DNA sequence data and morphology define *Cryphonectria* species in Europe, China, and Japan

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Abstract: *Cryphonectria* includes important tree pathogens as well as species believed to be saprophytes. Recent phylogenetic studies have concentrated on North American and southern hemisphere *Cryphonectria* spp., but little is known about Asian and European taxa. In this study we identify and differentiate among the species occurring in Europe, China, and Japan using morphological and phylogenetic comparisons among the *Cryphonectria* species. Phylogenetic comparisons were based on sequence data from the ribosomal ITS operon and two regions in the β-tubulin gene. Unknown Japanese and Chinese isolates showing different cultural features than those of *Cryphonectria parasitica* (Murrill) M.E. Barr from Japan and the USA, grouped with isolates of *Cryphonectria nitschkei* (G.H. Otth) M.E. Barr from *Quercus* spp. and *Rhus javanica* L. Isolates of *Cryphonectria havanensis* (Bruner) M.E. Barr from *Quercus grosseserrata* Blume, *Castanopsis cuspidata* Schottky, *Pyrus sinensis* Lindl., and *Eucalyptus globulus* Labill. also grouped in this phylogenetic clade. We propose that *Cryphonectria nitschkei* and the fungus that has been referred to as *Cryphonectria havanensis* in Japan should be treated as a single taxon. Phylogenetic and morphological data also suggest that there are two species currently representing *Cryphonectria radicalis* (Schwein.: Fr.) M.E. Barr in Europe. One of these species is similar to the type specimen of *Cryphonectria radicalis*, while the other species probably is new.

Key words: Cryphonectria parasitica, Cryphonectria radicalis, Cryphonectria havanensis, Cryphonectria macrospora, Cryphonectria nitschkei, Diaporthales.

Résumé : Le genre *Cryphonectria* comporte d'importants pathogènes des arbres, ainsi que des espèces apparemment saprophytes. Les récentes études phylogénétiques ont porté surtout sur les espèces de *Cryptonectria* nord-américaines et de l'hémisphère sud, mais on connaît peu de choses sur les taxons asiatiques et européens. Dans cette étude, les auteurs identifient et différencient des espèces venant en Europe, en Chine et au Japon, en utilisant des comparaisons morphologiques et phylogénétiques entre les espèces de *Cryphonectria*. Les comparaisons phylogénétiques sont basées sur les données de séquençage de l'opéron de l'ITS ribosomal et de deux régions du gène de la β-tubuline. Des isolats inconnus du Japon et de la Chine, montrant des caractères culturaux différents de ceux du *Cryphonectria partasitica* (Murrill) M.E. Barr du Japon et des Etats-Unis, se regroupent avec des isolats du *Cryphonectria nitschkei* (G.H. Otth) M.E. Barr venant sur des *Quercus* spp. et sur le *Rhus javanica* L. Des isolats du *Cryphonectria havanensis* (Bruner) M.E. Barr provenant des *Quercus grosseserrata* Blume, *Castanopsis cuspidata* Schottky, *Pyrus sinensis* Lindl. et *Eucalyptus globulus* Labill. se retrouvent également dans ce clade phylogénétique. Les auteurs proposent que le *Cryphonectria nitschkei* et le champignon correspondant au *Cryphonectria havanensis* du Japon soient traités comme un même taxon. Les données morphologiques et phylogénétiques suggèrent également qu'il s'agît de deux espèces représentant couramment le *Cryphonectria radicalis* (Schwein.: Fr.) M.E. Barr en Europe. Une de ces espèces est semblable au spécimen type du *Cryphonectria radicalis,* alors que l'autre espèce est probablement nouvelle.

Mots clés : Cryphonectria parasitica, Cryphonectria radicalis, Cryphonectria havanensis, Cryphonectria macrospora, Cryphonectria nitshkei, Diaporthales.

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Introduction

Cryphonectria and *Endothia* include fungal species that are both tree pathogens and saprophytes. A number of challenges exist to distinguish among these genera and the species assigned to them. For example, the orange stromata of *Cryphonectria* and *Endothia* species are superficially similar. Additionally, differentiation of *Cryphonectria* and *Endothia* species is hindered by the fact that their ranges of spore size commonly overlap, and that ascospores for specimens are not always fully developed, resulting in variable measurements. Identification is further complicated when specimens or cultures do not produce spores. Taxonomic information is needed from both teleomorph and anamorph states and when only one morph is present, especially the anamorph, conclusive identification is difficult. Furthermore, fruiting structures, especially perithecia, are rarely produced in culture, and culture morphology is not sufficient to be used as the only means of identification.

Recent taxonomic studies, based on DNA sequence comparisons, have resolved a number of questions pertaining to the identification and differentiation of *Cryphonectria* and *Endothia* species (Myburg et al. 1999, 2004; Venter et al. 2002). A comprehensive phylogenetic study on representative species of *Cryphonectria* and *Endothia*, for which cultures were available, indicated that these genera should be considered as separate taxonomic entities, even though they are closely related (Myburg et al. 2004; Venter et al. 2002). However, studies such as those of Myburg et al. (1999), Myburg et al. (2004), and Venter et al. (2002) focussed primarily on species of *Cryphonectria* and *Endothia* that originated from the USA, Europe, and countries in the Southern Hemisphere. Therefore, a similar study that is directed towards the taxonomic and phylogenetic relationships of Asian *Cryphonectria* and *Endothia* species is required.

The best-known species in *Cryphonectria* is *Cryphonectria parasitica* (Murrill) M.E. Barr, the causal agent of chestnut blight. This disease practically eliminated native stands of American chestnut (*Castanea dentata* Borkh.) during the last century (Anagnostakis 1987; Griffin 1986) after being introduced from eastern Asia, where *Cryphonectria parasitica* is native (Anagnostakis 1992; Milgroom et al. 1996; Shear and Stevens 1913, 1916). *Cryphonectria parasitica* also occurs on European chestnuts (*Castanea sativa* Mill.), although the disease has not been as severe as it has been in North America (Bazzigher and Miller 1991; Bissegger and Heiniger 1991; Heiniger and Rigling 1994). This is attributed to greater resistance in European chestnuts (Clapper 1952; Heiniger and Rigling 1994; Metcalf 1908), differences in environmental conditions, and the presence of naturally occurring hypovirulent *Cryphonectria parasitica* strains in Europe (Grente 1965, 1975; Heiniger and Rigling 1994).

Cryphonectria radicalis (Schwein.: Fr.) M.E. Barr is a colonist of *Castanea* and *Quercus* species in the Northern Hemisphere (Anderson and Anderson 1912; Shear et al. 1917). The fungus was first described in 1814 from England (Sowerby 1814) and later from the USA (Fries 1828; Shear et al. 1917). It was reported from southern Europe in 1863 (Shear et al. 1917) and from Japan in 1914 (Shear et al. 1917; Kobayashi 1970). *Cryphonectria radicalis* was, therefore, known in North America and Europe before *Cryphonectria parasitica* was introduced. *Cryphonectria radicalis* has an association with *Cryphonectria parasitica* (Anagnostakis 1983, 1995; Hoegger et al. 2002) in that both species occur on the same hosts. Previous studies report species of *Castanea* and *Quercus* (Fagaceae) as the most important hosts of *Cryphonectria parasitica* and *Cryphonectria radicalis* in Europe, North America (Shear et al. 1917; Roane 1986*a*), and eastern Asia (Kobayashi and Itô 1956*a*; Kobayashi 1970). In recent studies (Hoegger et al. 2002; Sotirovski et al. 2004) aimed at isolating *Cryphonectria parasitica* from dead chestnut stems in Switzerland, Greece, and Macedonia, *Cryphonectria radicalis* isolates were unintentionally collected. Identification of these isolates as *Cryphonectria radicalis* was based on comparisons of morphology in culture (Hoegger et al. 2002; Sotirovski et al. 2004), ascospore dimensions, mating behaviour, pathogenicity to chestnut plants (Hoegger et al. 2002), and polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) banding patterns (Sotirovski et al. 2004).

