequence analyses

Original label name of taxon	Isolate numbers[a]	Additional numbers[a]	Host	Origin	Collector	GenBank Accession numbers[b]	
						ITS, β-tubulin1 and 2	LSU
Chrysoporthe cubensis	CMW 8758	—	*Eucalyptus* sp.	Indonesia	MJ Wingfield	AF 046898, AF 273068, AF 273463	**AY 194098**
	CMW 2632	—	*Eucalyptus marginata*	Australia	E Davison	AF 046893, AF 273078, AF 375607	—
	CMW 1853	—	*Syzygium aromaticum*	Brazil	—	AF 036891, AF 273070, AF 273465	—
Cryphonectria havanensis[c]	CMW 10453	E40, CBS 505.63	*Eucalyptus saligna*	Demographic Republic of Congo	—	AY 063476, AY 063478, AY 063480	AF 408339
Chrysoporthe austroafricana	CMW 62	—	*E. grandis*	South Africa	MJ Wingfield	AF 292041, AF 273063, AF 273458	**AY 194097**
	CMW 2113	CBS 112916	*E. grandis*	South Africa	MJ Wingfield	AF 046892, AF 273067, AF 273462	—
Chrysoporthella hodgesiana	CMW 9929	—	*Tibouchina urvilleana*	Colombia	C Rodas, MJ Wingfield	AF 265656, AF 292036, AF 292039	—
	CMW 10641	CBS 115854	*Tibouchina semidecandra*	Colombia	R Arbaleaz	AY 692322, AY 692326, AY 692325	—
Rostraureum tropicale	CMW 9972	—	*Terminalia ivorensis*	Ecuador	MJ Wingfield	AY 167426, AY 167431, AY 167436	**AY 194092**
	CMW 10796	CBS 115757	*T. ivorensis*	Ecuador	MJ Wingfield	AY 167428, AY 167433, AY 167438	—
Unidentified	CMW 14853	CBS 534.82	*Eugenia aromatica*	Indonesia	S Mandang	**DQ120759, DQ120763, DQ120764**	AF 277142
	CMW 10780	—	*E. aromatica*	Indonesia	MJ Wingfield	AY 084008, AY 084020, AY 084032	—
	CMW 10781	CBS 115844	*E. aromatica*	Indonesia	MJ Wingfield	AY 084009, AY 084021, AY 084033	**AY 194093**
Cryphonectria radicalis	CMW 10436	E14, CBS 165.30	*Quercus suber*	Portugal	B d'Oliveira	AF 452117, AF 525703, AF 525710	—
	CMW 10455	E42, CBS 238.54	*Q. suber*	Italy	A Biraghi	AF 452113, AF 525705, AF 525712	**AY 194101**
	CMW 10477	E76, CBS 240.54	*Q. suber*	Italy	A Biraghi	AF 368328, AF 368347, AF 368347	**AY 194102**
	CMW 10484	E83, CBS 112918	*Q. suber*	Italy	A Biraghi	AF 368327, AF 368349, AF 368349	—
Cryphonectria parasitica	CMW 7048	E9, ATCC 48198	*Q. virginiana*	USA	RJ Stipes	AF 292043, AF 273076, AF 273470	**AY 194100**
	CMW 13749	MAFF 410158, TFM:FPH Ep1	*Castanea mollisima*	Japan	Unknown	AY 697927, AY 697943, AY 697944	—
Cryphonectria nitschkei	CMW 10786		*Quercus* sp.	Japan	M Milgroom, S Kaneko	AF 140247, AF 140251, AF 140259	**AY 194099**
	CMW 13742	MAFF 410570, TFM:FPH E19	*Quercus grosseserrata*	Japan	T Kobayashi	AY 697936, AY 697961, AY 697962	—
	CMW 10527	AR 3433, CBS 109776	*Quercus mongolica*	Russia	L Vasilyeva	**DQ120761, DQ120767, DQ120768**	AF 408341
Cryphonectria macrospora[c]	CMW 10528	AR 3444, CBS 109764	*Q. mongolica*	Russia	L Vasilyeva	**DQ120760, DQ120765, DQ120766**	AF 408340
Cryphonectria macrospora	CMW 10463	E54, CBS 112920	*C. cuspidata*	Japan	T Kobayashi	AF 368331, AF 368351, AF 368350	—
	CMW 10914	TFM: FPH E55	*C. cuspidata*	Japan	T Kobayashi	AY 697942, AY 697973, AY 697974	—
Cryptodiaporthe corni	CMW 10526	AR 2814, CBS 245.90	*Cornus alternifolia*	Maine, USA	S Redlin	**DQ120762, DQ120769, DQ120770**	AF 408343
Endothia gyrosa	CMW 2091	E13, CBS 112915	*Quercus palustris*	USA	RJ Stipes	AF 046905, AF 368337, AF 368336	**AY 194114**
	CMW 10442	E27	*Q. palustris*	USA	RJ Stipes	AF 368326, AF 368339, AF 368338	**AY 194115**

