CHAPTER 4

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

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***Cryphonectriaceae* (*Diaporthales*), a new family including *Cryphonectria, Endothia, Chrysoporthe* 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 have also 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 color reaction in KOH and a yellow reaction in lactic acid associated with pigments in the stromatic tissue or in culture.

Taxonomic novelty: *Cryphonectriaceae* Gryzenh. & M. J. Wingf. fam. nov. nom. prov.

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Key words: *Amphilogia*, *Chrysoporthe*, *Cryphonectria*, *Cryphonectriaceae*, *Cryptodiaporthe corni*, *Endothia*, *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 is *Cryphonectria parasitica* (Murrill) M. E. Barr, which has devastated American chestnut (*Castanea dentata*) populations in North America (Anagnostakis 1987, Heiniger & Rigling 1994), and *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., the causal agent of stem canker of soybeans (Kulik 1984).

Members of the *Diaporthales* are morphologically united by a *Diaporthe*-type centrum (Alexopoulos & 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 & 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 are currently recognised 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

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emerged from recent DNA sequence comparisons of Castlebury *et al.* (2002), who compared genera representing the families previously recognised 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 yet been confirmed using DNA sequence data (Castlebury *et al.* 2002).

In addition to the described families, groups not recognised previously were also 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. and 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 (Castlebury *et al.* 2002, Lee *et al.* 2004). This group could not be described as a family since the status of *Wuestneia* Auersw. ex Fuckel as the teleomorph of this coelomycete genus must still be confirmed (Lee *et al.* 2004). Besides 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/Chapter 1 in this thesis, Myburg *et al.* 2004a,

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Gryzenhout *et al.* 2005a/Chapter 7 in this thesis, 2005b/Chapter 6 in this thesis). 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 was subsequently 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* (clove) in Indonesia, was recognised 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 data set for the *Cryphonectria-Endothia* complex defined by Castlebury *et al.* (2002). The expanded LSU sequence data set was ultimately used to characterise and describe a family for species and genera in the *Cryphonectria-Endothia* complex of genera. The LSU sequences were also

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supplemented with more variable sequences of the ribosomal ITS region and β-tubulin genes, to show infra-familial 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, 2004b, Gryzenhout *et al.* 2005a), were included in sequence data analyses (Table 1). Isolates (CMW 10779–10781) that represented the undescribed genus from *S. aromaticum* in Indonesia (Myburg *et al.* 2003), were also included (Table 1).

Isolates of some of the species studied by Castlebury *et al.* (2002) and Zhang & 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, USA). *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 & Blackwell (2001) and used by Castlebury *et al.* (2002), was acquired from the Centraalbureau voor

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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 previously been shown (as E40 or CMW 10453) to represent *Chr. cubensis* (Hodges *et al.* 1986, Micales *et al.* 1987, Myburg *et al.* 2004a).

Isolates used in this study (Table 1) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A representative sub-set of these isolates not in other internationally recognised culture collections, is also stored in the culture collection of the Centraalbureau voor Schimmelcutures, Utrecht, Netherlands (Table 1). Background information pertaining to other isolates included in the phylogenetic analyses can be found in the studies of Zhang & 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 characterise 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 using previously described methods (Myburg *et al.* 1999, Myburg *et al.* 2002).

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DNA from isolates representing key species of *Cryphonectria*, *Endothia*, *Chrysoporthe*, *Rostraureum*, *Amphilogia* and the undescribed fungi from Indonesia (Table 1), was used to amplify a region of the LSU rDNA gene. Primers pairs ITS3 (White *et al.* 1990) and LR3 (Vilgalys & 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 ºC for 3 min (denature), 30 cycles of 95 ºC for 30 s (denature), 56 ºC for 45 s (anneal), 72 ºC for 1 min (elongation) and a final elongation step of 72 ºC for 4 min. Amplification products were purified using 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, United Kingdom). Nucleotide sequence data were generated using an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, United Kingdom). The primer pairs used in the respective sequencing reactions were as follows: 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).

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

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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 1). These sequences were aligned with the 650 bp data set 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 were also 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 & Blackwell (2001).

Phylogenetic trees were generated by parsimony and distance analyses. LSU sequence data generated in this study were deposited in GenBank (Table 1) and the datamatrix in TreeBase (SN 2390). 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) index 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 since bootstrap analysis was inordinately extensive using the parameters defined to generate the trees and could not run to completion (Castlebury

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et al. 2002). For the distance analyses, a neighbour-joining tree was generated with the Tamura-Nei (TrNef+I+G) model (Tamura & 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 the program Modeltest version 3.5 (Posada & Crandall 1998).