In Japan, *Cryphonectria havanensis* (Bruner) M.E. Barr, *Cryphonectria nitschkei* (G.H. Otth) M.E. Barr, *Cryphonectria radicalis*, and *Cryphonectria parasitica* have been recorded on *Castanea* and *Quercus* spp. (Kobayashi and Itô 1956*a*, 1956*b*; Kobayashi 1970), making identification of the fungi on these hosts a challenge. Furthermore, *Endothia singularis* (Syd. & P. Syd.) Shear & N.E. Stevens, a fungus with orange stromata typical of *Cryphonectria* species, but with aseptate ascospores, also occurs on *Castanea* and *Quercus* spp. in Japan (Kobayashi and Itô 1956*a*; Kobayashi 1970). A fungus similar to *Endothia gyrosa* (Schwein.: Fr.) Fr. has also been reported on *Quercus* from China (Teng 1934). Minor host species for this group of fungi in eastern Asia include *Castanopsis cuspidata* Schottky, a reported host for *Cryphonectria macrospora* (Tak. Kobay. & Kaz. Itô) Barr and *Endothia singularis* (Kobayashi and Itô 1956*a*, 1956*b*; Kobayashi 1970). Of the above-mentioned species, only *Endothia gyrosa* is regarded as a pathogen, while *Cryphonectria havanensis*, *Cryphonectria nitschkei*, *Cryphonectria macrospora*, and *Endothia singularis* are considered saprophytes (Roane 1986*b*; Kobayashi 1970; Shear et al. 1917).

A collection of isolates and specimens identified as *Cryphonectria* spp. and originating from *Quercus* and *Castanea* spp. in Greece, Japan, and China form the basis of this study. A number of these isolates were used by Liu et al. (2003) in their recent study, but they have not been described taxonomically. The objectives of this study were, therefore, to identify the isolates in the collection from Greece, Japan, and China by comparing them to *Cryphonectria* spp. from various hosts. DNA sequence and morphological data are used to facilitate differentiation among the species occurring in Europe and Asia.

Materials and methods

Collection of isolates and specimens

This study includes 42 isolates (Table 1). Eight (CMW 10782 to CMW 10789) of these represented an unidentified fungus, sampled from *Castanea* and *Quercus* spp. from Japan, China, and Greece, that produces less orange pigmentation than is characteristic of *Cryphonectria parasitica*; three

Table 1. List of isolates included in this study.

Isolate number[a]	Alternative isolate numbers[b]	Species	Host	Origin	Collector	GenBank accession Nos.
CMW 10782	—	*Cryphonectria nitschkei*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140242, AF 140248, AF 140254
CMW 10783	—	*Cryphonectria nitschkei*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140244, AF 140250, AF 140256
CMW 10784	—	*Cryphonectria nitschkei*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140245, AF 140249, AF 140257
CMW 10785	09494	*Cryphonectria nitschkei*	*Quercus* sp.	China	M. Milgroom and K. Wang	AF 140246, AF 140252, AF 140258
CMW 10786	KB1	*Cryphonectria nitschkei*	*Castanea crenata*	Japan	M. Milgroom and S. Kaneko	AF 140247, AF 140251, AF 140259
CMW 10787	CD28	*Cryphonectria nitschkei*	*Castanea crenata*	Japan	M. Milgroom and S. Kaneko	AF 214212, AF 214214, AF 214216
CMW 5877	YM2	*Cryphonectria nitschkei*	*Castanea crenata*	Japan	M. Milgroom and S. Kaneko	AY 697933, AY 697955, AY 697956
CMW 11294	TFM:FPH E57	*Cryphonectria havanensis*	*Quercus grosseserrata*	Japan	T. Kobayashi	AY 214211, AY 214213, AY 214215
CMW 13744	MAFF 410568, TFM:FPH E23	*Cryphonectria nitschkei*	*Quercus grosseserrata*	Japan	T. Kobayashi	AY 697934, AY 697957, AY 697958
CMW 13741	MAFF 410156, TFM:FPH E18	*Cryphonectria nitschkei*	*Quercus grosseserrata*	Japan	T. Kobayashi	AY 697935, AY 697959, AY 697960
CMW 13747	MAFF 410569, TFM:FPH E25	*Cryphonectria nitschkei*	*Quercus serrata*	Japan	T. Kobayashi	AY 697937, AY 697963, AY 697964
CMW 13742	MAFF 410570, TFM:FPH E19	*Cryphonectria nitschkei*	*Quercus grosseserrata*	Japan	T. Kobayashi	AY 697936, AY 697961, AY 697962
CMW 10910	TFM:FPH E11	*Cryphonectria havanensis*	*Eucalyptus globulus*	Japan	T. Kobayashi	AY 697941, AY 697971, AY 697972
CMW 13736	MAFF 410154, TFM:FPH E30	*Cryphonectria havanensis*	*Pyrus serotina*	Japan	T. Kobayashi	AY 697938, AY 697965, AY 697966
CMW 13737	MAFF 410556, TFM:FPH E12	*Cryphonectria havanensis*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AY 697939, AY 697967, AY 697968
CMW 13745	MAFF 410572, TFM:FPH E38	*Cryphonectria nitschkei*	*Rhus javanica*	Japan	Y. Zinno	AY 697940, AY 697969, AY 697970
CMW 7047	ATCC 48197, E5	*Cryphonectria parasitica*	*Quercus virginiana*	USA	R.D. Wolfe	AF 368329, AF 273073, AF 273469
CMW 7048	ATCC 48198, E9	*Cryphonectria parasitica*	*Quercus virginiana*	USA	F.F. Lombard	AF 368330, AF 273076, AF 273470
CMW 8436	DY23	*Cryphonectria parasitica*	*Castanea dentata*	USA	M. Milgroom	AY 697931, AY 697951, AY 697952
CMW 13749	MAFF 410158, TFM:FPH Ep1	*Cryphonectria parasitica*	*Castanea mollisima*	Japan	—	AY 697927, AY 697943, AY 697944
CMW 13750	MAFF 410159, TFM:FPH Ep2	*Cryphonectria parasitica*	*Castanea crenata*	Japan	—	AY 697928, AY 697945, AY 697946
CMW 13751	MAFF 410160, TFM:FPH Ep3	*Cryphonectria parasitica*	*Castanea crenata*	Japan	T. Kobayashi	AY 697929, AY 697947, AY 697948
CMW 10916	TFM:FPH Ep4	*Cryphonectria parasitica*	*Castanea crenata*	Japan	T. Kobayashi	AY 697930, AY 697949, AY 697950
CMW 10790	—	*Cryphonectria parasitica*	*Quercus serrata*	Japan	M. Kusunoki	AF 140243, AF 140253, AF 140255
CMW 13754	MAFF 410152, TFM:FPH E8	*Cryphonectria radicalis*	*Fagus japonica*	Japan	T. Kobayashi	AY 697932, AY 697953, AY 697954
CMW 10788	D 15	*Cryphonectria radicalis*	*Quercus* sp.	Greece	P. Cortesi	AY 143075, AY 143077, AY 143079
CMW 10789	D 31	*Cryphonectria radicalis*	*Quercus* sp.	Greece	P. Cortesi	AY 143076, AY 143078, AY 143080
CMW 10791	M 285	*Cryphonectria radicalis*	*Quercus suber*	Italy	M. Orsenigo	AF 548742, AF 548746, AF 548750
CMW 10792	M 2268	*Cryphonectria radicalis*	*Castanea sativa*	Switzerland	P. Hoegger	AF 548743, AF 548747, AF 548751
CMW 10793	M 2269	*Cryphonectria radicalis*	*Castanea sativa*	Switzerland	P. Hoegger	AF 548744, AF 548748, AF 548752
CMW 10794	M 2270	*Cryphonectria radicalis*	*Castanea sativa*	Switzerland	D. Rigling	AF 548745, AF 548749, AF 5487503

Table 1 (*concluded*).