TABLE. I Continued

Original label name of taxon	Isolate numbers[a]	Additional numbers[a]	Host	Origin	Collector	GenBank Accession numbers[b]	
						ITS, β-tubulin1 and 2	LSU
Amphilogia gyrosa	CMW 10469	E67, CBS 112922	*Elaeocarpus dentatus*	New Zealand	GJ Samuels	AF 452111, AF 525707, AF 525714	**AY 194107**
	CMW 10470	E68, CBS 112923	*E. dentatus*	New Zealand	GJ Samuels	AF 452112, AF 525708, AF 525715	**AY 194108**
Diaporthe ambigua	CMW 5288	CBS 112900	*Malus domestica*	South Africa	WA Smit	AF 543817, AF 543819, AF 543821	—
	CMW 5587	CBS 112901	*Malus domestica*	South Africa	WA Smit	AF 543818, AF 543820, AF 543822	—

[a] CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; E, from the culture collection of Prof. R.J. Stipes (Department of Plant Pathology, Virginia Polytechnic Institute & State University, Blacksburg, Virginia, USA) now housed in the culture collection (CMW) of FABI; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MAFF, Microorganisms Section, MAFF GENEBANK, National Institute of Agrobiological Sciences (NIAS), MAFF Gene Bank, Ibaraki, Japan; TFM: FPH, Forestry and Forest Products Research Institute, Danchi-Nai, Ibaraki, Japan, while E or Ep refers to an isolate; AR, collection of Dr A. Rossman, U. S. National Fungus Collections, Systematic Botany and Mycology, Beltsville, Maryland.

[b] Sequences in bold were derived from cultures in this study. Other sequences were acquired from these previous studies: Zhang ans Blackwell 2001; Castlebury et al 2002; Myburg et al 2002; Venter et al 2002; Myburg et al 2003; Gryzenhout et al 2004; Myburg et al 2004a, b; Gryzenhout et al 2005a.