The probabilities of branches occurring were also tested using Bayesian inference employing the Markov chain Monte Carlo (MCMC) algorithm (Larget & Simon 1999). The program Mr. Bayes vers. 3.1.1 (Huelsenbeck & Ronquist 2001) was used with the following parameters: number of generations = 1000000, sample frequency = 100, number of chains = 4 (1 cold, 3 hot) and a burnin of 1000. Four independent analyses were run, with one of these having 3000000 generations. The likelihood model and settings used 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 1) 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, since 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 using both parsimony and distance methods. All the sequence characters were unordered. Gaps were treated as characters with the

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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 re-weighted according to their individual CI to reduce the number of trees.

The distance analysis was done using 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 & 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 using the same 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 5000000 generations. GenBank accession numbers of sequences generated in this study as well as those from previous phylogenetic studies are listed in Table 1. The resulting dataset and trees have been deposited in TreeBase as SN 2390.

RESULTS

Analyses of LSU rDNA sequences

The LSU sequence data set included 71 taxa, of which *Magnaporthe grisea* (T.T. Herbert) Yaegashi & Udugawa (AB 026819), *Pyricularia grisea* (Cooke) Sacc. (AF

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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 data set 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 the 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 & Blackwell 2001, Castlebury *et al.* 2002, Réblová *et al.* 2004).

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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 the outgroup. Results generated with the PHT analyses (P = 0.306) indicated that the rDNA and β-tubulin sequence data sets 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 data sets. The aligned ribosomal DNA sequence dataset (566 characters) consisted of 315 constant, 12 parsimony-uninformative and 239 parsimony-informative chararacters (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 chararacters (g1 = –0.535 after exclusion of uninformative characters). The combined data set 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 using 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,

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2004a, 2004b, 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 & 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 also 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 can also be distinguished from other taxa in the *Diaporthales* by the purple discolouration of the stromatic tissue in 3 % KOH and a yellow colour reaction in lactic acid (Castlebury *et al.* 2002). This discolouration is

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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 would thus represent an ideal foundation for a new family name. In the case of *Endothia*, *E. gyrosa*, the type species of the genus, is well characterised morphologically and based on DNA sequences (Shear *et al.* 1917, Roane 1986, Micales & Stipes 1987, Venter *et al.* 2002, Myburg *et al.* 2004a). However, the type specimen of this species is old and only anamorph structures are 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* has recently been revised. This was necessary because of nomenclatural problems with *Cryphonectria gyrosa* as type (Myburg *et al.* 2004a, Gryzenhout *et al.* 2005c/Chapter 5 in this thesis), and the fact that this fungus belongs in *Amphilogia* (Myburg *et al.* 2004a, Gryzenhout *et al.* 2005b, 2005c). 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. The following description is thus provided:

Cryphonectriaceae Gryzenh. & M. J. Wingf., **fam. nov., nom. prov.**

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

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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 colours 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 have previously 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 have recently been assigned to this group. In this way,

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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* can clearly be set aside from other families in the *Diaporthales* based on DNA sequence data, particularly for the LSU region. Their unique nature can also be recognised 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 colour 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 colour 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 was also 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 & 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

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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, and 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*, nor with the isolates from Indonesia that represent an undescribed genus. The fungus does, however, 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 & Rossman 1991, Castlebury *et al.* 2002). This fungus appears to represent an undescribed genus in the *Cryphonectriaceae*, since its morphology does not correspond with any of the genera currently known for this family (Myburg *et al.* 2004a, Gryzenhout *et al.* 2005a, 2005b). For instance, conidiomata of the anamorph, *Myxosporium nitidum* Berk. & Curtis, are fully immersed in the bark and emerge through lenticles as orange, sub-spherical 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

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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 characterisation of the *Crypto. corni* isolate in this study. The description of a new family encompassing *Chrysoporthe*, *Cryphonectria*, *Endothia* and allied genera should facilitate identification and taxonomic studies on these fungi.

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Table 1. 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	M.J. 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	M.J. Wingfield	AF 292041, AF 273063, AF 273458	**AY 194097**
	CMW 2113	CBS 112916	*E. grandis*	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462	—

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Chrysoporthella hodgesiana	CMW 9929	—	*Tibouchina urvilleana*	Colombia	C. Rodas, M.J. 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	M.J. Wingfield	AY 167426, AY 167431, AY 167436	**AY 194092**
	CMW 10796	CBS 115757	*T. ivorensis*	Ecuador	M.J. 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	M.J. Wingfield	AY 084008, AY 084020, AY 084032	—
	CMW 10781	CBS 115844	*E. aromatica*	Indonesia	M.J. 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	—

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	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	*Quercus virginiana*	USA	R.J. 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