Isolate number[a]	Alternative isolate numbers[b]	Species	Host	Origin	Collector	GenBank accession Nos.
CMW 10436	CBS 165.30, E14	*Cryphonectria radicalis*	*Quercus suber*	Portugal	B. d'Oliviera	AF 452117, AF 525703, AF 525710
CMW 10455	CBS 238.54, E42	*Cryphonectria radicalis*	*Castanea dentata*	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	CBS 240.54, E76	*Cryphonectria radicalis*	*Quercus suber*	Italy	M. Orsenigo	AF 368328, AF 368347, AF 368346
CMW 10484	E83, CBS 112918	*Cryphonectria radicalis*	*Castanea sativa*	Italy	A. Biraghi	AF 368327, AF 368349, AF 368349
CMW 10463	E54, CBS 112920	*Cryphonectria macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350
CMW 10914	TFM: FPH E55	*Cryphonectria macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AY 697942, AY 697973, AY 697974
CMW 10465	E58, CBS 112921	*Endothia singularis*	—	USA	R.J. Stipes	AF 368323, AF 368333, AF 368332
CMW 10442	E27	*Endothia gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 368326, AF 368339, AF 368338
CMW 2091	ATCC 48192, E13	*Endothia gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 046905, AF 368337, AF 368336
CMW 5288	CBS 112900	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821
CMW 5587	CBS 112901	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543818, AF 543820, AF 543822

Notes: All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

[a]Taxa presented in boldface type represent isolates sequenced in this study. Sequence data for the other taxa are from the studies of Myburg et al. (2002, 2004) and Venter et al. (2002).

[b]ATCC, American Type Culture Collection, Manassas, VA 20108, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; TFM:FPH, Forestry and Forest Products Research Institute, Danchi-Nai, Ibaraki, Japan. E or Ep. refers to an isolate: M, isolates used in Hoegger et al. (2002); D15, D31; isolates used in Cortesi et al. (1997); 09494, KB1, CD28, YM2; isolates used in Liu et al. (2003); DY23, culture collection of Michael Milgroom, Department of Plant Pathology, Cornell University, Ithaca, NY 14853-4203, USA; MAFF, Microorganisms Section, MAFF GENEBANK, National Institute of Agrobiological Sciences (NIAS), MAFF Gene Bank, Ibaraki, Japan; E, from the culture collection of Prof. R.J. Stipes (Department of Plant Pathology, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061-0331, USA) now housed in the culture collection (CMW) of FABI.

of these (CMW 10785 to CMW 10787) are from the study by Liu et al. (2003). One Japanese isolate from *Quercus serrata* Thunb. (CMW 10790) was morphologically similar to *Cryphonectria parasitica*. Isolates of *Cryphonectria* and *Endothia* species studied previously (Myburg et al. 2004; Venter et al. 2002) were included for comparative purposes: *Cryphonectria parasitica* (CMW 7047, CMW 7048), *Cryphonectria radicalis* (CMW 10436, CMW 10455, CMW 10477, CMW 10484), *Cryphonectria macrospora* (CMW 10463), *Endothia gyrosa* (CMW 10442, CMW 2091), and *Endothia singularis* (CMW 10465). Four *Cryphonectria radicalis* isolates (CMW 10791 to CMW 10794) from Europe, recently studied by Hoegger et al. (2002) were incorporated. The remaining isolates were *Cryphonectria parasitica*, *Cryphonectria radicalis*, *Cryphonectria nitschkei*, *Cryphonectria macrospora*, and *Cryphonectria havanensis* from various host families in Japan and are linked to the studies of Kobayashi and Itô (1956*a*, 1956*b*) and Kobayashi (1970) (Table 1). Two *Diaporthe ambigua* Nitschke isolates (CMW 5288, CMW 5587) were included to serve as outgroup taxa in the phylogenetic analyses. Isolates of *Cryphonectria radicalis* from North America unfortunately were not available and could not be included.

The majority of isolates from Japan were obtained from TFM:FPH (Forestry and Forest Products Research Institute, Danchi-Nai, Ibaraki, Japan) and MAFF GENEBANK (Microorganisms Section, MAFF GENEBANK, National Institute of Agrobiological Sciences (NIAS), MAFF Gene Bank, Tsukuba, Ibaraki, Japan) collections. All isolates from TFM:FPH have a number preceded by E or Ep, linking them to the studies of Kobayashi and Itô (1956*a*, 1956*b*) and Kobayashi (1970). These numbers distinguished them from the herbarium specimens. All isolates (CMW) are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Duplicates of cultures that are not already in other international culture collections have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

Herbarium specimens of the unidentified *Cryphonectria* species occurring in Japan and China (Table 2) and studied by Liu et al. (2003) were examined. Three of these specimens (TFM:FPH 7609, TFM:FPH 7610, TFM:FPH 7747) are linked to isolates, that is, CMW 10786, CMW 10787, and CMW 5877. These specimens have been deposited in the herbarium of the Forestry and Forest Products Research Institute (TFM:FPH), Danchi-Nai, Ibaraki, Japan.

DNA isolations and amplification

DNA was isolated from cultures as described in Myburg et al. (1999). The ITS1 and ITS2 regions of the ribosomal RNA operon, as well as the conserved 5.8S gene, were amplified using the primer set ITS1 and ITS4 (White et al. 1990). Two regions within the β-tubulin gene were amplified using primer pairs Bt1a with Bt1b and Bt2a with Bt2b (Glass and Donaldson 1995). These two gene regions were selected to ensure consistency between the present study and previously published phylogenetic data, where these gene regions (in combination with ITS1 and ITS2 sequence data) were successfully used to delimit phylogenetic relationships between *Cryphonectria* and *Endothia* spp. (Myburg et al.

Table 2. Specimens of *Cryphonectria* species used in the morphological comparisons.