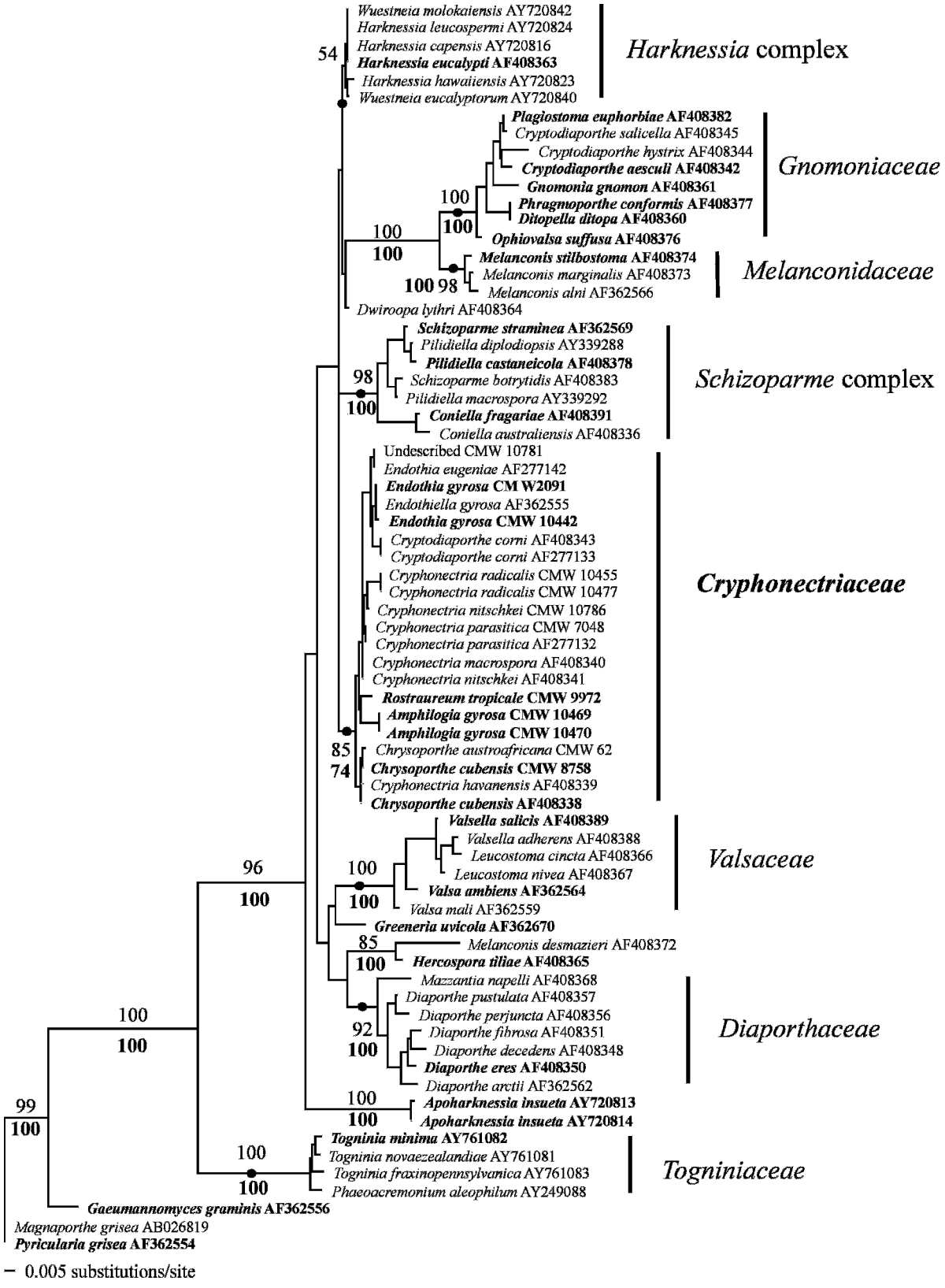
[c] The *C. havanensis* isolate represents *Chrysoporthe cubensis*, and the *C. macrospora* isolate represents *C. nitschkei*.

parameters and methodology as those in the LSU analysis, with the exception that the distance settings were those determined for this particular dataset by Modeltest and the long run had 5 000 000 generations. GenBank accession numbers of sequences generated in this study as well as those from previous phylogenetic studies are listed (TABLE I). The resulting dataset and trees have been deposited in TreeBase as S1487, M2671.