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Cryphonectria macrospora [c]	CMW 10528	AR 3444, CBS 109764	*Q. mongolica*	Russia	L. Vasilyeva	**DQ120760, DQ120765, DQ120766**	AF 408340
C. macrospora	CMW 10463	E54, CBS 112920	*Castanopsis cuspidate*	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	R.J. Stipes	AF 046905, AF 368337, AF 368336	**AY 194114**
	CMW 10442	E27	*Q. palustris*	USA	R.J. Stipes	AF 368326, AF 368339, AF 368338	**AY 194115**
Amphilogia gyrosa	CMW 10469	E67, CBS 112922	*Elaeocarpus dentatus*	New Zealand	G.J. Samuels	AF 452111, AF 525707, AF 525714	**AY 194107**
	CMW 10470	E68, CBS 112923	*E. dentatus*	New Zealand	G.J. Samuels	AF 452112, AF 525708, AF 525715	**AY 194108**

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Diaporthe ambigua	CMW 5288	CBS 112900	*Malus domestica*	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821	—
	CMW 5587	CBS 112901	*M. domestica*	South Africa	W.A. 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, USA.

[b] Sequences in bold were derived from cultures in this study. Other sequences were acquired from previous studies as follows: Zhang & 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, 2004b, Gryzenhout *et al.* 2005a.

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

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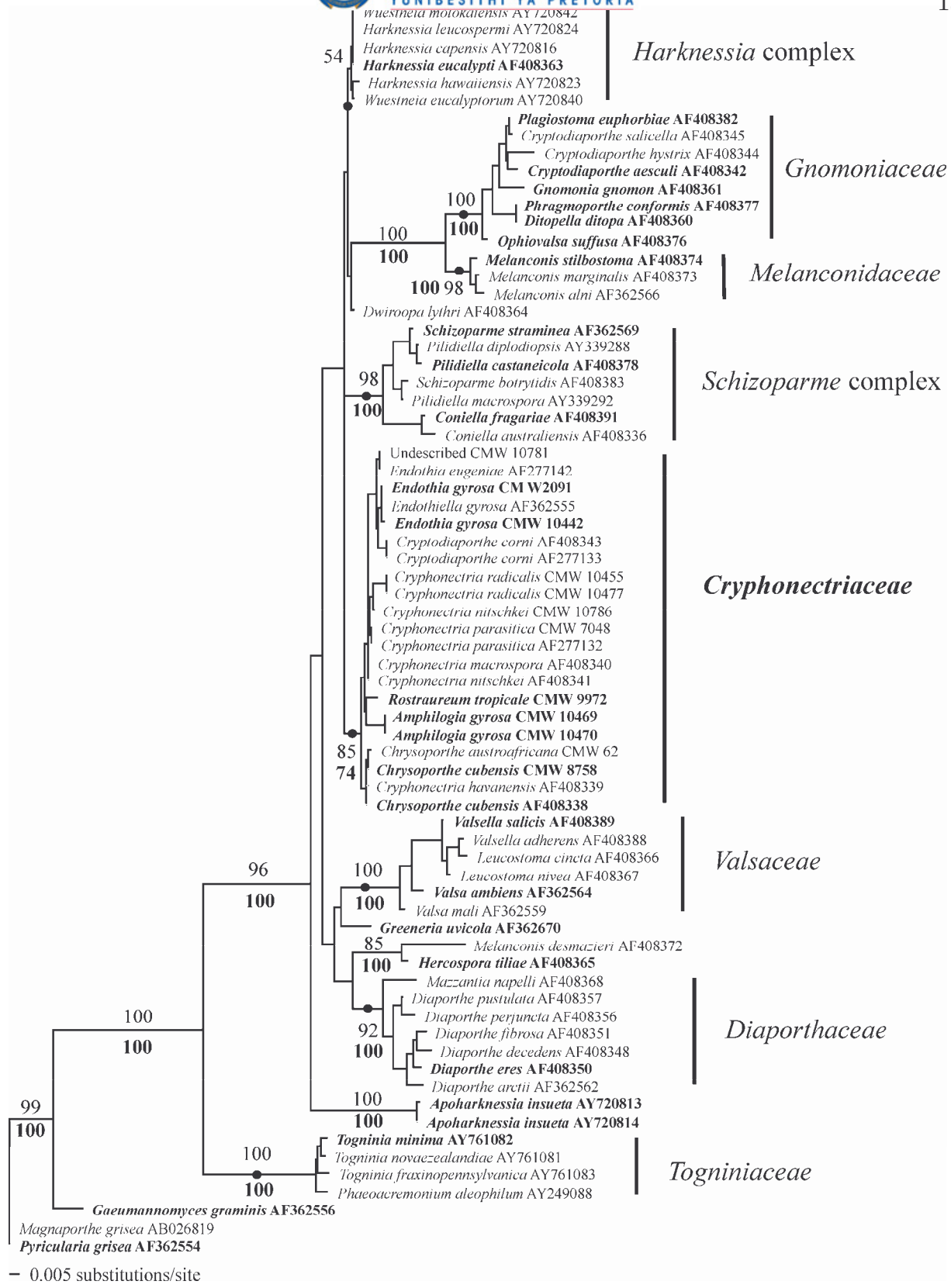

Fig. 1. LSU phylogram based on neighbor-joining analysis of the *Diaporthales*. Taxa in bold represent the type species of the genus. Branches representing families are indicated with dots. Bootstrap values (50%) of only these branches are shown above the branch, with the posterior probabilities given 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.