Identification	Herbarium allocation[a]	Isolate linked to specimen[b]	Host	Origin	Collector	Date
Cryphonectria parasitica	TFM:FPH 1326	—	*Castanea crenata*	Tsurukawa, Japan	T. Kobayashi	1953
Cryphonectria parasitica	TFM:FPH 629	—	*Castanea crenata*	Koganei, Japan	T. Kobayashi	1953
Cryphonectria parasitica	TFM:FPH 608	CMW 10916	*Castanea crenata*	Matsudo, Japan	T. Kobayashi	1953
Cryphonectria parasitica	TFM:FPH 600	—	*Castanea crenata*	Seki, Japan	T. Kobayashi	1953
Cryphonectria parasitica	TFM:FPH 351	CMW 13751	*Castanea crenata*	Inagi, Japan	T. Kobayashi	1953
Cryphonectria radicalis	BPI 612660	—	—	Como, Italy	C.L. Shear	1912
Cryphonectria radicalis	BPI 612672	—	*Castanea sativa*	Etremblieres, Switzerland	C.L. Shear	1913
Cryphonectria radicalis	BPI 613739	—	*Castanea sativa*	Stresa, Italy	C.L. Shear	1913
Cryphonectria radicalis	BPI 1112743	—	*Quercus* sp.	Bois Bastard, France	F. Candoussau	1992
Cryphonectria radicalis	BPI 797696	—	*Castanea* sp.	Rome, Italy	Prof. Liropoli	1877
Cryphonectria radicalis	BPI 797697	—	*Castanea sativa*	Locarno, Switzerland	—	1862
Cryphonectria radicalis	BPI 797698	—	—	Sciolze, Italy	—	1873
Cryphonectria radicalis	BPI 797692	—	*Carpinus betulus*	Abkchazia	Woronin	—
Cryphonectria radicalis	BPI 797693	—	*Castanea* sp.	Locarno, Switzerland	Denotaris	1862
Cryphonectria radicalis	BPI 797694	—	*Castanea* sp.	Locarno, Switzerland	Daldini	1862
Cryphonectria radicalis	BPI 797695	—	*Castanea* sp.	Como, Italy	—	—
Cryphonectria radicalis	TFM:FPH 1200	—	*Quercus variabilis*	Meguro, Japan	T. Kobayashi	1953
Cryphonectria radicalis	TFM:FPH 1072	—	*Quercus serrata*	Machida, Japan	T. Kobayashi	1954
Cryphonectria radicalis	TFM:FPH 1202	CMW 13754	*Fagus japonica*	Meguro, Japan	T. Kobayashi	1953
Cryphonectria radicalis	TFM:FPH 2483	—	*Quercus salicina*	Komayama, Japan	T. Kobayashi	1959
Cryphonectria radicalis	TFM:FPH 601	—	*Alnus firma*	Nishina, Japan	T. Kobayashi	1955
Cryphonectria radicalis	TFM:FPH 652	—	*Carpinus japonica*	Asakawa, Japan	T. Kobayashi	1962
Cryphonectria radicalis	NY 1963	—	*Quercus* sp.	Glatfelter, Penn., USA	C.L. Shear and N.E. Stevens	1913
Cryphonectria radicalis	CUP 6178	—	Chestnut stump	Connellsville, Penn., USA	P.J. Anderson and H.W. Anderson	1912
Cryphonectria radicalis (type)	K 109808	—	Bark (possibly *Quercus*)	USA	Schweinitz	1828
Cryphonectria havanensis	TFM:FPH 633	CMW 10910	*Eucalyptus globulus*	Meguro, Japan	T. Kobayashi	1954
Cryphonectria havanensis	TFM:FPH 2300	—	*Betula* sp.	Yoshiwara, Japan	Zinno	1963
Cryphonectria havanensis	TFM:FPH 1270	CMW 13736	*Pyrus sinensis*	Inagi, Japan	T. Kobayashi	1960
Cryphonectria havanensis	TFM:FPH 1203	—	*Quercus variabilis*	Seto, Japan	T. Kobayashi	1953
Cryphonectria havanensis	TFM:FPH 1047	—	*Quercus glandulifera*	Japan	T. Kobayashi	1954
Cryphonectria macrospora (type)	TFM:FPH 1057	—	*Castanopsis cuspidata*	Shinagawa, Japan	T. Kobayashi	1954
Cryphonectria macrospora	TFM:FPH 1058	—	*Castanopsis cuspidata*	Shinagawa, Japan	T. Kobayashi	1954
Cryphonectria nitschkei (type)	TFM:FPH 1045	—	*Quercus grosseserrata*	Meguro, Japan	T. Kobayashi	1954
Cryphonectria nitschkei	TFM:FPH 1046	—	*Quercus grosseserrata*	Mt. Fuji, Japan	T. Kobayashi	1954
Cryphonectria nitschkei	TFM:FPH 1049	CMW 13744	*Quercus grosseserrata*	Mt. Ôtake, Japan	T. Kobayashi	1954
Cryphonectria nitschkei	TFM:FPH 1064	CMW 13747	*Quercus glandulifera*	Kano, Japan	T. Kobayashi	1954
Cryphonectria nitschkei	TFM:FPH 2225	CMW 13745	*Rhus javanica*	Hanawa, Japan	Y. Zinno	1963
Cryphonectria nitschkei	TFM:FPH 632	CMW 13742	*Quercus grosseserrata*	Agematsu, Japan	T. Kobayashi	1955
Cryphonectria nitschkei	TFM:FPH 586	—	*Quercus grosseserrata*	Wada, Japan	T. Kobayashi	1955
Cryphonectria nitschkei	TFM:FPH 570	CMW 13741	*Quercus grosseserrata*	Nagato, Japan	T. Kobayashi	1955

Table 2 (*concluded*).

Identification	Herbarium allocation[a]	Isolate linked to specimen[b]	Host	Origin	Collector	Date
Cryphonectria nitschkei	TFM:FPH 7609	CMW 10786	*Castanea crenata*	Kobuchizawa, Japan	M. Milgroom and S. Kaneko	1998
Cryphonectria nitschkei	TFM:FPH 7610	CMW 10787	*Castanea crenata*	Chudai, Japan	M. Milgroom and S. Kaneko	1998
Cryphonectria nitschkei	TFM:FPH 7747	CMW 8577	*Castanea crenata*	Yamada, Japan	M. Milgroom and S. Kaneko	1998

[a]BPI, US National Fungus Collections, Systematic Botany and Mycology, Bellsville, MD 20705-2350, USA; NY, William and Lynda Steere Herbarium, New York Botanical Garden, Bronx, NY 10458-5126, USA; TFM:FPH, Forestry and Forest Products Research Institute, Danchi-Nai, Ibaraki, Japan; CUP, Plant Pathology Herbarium, Cornell University, Ithaca, NY 14853, USA; K, Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey, UK.
[b]Isolates linked to these specimens are listed in Table 1.

2004; Venter et al. 2002). The amplification reaction mixes, as well as the reaction conditions, were the same as those described in Myburg et al. (2002).

DNA sequencing and analyses

PCR products were sequenced in both directions using the same primer pairs that were used in the amplification reactions. Sequencing reactions were done using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, UK). DNA sequences were determined using an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer).

Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software was used to edit the DNA sequences. The sequences were manually aligned with sequence data sets from previous studies (Myburg et al. 2004; Venter et al. 2002). Phylogenetic analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony) software version 4.0b (Swofford 1998). After the exclusion of uninformative sites, the ribosomal DNA (ITS1, 5.8S, ITS2) and the β-tubulin sequence data sets were subjected to a 500 replicate partition-homogeneity test to test the null hypothesis that the different data sets were homogenous, thus indicating whether the different sets could be combined in the subsequent phylogenetic analyses (Farris et al. 1994). Phylogenetic analyses were done using heuristic searches with the tree-bisection-reconnection and MULTREES (saving all optimal trees) options. Gaps were treated as fifth characters (NEWSTATE) in the heuristic searches. Distance analyses were also performed on the data set using the Tamura–Nei parameter model (Tamura and Nei 1993) with the Gamma distribution shape parameter set to 0.2271. This model (TrN+G) was shown to be the appropriate distance model for these datasets by MODELTEST version 3.5 (Posada and Crandall 1998). The reproducibility of the branching nodes was determined by a bootstrap analysis (1000 replications). Although various authors use different bootstrap values to infer clades (Zander 2004), we have opted in the present study to consider groups with bootstrap values above 80% a clade. Sequence data from two *D. ambigua* isolates were used to root the phylogenetic tree. This taxon was chosen because *Diaporthe* resides in the same order as, but in a different phylogenetic complex than, *Cryphonectria* and *Endothia* species (Castlebury et al. 2002). Sequences generated in this study have been deposited in GenBank and accession numbers are listed in Table 1, together with accession numbers of previously deposited sequences (Myburg et al. 2004; Venter et al. 2002). The DNA sequence alignments were submitted in TreeBase (study accession No. S1146, matrix accession No. M1969).

Morphological comparisons

Fruiting structures on herbarium specimens were cut from the bark and rehydrated for 1 min in boiling water. The structures were sectioned at –20 °C with a Leica CM1100 cryostat after embedding in Leica mountant (Setpoint Premier, Johannesburg, South Africa). Sections, 12–16 µm thick, were mounted on microscope slides in lactophenol. Spores, asci, and conidiophores from the various specimens were measured in 3% KOH and lactophenol, respectively. At

least 20 spores for each specimen were measured. The distribution of measurements (µm) for each specimen is expressed as the range and the mean (± SD) using the notation: (min–) (mean – SD) – (mean + SD) (–max). Standard colour notations of Rayner (1970) were used.

Results

DNA sequencing and analyses

Amplification of the ITS1 and ITS2 ribosomal RNA operons and β-tubulin gene regions resulted in PCR products between 550 and 600 bp (base pairs) in size (data not shown). The DNA sequence of the partial ITS1 and ITS2 regions (569 bp) consisted of 339 constant characters, 11 parsimony-uninformative, and 219 parsimony-informative characters, while the sequence of the β-tubulin gene regions (965 bp) consisted of 561 constant characters, 25 parsimony-uninformative, and 379 parsimony-informative characters. The partition-homogeneity test, which was performed on the partitions ribosomal DNA and β-tubulin, generated a P value of 0.586, indicating that the null hypothesis could not be rejected and that the respective gene regions could be combined in subsequent analyses. Sequences from a total of 42 isolates were included in the combined data set. The two *D. ambigua* isolates were used as outgroup taxa. Using parsimony, the combined data set consisted of a total of 1534 characters, of which 900 were constant, 36 were parsimony-uninformative, and 598 parsimony-informative. Of these, 11 ambiguous base pairs were excluded. The heuristic search produced 100 most parsimonious trees (tree length = 1173 steps, consistency index = 0.792, retention index = 0.942), which differed only in the length of the internal branches (Fig. 1). The tree derived from the distance analyses (Fig. 2) resulted in the same clades as the trees derived with parsimony, although the relationships among the clades were represented slightly differently.

Cryphonectria and *Endothia* were resolved into distinct clades in the phylogenetic trees (Figs. 1 and 2). The greater *Cryphonectria* group included several clades representing various species (Figs. 1 and 2). The first of these includes *Cryphonectria parasitica* isolates originating from the USA and Japan (bootstrap support 100% in both analyses). A second clade included isolates from Europe that have been referred to as *Cryphonectria radicalis* (bootstrap support 100% in both analyses). These *Cryphonectria radicalis* isolates from Italy (CMW 10455, CMW 10477, CMW 10791), Greece (CMW 10788, CMW 10789), and Switzerland (CMW 10792, CMW 10793, CMW 10794) showed a high degree of sequence similarity (bootstrap support 100% in both analyses). An isolate from *Fagus japonica* Maxim. (Fagaceae) in Japan (CMW 13754) grouped close to these isolates. However, two isolates, CMW 10436 from Portugal and CMW 10484 from Italy, previously identified as *Cryphonectria radicalis* (Myburg et al. 2004), grouped separately from the other *Cryphonectria radicalis* isolates from Italy, Greece, Switzerland, and Japan (bootstrap support 100% in both analyses).

The unidentified fungus represented by the isolates (CMW 10782 to CMW 10787) from *Quercus* and *Castanea* spp. in China and Japan grouped closely with other isolates of *Cryphonectria nitschkei* (bootstrap support 100% in both analyses). Japanese isolates of *Cryphonectria nitschkei* from *Quercus* spp. (CMW 13744, CMW 5877, CMW 13741, CMW 13742, CMW 13747, CMW 10581) and an isolate of *Cryphonectria havanensis* from *Quercus grosseserrata* Blume (CMW 11294) grouped in this clade. Isolates of *Cryphonectria havanensis* from *Castanopsis cuspidata* (CMW 13737), *Pyrus sinensis* Lindl. (*Rosaceae*) (CMW 13736), and *Eucalyptus globulus* Labill. (*Myrtaceae*) (CMW 10910) also grouped in this clade, as did a *Cryphonectria nitschkei* isolate from *Rhus javanica* L. (*Anacardiaceae*) (CMW 13745). Isolates of *Cryphonectria macrospora* from Japan (CMW 10463, CMW 10914) grouped closer to isolates of *Cryphonectria nitschkei* and *Cryphonectria havanensis* than to isolates in any of the other clades (bootstrap support 100%) but still formed a distinct clade.