RESULTS

Analyses of LSU rDNA sequences.—The LSU sequence dataset included 71 taxa, of which *Magnaporthe grisea* (T.T. Herbert) Yaegashi & Udugawa (AB 026819), *Pyricularia grisea* (Cooke) Sacc. (AF 362554), *Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver (AF 362556) and *Gaeumannomyces graminis* (AF 362557) were defined as outgroup taxa. These species do not reside in the Diaporthales. The LSU sequence dataset consisted of a total of 655 bases of which 468 were constant, 24 were parsimony uninformative and 163 were parsimony informative (g1 = −1.1078 after exclusion of uninformative characters). The heuristic search for the MP analyses resulted in 74 trees (tree length = 222.64762 steps, CI = 0.629, RI (retention index) = 0.886), which did not differ markedly in the grouping of the major lineages but differed in branch lengths and the topology within clades. The phylogram obtained with distance analyses (FIG. 2) showed the same lineages, although relationships between the lineages differed. Reasonably high bootstrap values (85%) were obtained for the *Cryphonectria-Endothia* complex in the distance analyses, although bootstrap values were below 50% for parsimony analyses. Bayesian analyses showed the same groupings and topology than those obtained in the distance and parsimony analyses with high posterior probability values for the different families (FIG. 2). This included the clade representing the *Cryphonectria-Endothia* complex (posterior probability 74%).

The LSU phylogenetic tree based on our analyses (FIG. 1) was similar to the trees presented by Castlebury et al (2002), although the present study included a substantially greater number of taxa representing the *Cryphonectria-Endothia* complex. These included at least six genera. Inclusion of these additional taxa did not affect the structure of the *Cryphonectria-Endothia* group, which remained a distinct lineage. Other lineages in the phylogram represent the families Gnomoniaceae, Melanconidaceae, Valsaceae, Diaporthaceae, Togniniaceae and the *Schizoparme* complexes as previously defined (Zhang and


Wuestneia molokaiensis AY720842
Harknessia leucospermi AY720824
Harknessia capensis AY720816
Harknessia eucalypti AF408363
Harknessia hawaiiensis AY720823
Wuestneia eucalyptorum AY720840
Harknessia complex
Plagiostoma euphorbiae AF408382
Cryptodiaporthe salicella AF408345
Cryptodiaporthe hystrix AF408344
Cryptodiaporthe aesculi AF408342
Gnomonia gnomon AF408361
Phragmoporthe conformis AF408377
Ditopella ditopa AF408360
Ophiovalsa suffusa AF408376
Gnomoniaceae
Melanconis stilbostoma AF408374
Melanconis marginalis AF408373
Melanconis alni AF362566
Melanconidaceae
Dwiroopa lythri AF408364
Schizoparme straminea AF362569
Pilidiella diplodiopsis AY339288
Pilidiella castaneicola AF408378
Schizoparme botrytidis AF408383
Pilidiella macrospora AY339292
Coniella fragariae AF408391
Coniella australiensis AF408336
Schizoparme complex
Undescribed CMW 10781
Endothia eugeniae AF277142
Endothia gyrosa CMW 2091
Endothiella gyrosa AF362555
Endothia gyrosa CMW 10442
Cryptodiaporthe corni AF408343
Cryptodiaporthe corni AF277133
Cryphonectria radicalis CMW 10455
Cryphonectria radicalis CMW 10477
Cryphonectria nitschkei CMW 10786
Cryphonectria parasitica CMW 7048
Cryphonectria parasitica AF277132
Cryphonectria macrospora AF408340
Cryphonectria nitschkei AF408341
Rostraureum tropicale CMW 9972
Amphilogia gyrosa CMW 10469
Amphilogia gyrosa CMW 10470
Chrysoporthe austroafricana CMW 62
Chrysoporthe cubensis CMW 8758
Cryphonectria havanensis AF408339
Chrysoporthe cubensis AF408338
Cryphonectriaceae
Valsella salicis AF408389
Valsella adherens AF408388
Leucostoma cincta AF408366
Leucostoma nivea AF408367
Valsa ambiens AF362564
Valsa mali AF362559
Valsaceae
Greeneria uvicola AF362670
Melanconis desmazieri AF408372
Hercospora tiliae AF408365
Mazzantia napelli AF408368
Diaporthe pustulata AF408357
Diaporthe perjuncta AF408356
Diaporthe fibrosa AF408351
Diaporthe decedens AF408348
Diaporthe eres AF408350
Diaporthe arctii AF362562
Diaporthaceae
Apoharknessia insueta AY720813
Apoharknessia insueta AY720814
Togninia minima AY761082
Togninia novaezealandiae AY761081
Togninia fraxinopennsylvanica AY761083
Phaeoacremonium aleophilum AY249088
Togniniaceae
Gaeumannomyces graminis AF362556
Magnaporthe grisea AB026819
Pyricularia grisea AF362554
0.005 substitutions/site

Blackwell 2001, Castlebury et al 2002, Réblová et al 2004).