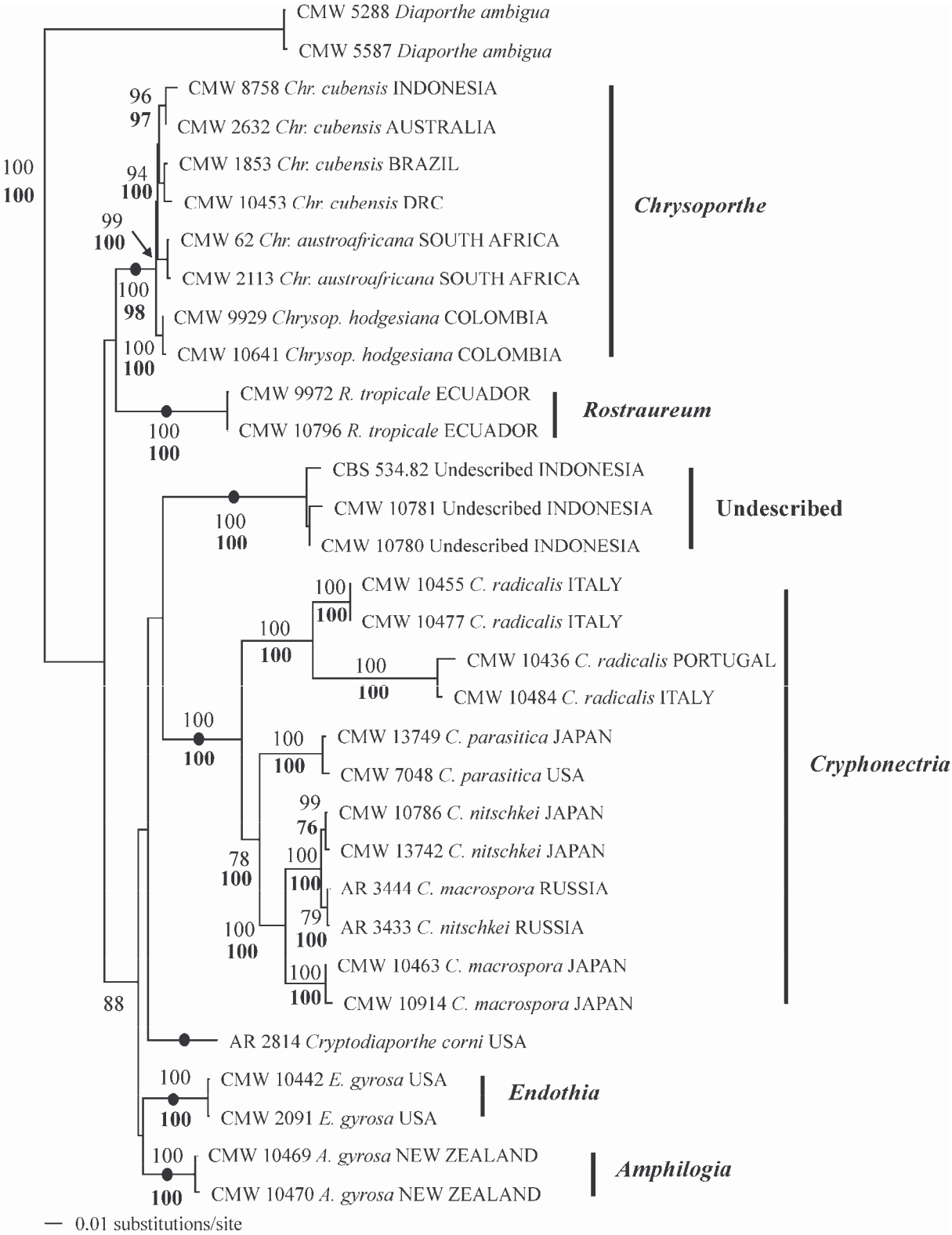

Fig. 2. ITS/β-tubulin phylogram based on neighbor-joining analysis of members of the *Cryphonectriaceae*. Confidence levels of the tree branch nodes are indicated and were determined by a 70% bootstrap analysis (1000 replicates). Posterior probabilities are given as a percentage in bold typeface. Species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

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