Morphological comparisons

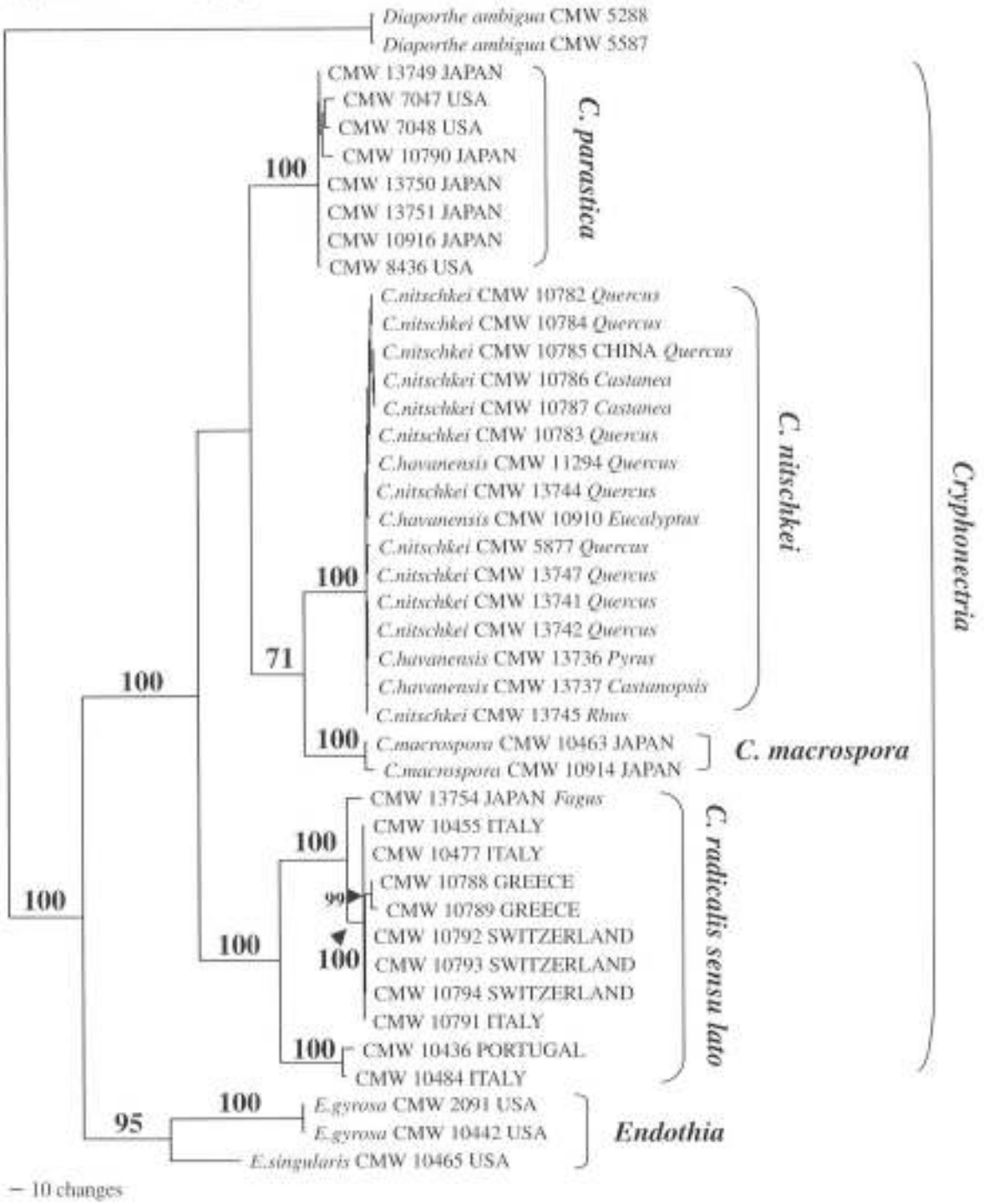
Identification of the unknown Cryphonectria *sp.*

The morphology of the fungus represented by isolate CMW 10786 (annotated KB1 in Liu et al. (2003)) that grouped with the other *Cryphonectria nitschkei* isolates in the phylogenetic tree, is linked to specimen TFM:FPH 7609. The ascospores of this fungus were (8.5–)10–11.5(–12.5) × (3.5–)4–4.5(–5) µm. The conidia were 4–5.5(–6) × (1–)1.5(–2) µm (Table 3). Specimens TFM:FPH 7610, linked to isolate CMW 10787 (annotated CD 28 in Liu et al. (2003)), and TFM:FPH 7747 (linked to isolate CMW 5877/YM2) had slightly longer conidia (up to 7.5 µm). Despite this significant morphological difference, these isolates grouped together with CMW 10786 in the phylogenetic tree.

Of all of the species previously reported on woody hosts in Japan (Kobayashi and Itô 1956*a*; Kobayashi 1970), the ascospore and conidial dimensions of the unidentified specimens (TFM:FPH 7609, TFM:FPH 7610, TFM:FPH 7747) most closely resembled those of *Cryphonectria nitschkei* and *Cryphonectria havanensis* (Table 3). The ascospore and conidial sizes of *Cryphonectria nitschkei* and *Cryphonectria havanensis* overlapped (Table 3). Specimens labeled as *Cryphonectria havanensis* that originated from fagaceous and non-fagaceous hosts also had comparable ascospore sizes. These included two specimens from *Quercus* spp. (TFM: FPH 1203 from *Quercus variabilis* Blume and TFM:FPH 1047 from *Quercus glandulifera* Blume), specimen TFM:FPH 2300 from a *Betula* sp. (*Betulaceae*), and specimen TFM:FPH 1270 from *Pyrus sinensis* (Table 3). The ascospore measurements for the above-mentioned specimens were similar to those given by Kobayashi (1970), which were an average of 8–12.5 × 3–4 µm for *Cryphonectria havanensis* and 10–13 × 4–4.5 µm for *Cryphonectria nitschkei*. The morphological similarities corresponded with the grouping of isolates of these taxa in the same phylogenetic clade (Figs. 1 and 2).

Only one specimen (TFM:FPH 633) of *Cryphonectria havanensis* from *Eucalyptus globulus* (*Myrtaceae*) was available for this study. This specimen is connected to isolate CMW 10910 included in the phylogenetic analysis. Ascospore morphology for TFM:FPH 633 was similar to the other *Cryphonectria havanensis*-labeled specimens (designated as "A. *Cryphonectria havanensis*" in Table 3). The ascospore similarity to other specimens of *Cryphonectria havanensis* and *Cryphonectria nitschkei* supports the phylogenetic grouping of isolate CMW 10910 with isolates of

Fig. 1. One of 100 most parsimonious phylogenetic trees (length = 1244 steps, consistency index = 0.7709, retention index = 0.9390) showing phylogenetic relationships among different species of *Cryphonectria* from Europe, China, and Japan. The tree was generated with the heuristic algorithm (tree-bisection-reconnection option) from sequence variation within a combined ribosomal (ITS1, 5.8S, ITS2) and β-tubulin (1a/b, 2a/b) sequence data set. Bootstrap values (1000 replicates) greater than 70% are indicated at the branch nodes. Clades with bootstrap values above 80% were considered significant. All of the *Cryphonectria nitschkei* and *Cryphonectria havanensis* isolates originated from Japan except for CMW 10785, which originated from China. The *Diaporthe ambigua* isolates were used as outgroup taxa to root the phylogenetic tree.