In this study a core group of *Harknessia* and *Wuestneia* species formed a discrete clade (bootstrap 54%). Species of the closely related genera *Dwiroopa* and *Apoharknessia* however did not group in this clade. Some other species such as *G. uvicola*, *Melanconis desmazieri* Petr. and *Hercospora tiliae* (Pers.) Tul. & C. Tul. retained their groupings separate from the major lineages (Castlebury et al 2002).

Analyses of ITS rDNA and β-tubulin sequences.—The dataset consisted of 32 taxa of which the two *D. ambigua* isolates were defined as outgroup. Results generated with the PHT analyses ($P = 0.306$) indicated that the rDNA and β-tubulin sequence datasets were significantly congruent and that they could be combined; this is in accordance with the trees of similar topology and having strong support generated from the separate datasets. The aligned ribosomal DNA sequence dataset (566 characters) consisted of 315 constant, 12 parsimony uninformative and 239 parsimony informative characters (g1 = −0.7185 after exclusion of uninformative characters), and the β-tubulin alignment (955 characters) consisted of 538 constant, 21 parsimony uninformative and 396 parsimony informative characters (g1 = −0.535 after exclusion of uninformative characters). The combined dataset consisted of 1521 characters. The heuristic search resulted in a single most parsimonious tree (tree length = 1223.91668, CI = 0.741, RI = 0.905). Both the distance and Bayesian analyses showed the same grouping of isolates. Exclusion of ambiguously aligned sequences representing the introns in the β-tubulin alignment and the ITS1 regions also resulted in similar trees. The tree obtained with distance analyses was chosen for presentation (FIG. 2).

Phylogenetic analyses based on the ITS region and β-tubulin sequences, showed the same clades as those observed in previous studies (Myburg et al 2003, 2004a, b; Gryzenhout et al 2005a). *Endothia*, *Cryphonectria*, *Chrysoporthe*, *Amphilogia* and *Rostraureum* formed distinct and well supported clades, while the isolates representing an apparently undescribed genus from clove in Indonesia also formed a discrete group (FIG. 2). The isolate of *E. eugeniae* (CBS 534.82) included in the study of Zhang and Blackwell (2001), grouped in the clade representing this undescribed genus (bootstrap 100%). The isolates of *C. nitschkei* and *C. macrospora* from Russia included in the study of Castlebury et al (2002), grouped with Japanese isolates of *C. nitschkei* in the *Cryphonectria* clade (bootstrap 100%, posterior probability 100%). The isolate of *Crypto. corni* did not reside in any of the clades resulting from the phylogenetic analyses but grouped closely to them.

TAXONOMY

Addition of a more representative taxon set to that analyzed by Castlebury et al (2002) showed that *Cryphonectria*, *Endothia* and closely related genera represent a distinct monophyletic lineage in the Diaporthales. This has also been shown based on analyses of a larger LSU sequence data as the one used in this study (Castlebury et al 2002) and it is supported by easily defined morphological characteristics. These findings provide strong justification for the establishment of a new family in the Diaporthales.

Genera in this complex have distinct orange stromatic tissue in the teleomorph state and usually in the anamorph state, which is different from any other species in the Diaporthales. Members of this group also can be distinguished from other taxa in the Diaporthales by the purple discoloration of the stromatic tissue in 3% KOH and a yellow reaction in lactic acid (Castlebury et al 2002). This discoloration is due to pigments in the stromatic tissue, often responsible for the orange colour, and that is also produced in culture (Roane 1986, Castlebury et al 2002).