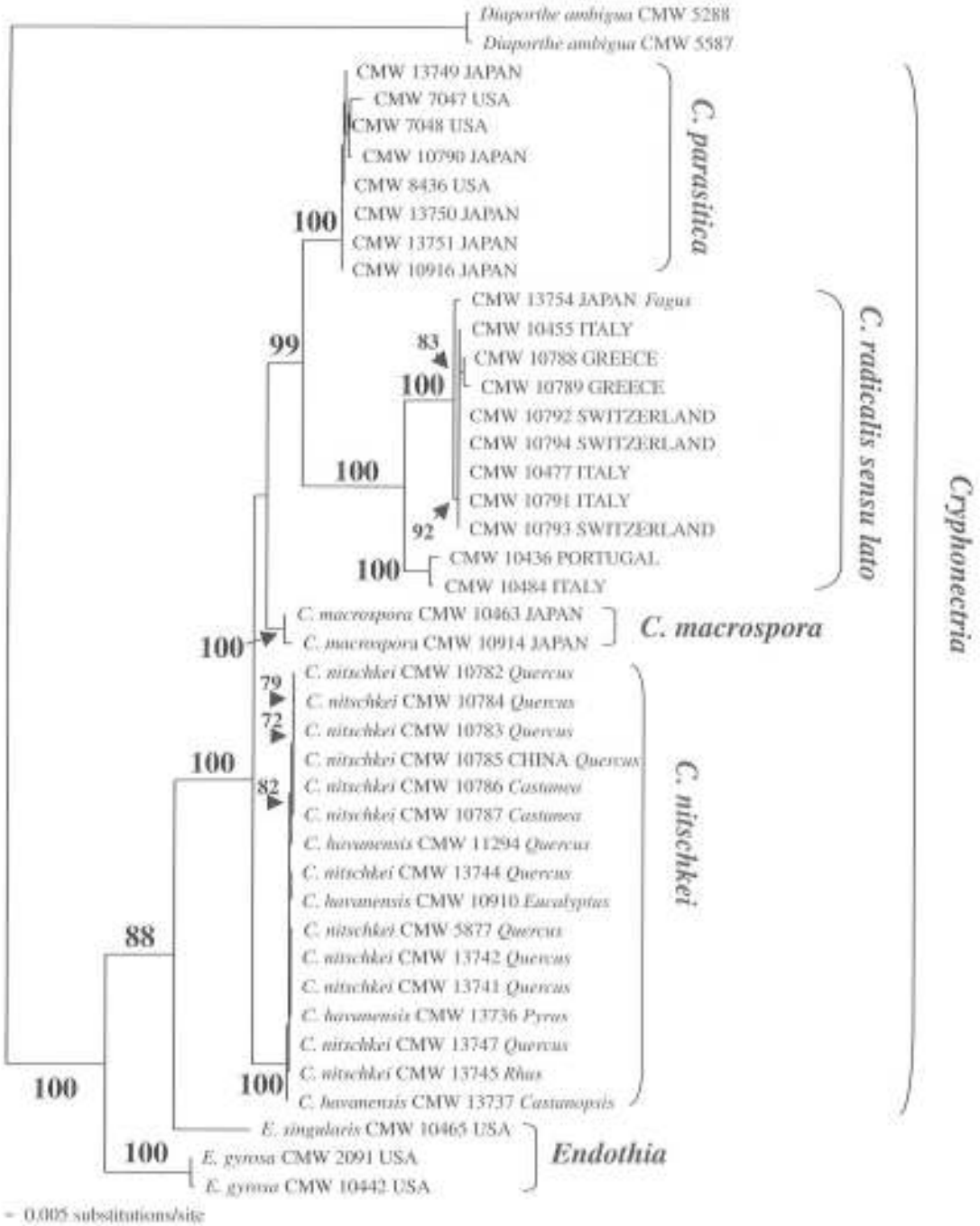


Cryphonectria havanensis and *Cryphonectria nitschkei* (Figs. 1 and 2). Other fruiting structures were, however, found on specimen TFM:FPH 633 (designated as "B. *Cryphonectria havanensis*" in Table 3) with ascospores different ((6–)7–9(–10.5) × (3–)3.5–4(–4.5) µm) from those of the other fruiting structures on the same specimen.

Distinction among Cryphonectria radicalis *groups in Europe*

Two groups could be distinguished for European specimens labeled as *Cryphonectria radicalis* based on ascospore size (Table 3). This is consistent with the phylogenetic analyses (Figs. 1 and 2) showing two distinct groups for *Cry-*

Fig. 2. Distance phylogram showing phylogenetic relationships among different species of *Cryphonectria* from Europe, China, and Japan. The phylogram was obtained with the Tamura-Nei parameter model ($G = 0.2271$) on the combined data set of ribosomal DNA and β-tubulin gene sequences. Bootstrap values greater than 70% (1000 replicates) are indicated at the branch nodes. Clades with bootstrap values above 80% were considered significant. All of the *Cryphonectria nitschkei* and *Cryphonectria havanensis* isolates originated from Japan except for CMW 10785, which originated from China. The *Diaporthe ambigua* isolates were used as outgroup taxa to root the phylogenetic tree.



phonectria radicalis isolates from Europe. However, none of the specimens are linked to isolates in the phylogenetic tree. Thus, no specific connections among groups defined by sequence data and spore morphology are possible at present.

One group of *Cryphonectria radicalis* specimens (BPI 797697, BPI 613739, BPI 612672, BPI 797693; Table 2), originating from *Castanea sativa* in Italy and Switzerland, had smaller ascospores, (6–)7–8.5 × (2–)2.5–3 µm (Table 3). These dimensions are similar to those given for the European *Cryphonectria radicalis* isolates (CMW 10792, CMW 10793, CMW 10794) by Hoegger et al. (2002) and to the Japanese *Cryphonectria radicalis* specimens (Table 3).

Table 3. Spore sizes for the different species studied.

Label name	Specimens	Ascospore length	Ascospore width	Specimens	Conidial length	Conidial width
Cryphonectria macrospora	TFM:FPH 1057	14–17(–19)	(4.5–)5.5–7(–8)	TFM:FPH 1057 TFM:FPH 1058	3.5–4.5(–5)	1–1.5
Cryphonectria nitschkei	TFM:FPH 1045 (type) TFM:FPH 1046 TFM:FPH 1064 TFM:FPH 586	(8–)9.5–11.5(–12.5)	(3–)3.5–4.5(–5)	TFM:FPH 1045 (type) TFM:FPH 1064 TFM:FPH 2225	(3–)3.5–5(–6)	1–1.5(–2)
Cryphonectria havanensis (*Quercus*)	TFM:FPH 1203 TFM:FPH 1047	(8–)9.5–11.5(–13)	(3.5–)4–5(–5.5)	TFM:FPH 1047	(3–)3.5–4.5(–5)	1.5(–2)
Cryphonectria nitschkei[a]	TFM:FPH 7609	(8.5–)10–11.5(–12.5)	(3.5–)4–4.5(–5)	TFM:FPH 7609	4–5.5(–6)	(1–)1.5(–2)
Cryphonectria nitschkei[a]	TFM:FPH 7610	—	—	TFM:FPH 7610	(4.5–)5–6.5(–7)	1.5
Cryphonectria havanensis (*Betula* sp.)	TFM:FPH 2300	(8–)9.5–11(–12.5)	(3–)3.5–4(–4.5)	—	—	—
Cryphonectria havanensis (*Pyrus sinensis*)	TFM:FPH 1270	10–12(–13.5)	(3–)3.5–4(–4.5)	—	—	—
"A. *Cryphonectria havanensis*" (*Eucalyptus globulus*)	TFM:FPH 633	(9.5–)10–12(–13.5)	3–4(–4.5)	—	—	—
"B. *Cryphonectria havanensis*" (*Eucalyptus globulus*)	TFM:FPH 633	(6–)7–9(–10.5)	(3–)3.5–4(–4.5)	—	—	—
Cryphonectria parasitica	TFM:FPH 629 TFM:FPH 1326	(7.5–)8–9(–9.5)	3.5–4(–4.5)	TFM:FPH 600 TFM:FPH 608 TFM:FPH 1326	(3–)3.5–4(–4.5)	1–1.5
Cryphonectria radicalis longer ascospores (Europe)	BPI 797696	(7–)8–10(–12)	(2–)2.5–3.5(–4)	BPI 1112743	(3–)3.5–4(–4.5)	1–1.5(–2)
	BPI 797692 BPI 1112743 BPI 797698 BPI 612660			BPI 797698 BPI 612660		
Cryphonectria radicalis Japan	TFM:FPH 652 TFM:FPH 2483	(5.5–)6.5–8(–9.5)	(2–)2.5–3.5	TFM:FPH 601 TFM:FPH 652 TFM:FPH 1072 TFM:FPH 1200	3–4(–4.5)	1–1.5
Cryphonectria radicalis smaller ascospores (Europe)	BPI 797697	(6–)7–8.5	(2–)2.5–3	BPI 613739	(3–)3.5–4(–4.5)	1–1.5(–2)
	BPI 613739 BPI 612672 BPI 797693			BPI 612672 BPI 797693		
Cryphonectria radicalis NY 1963 (USA)	NY 1963	(5.5–)6.5–8.5(–10)	(2.5–)3–4	—	—	—
Cryphonectria radicalis CUP 6178 (USA)	CUP 6178	(5–)5.5–7(–7.5)	2.5–3(–3.5)	CUP 6178	(2.5–)3–3.5(–4)	1–1.5
Cryphonectria radicalis K 109808 (USA)	K 109808	(5.5–)6–7.5(–8.5)	2.5–3.5	K 109808	(3–)3.5–4(–4.5)	1–1.5

Note: Species and individual specimens that were considered separately are mentioned in order of decreasing ascospore length.
[a]Identified in this study.