Endothia and *Cryphonectria* represent the oldest and best known names in the group representing these and related fungi. They thus would represent an ideal foundation for a new family name. In the case of *Endothia*, *E. gyrosa*, the type species of the genus, is well characterized morphologically and based on DNA sequences (Shear et al 1917, Roane 1986, Micales and Stipes 1987, Venter et al 2002, Myburg et al 2004a). However the type specimen of this species is old and only anamorph structures are

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FIG. 1. LSU phylogram based on neighbor joining analysis of the Diaporthales. Taxa in bold represent the type species of the genus. Branches representing families are indicated with dots. Bootstrap values (50%) of only these branches are shown above the branch, with the posterior probabilities given as a percentage in bold typeface. GenBank accession numbers (AB, AF, AY or U) of isolates not sequenced in this study are indicated next to each taxon. The LSU sequence data for *Magnaporthe grisea*, *Pyricularia grisea* and *Gaeumannomyces graminis* generated in the study of Castlebury et al (2002) were used as outgroup taxa to root the LSU phylogenetic tree.

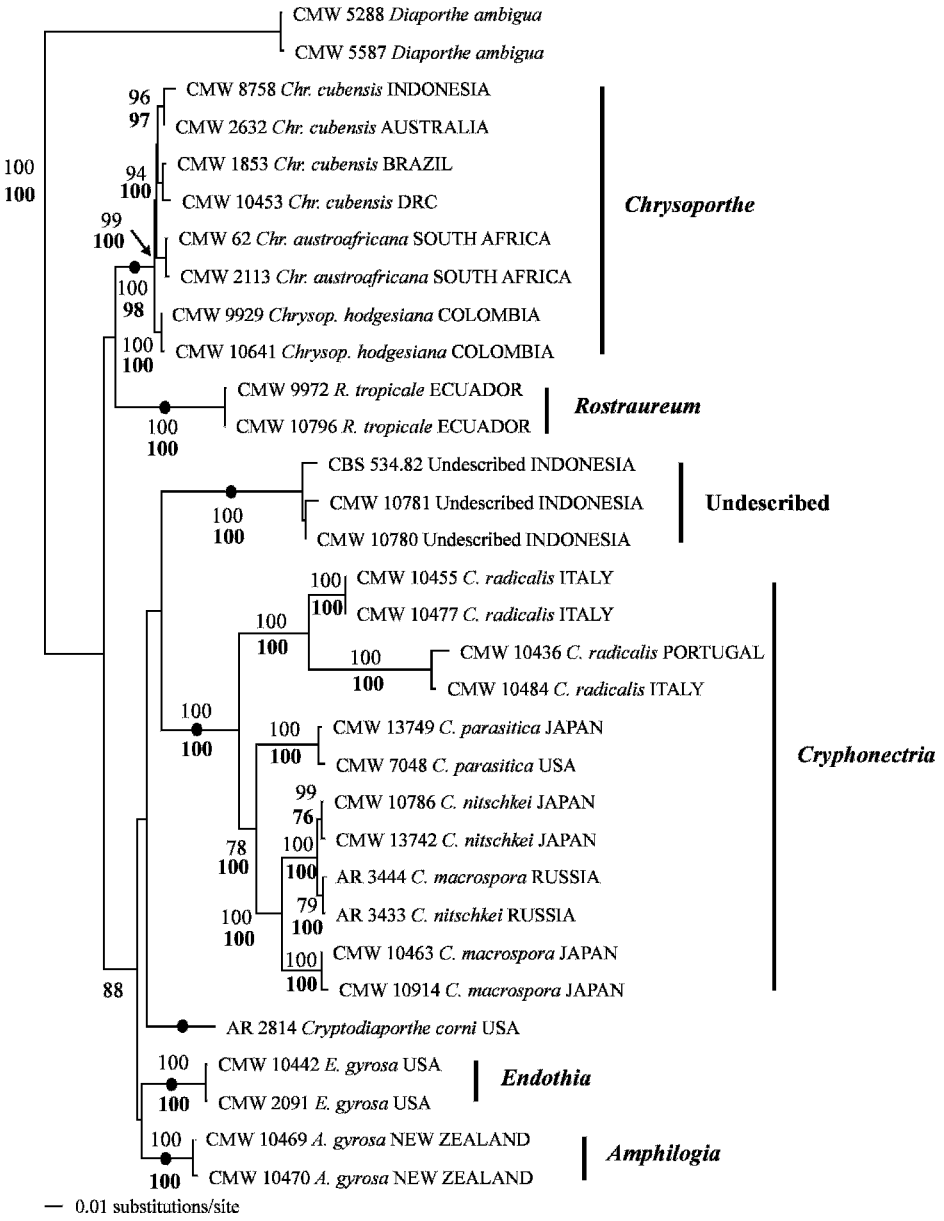

FIG. 2. ITS/β-tubulin phylogram based on neighbor joining analysis of members of the Cryphonectriaceae. Confidence levels of the tree branch nodes are indicated and were determined by a 70% bootstrap analysis (1000 replicates). Posterior probabilities are given as a percentage in boldface type. Species names in capital letters represent host species. Branches representing genera are accentuated with dots. *Diaporthe ambigua* isolates were used as outgroup.

present on it (Shear et al 1917). An epitype for this species is thus needed (Myburg et al 2004a) and it is not presently available.

The typification of *Cryphonectria* recently has been revised. This was necessary because of nomenclatural problems with *Cryphonectria gyrosa* as type (Myburg et al 2004a, Gryzenhout et al 2005c) and the fact that this fungus belongs in *Amphilogia* (Myburg et al 2004a; Gryzenhout et al 2005b, c). Hence *Cryphonectria* has been conserved with a new type, *C. parasitica* (Gryzenhout et al 2005c). Due to the importance and notoriety of this fungus, *Cryphonectria* represents an appropriate choice as type for a new family. This description thus is provided:

Cryphonectriaceae Gryzenh. & M. J. Wingf., fam. nov.

Ascostromata subimmersa vel superficialia, textura stromatica aurantiaca, collis peritheciorum cum textura stromatica aurantiaca vel fusconigra tectis. Asci fusoidei. Ascosporae ellipsoideae, fusoideae vel cylindricae, non septatae vel usque ad multiseptatae, hyalinae. Conidiomata eustromatica, subimmersa vel

superficialia, aurantiaca vel fusconigra. Cellulae conidiogenae phialidicae. Conidia perparvula, ovoidea vel cylindrica, non septata, hyalina. Textura stromatica in 3% KOH purpurascit, in acido lactico flavescit.

Ascostromata small to large, erumpent, semi-immersed to superficial, generally with orange stromatic tissue. Perithecia fuscous black to umber, occurring underneath bark surface or superficially in stroma, perithecial necks slender, covered with orange to fuscous-black stromatic tissue. Asci fusoid, aparaphysate, free floating. Ascospores generally ellipsoid to fusoid to cylindrical, aseptate to multiseptate, hyaline. Conidiomata eustromatic, semi-immersed to superficial, pyriform to pulvinate, orange to fuscous black, occasionally occurring in same stroma than perithecia. Conidiogenous cells phialidic, simple or branched. Conidia minute, generally ovoid to cylindrical, aseptate, hyaline. Stromatic tissue turns purple in 3% KOH and yellow in lactic acid.

Typus genus: *Cryphonectria* (Sacc.) Sacc., Syll. Fung. 17:783. 1905.

DISCUSSION

Results of this study have provided additional evidence to support the establishment of a new family in the Diaporthales accommodating species that previously have been treated in the *Cryphonectria-Endothia* complex. Early evidence for the existence of this distinct group was provided in a fundamental study by Castlebury et al (2002), which treated a large number of genera in the Diaporthales to delimit family relationships within the order. The aim of the present study was to focus specifically on genera in the *Cryphonectria-Endothia* complex and to include additional isolates, particularly new genera that recently have been assigned to this group. In this way we were able to further test the unique nature of the group and to show that it represents a distinct phylogenetic lineage, for which we have now provided family status.

Genera residing in the newly defined Cryphonectriaceae clearly can be set aside from other families in the Diaporthales based on DNA sequence data, particularly for the LSU region. Their unique nature also can be recognized based on a number of morphological features such as the formation of orange stromata and pigments in the stromatic tissue or in culture (Roane 1986) that can be tested with unique color reactions in KOH and lactic acid (Castlebury et al 2002). This is similar to the classifications of the Nectriaceae within the Hypocreales, where one of the distinguishing characteristics of taxa in this family is similar color reactions in KOH and lactic acid (Rossman et al 1999).

In this study problems were experienced with low bootstrap support for the Cryphonectriaceae in parsimony analyses. Similar low bootstrap support also was shown in parsimony analyses presented by Castlebury et al (2002). These authors however showed conclusively that the branch separating the *Cryphonectria-Endothia* complex from the other lineages was well supported based on additional distance and Bayesian analyses and a longer DNA sequence dataset. We have confirmed this in our study, where support for the clade representing the Cryphonectriaceae was adequately high based on distance and Bayesian analyses.

Analyses of the variable ITS region and β-tubulin genes in the present study have shown that isolates of *Crypto. corni* (AR 2814), *C. macrospora* (AR 3444) and *E. eugeniae* (CBS 534.82) used in the studies of Zhang and Blackwell (2001) and Castlebury et al (2002) represent taxa other than those assigned to them. Thus the *E. eugeniae* isolate was shown to group together with isolates representing an undescribed genus from clove in Indonesia (Myburg et al 2003). This isolate does not represent *Chr. cubensis*, of which *E. eugeniae* is a synonym to (Hodges et al 1986, Myburg et al 2003). The isolate of *C. macrospora* from Russia represents *C. nitschkei* (Myburg et al 2004b), confirming observations of Vasilyeva (1998) that *C. nitschkei* occurs in Russia, not only in China and Japan (Myburg et al 2004b).

In the present study the isolate of *Crypto. corni* treated by Castlebury et al (2002) did not group with any of the isolates of *Cryphonectria*, *Endothia*, *Chrysoporthe*, *Amphilogia* or *Rostraureum* or with the isolates from Indonesia that represent an undescribed genus. The fungus however does have a position in the Cryphonectriaceae based on the LSU sequence data and its orange/yellow stromatic tissue that turns purple in KOH and yellow in lactic acid (Redlin and Rossman 1991, Castlebury et al 2002). This fungus appears to represent an undescribed genus in the Cryphonectriaceae because its morphology does not correspond with any of the genera currently known for this family (Myburg et al 2004a; Gryzenhout et al 2005a, b). For instance conidiomata of the anamorph *Myxosporium nitidum* Berk. & Curtis are fully immersed in the bark and emerge through lenticels as orange, subspherical pycnidia (Redlin & Rossman 1991). More detailed studies with additional isolates and specimens of this fungus would be required before a name can be provided for it.

The new family Cryphonectriaceae defined in this study includes some of the most serious tree pathogens in the world. Notable examples are the causal agent of chestnut blight *C. parasitica* (Anagnostakis 1987) and *Chr. cubensis*, which is one of the most

serious pathogens of plantation-grown *Eucalyptus* spp. (Wingfield 2003). Many other members of the family are also pathogens. For example *R. tropicale* causes cankers on *Terminalia* spp. (Gryzenhout et al 2005a), although it does not appear to have a large ecological impact. It is likely that additional genera will be discovered that reside in the Cryphonectriaceae, as illustrated by the characterization of the *Crypto. corni* isolate in this study. The description of a new family encompassing *Chrysoporthe*, *Cryphonectria*, *Endothia* and allied genera should aid identification and taxonomic studies on these fungi.

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