Conidiomata in this first group of European specimens also had pale luteous cells lining the conidial locules, similar to Japanese *Cryphonectria radicalis* specimens.

The small ascospore group also included specimens of North American *Cryphonectria radicalis*. Ascospore sizes for a specimen from a *Quercus* sp. (NY 1963), another specimen (CUP 6178), and the type specimen of *Cryphonectria radicalis* (K 109808) fell within the size range given for *Cryphonectria radicalis* specimens from Japan and the European group with smaller ascospores (Table 3). Furthermore, for specimen CUP 6178, cells lining the conidial locules were pale luteous in colour. These features were similar to those in Japanese and the European specimens with smaller ascospores. Conidial sizes for the North American *Cryphonectria radicalis* specimens (CUP 6178, NY 2018, K 109808) were comparable with those from the rest of the world (Table 3).

The other group of specimens from Europe labeled *Cryphonectria radicalis* (BPI 797696, BPI 797692, BPI 1112743, BPI 797698, BPI 612660; Table 2) had longer ascospores than those of the first group but were similar in width (Table 3). These originated from Italy, Abkhazia, and France on *Castanea sativa*, a *Quercus* sp., and *Carpinus betulus* L., respectively. The ascospore sizes of the fungus residing in this group did not resemble those of any other *Cryphonectria* species examined in this study (Table 3). Conidia were similar in size to the first group of *Cryphonectria radicalis* from Europe with smaller ascospores (Table 3).

Discussion

The present study has provided morphological as well as phylogenetic analyses of DNA sequence data for the majority of *Cryphonectria* species known to occur on woody host species in Europe, China, and Japan. These species include *Cryphonectria parasitica*, *Cryphonectria radicalis*, *Cryphonectria nitschkei*, and *Cryphonectria macrospora*. The combination of morphological and DNA sequence data presented in this study should aid future researchers in making correct identifications of *Cryphonectria* species found in Eurasia. This is particularly so given the difficulty of making firm identifications of these fungi based on morphology alone. This is due to problems such as the variability of spores among specimens and the absence of either the sexual or asexual state.

The isolates identified for the first time in this study as *Cryphonectria nitschkei* were mentioned in a recent study of interspecies transmission of hypoviruses (Liu et al. 2003). While sampling *Cryphonectria parasitica* isolates in Japan, Liu et al. (2003) recognised that their collections included another *Cryphonectria* species that produced less pigment in culture than *Cryphonectria parasitica*, which in general was more orange in colour. This unknown sympatric species, along with *Cryphonectria parasitica*, also contained *Cryphonectria hypovirus* 1 (CHV-1), which could be transmitted between the two species in culture. Liu et al. (2003) showed, through DNA sequencing and RFLP data of the ribosomal ITS DNA region sequence, that isolates CMW 10785, CMW 10786, CMW 10787 (isolates 09494, KB1, and CD28, respectively, in Liu et al. (2003)) of this unknown species grouped separately from their *Cryphonectria parasitica* isolates. In the present study, we have been able to show conclusively, by additional sequence data and morphological comparisons, that this unknown *Cryphonectria* species represents *Cryphonectria nitschkei*. This confirms the discovery by Liu et al. (2003) that the virus transmission they observed both in the laboratory and in nature was among different fungal species.

The fungus previously treated as *Cryphonectria havanensis* from Japan was initially thought to represent two species occurring on two different hosts (Kobayashi and Itô 1956*a*; Kobayashi 1970). These were not newly described species but were named after already existing species. The first of these, annotated as *Endothia havanensis* Bruner (Kobayashi and Itô 1956*a*), was isolated from dead bark of *Eucalyptus globulus*. *Endothia havanensis* was originally described from *Eucalyptus* spp. in Cuba (Bruner 1916). The second fungus occurred on fagaceous hosts in Japan and was identified as *Endothia tropicalis* Shear & N.E. Stevens (Kobayashi and Itô 1956*a*). *Endothia tropicalis* is a synonym of *Cryphonectria gyrosa* (Berk. & Broome) Sacc., which is the type species of *Cryphonectria* (Barr 1978). *Cryphonectria gyrosa* is known from Sri Lanka (Berkeley and Broome 1875; Shear et al. 1917) occurring on *Elaeocarpus* spp. (Shear et al. 1917).

At the time when *Cryphonectria* was considered a synonym of *Endothia*, Kobayashi (1970) reduced *Endothia tropicalis* (now known as *Cryphonectria gyrosa*) to synonymy with *Endothia havanensis* (now known as *Cryphonectria havanensis*). This synonymy was based largely on the similar ascospore sizes of the two taxa. Hence, Japanese specimens of *Endothia tropicalis* and *Endothia havanensis* were amalgamated under the name *Endothia havanensis* (Kobayashi 1970). However, when Barr (1978) segregated *Cryphonectria* from *Endothia*, she also separated *Cryphonectria gyrosa* from *Cryphonectria havanensis*, but the fungus from Japan was retained under the name *Cryphonectria havanensis*, including specimens from both *Quercus* and *Eucalyptus*.

Distinction of *Cryphonectria havanensis* from the other species occurring in Japan was based on ascospore size (Kobayashi and Itô 1956*a*; Kobayashi 1970), but this species resembled *Cryphonectria nitschkei* most closely (Kobayashi 1970). Results of the phylogenetic analyses emerging from the present study showed that isolates of *Cryphonectria havanensis* from Japan are identical to those of *Cryphonectria nitschkei*. Morphological comparisons confirmed that these two taxa in Japan are the same. Furthermore, isolates and specimens previously known as *Cryphonectria havanensis* in Japan and collected from *Eucalyptus* and *Quercus* are identical based on morphological features and phylogenetic analyses.

Results of this study have shown that the Japanese isolates assigned the names *Cryphonectria havanensis* and *Cryphonectria nitschkei* are identical. Whether or not the isolates represented by the phylogenetic clade in this study are the same as the earlier discovered Cuban fungus known as *Cryphonectria havanensis* on *Eucalyptus*, cannot be resolved satisfactorily in the absence of cultures of the latter fungus. Although not the aim of this study, collections of *Cryphonectria nitschkei* and those labeled as *Cryphonectria*

havanensis from Japan, especially on *Eucalyptus*, should be compared with the type specimen of *Cryphonectria havanensis* and preferably cultures of this fungus from Cuba. This will aid in establishing whether *Cryphonectria nitschkei* in Japan represents a taxon distinct from *Cryphonectria havanensis* in Cuba or whether previous authors (Hodges 1980; Kobayashi 1970) were correct in reducing the two fungi to synonymy. We prefer to keep the taxa *Cryphonectria havanensis* and *Cryphonectria nitschkei* separate pending a more thorough study investigating these questions.

Isolates of *Cryphonectria havanensis* referred to in this study were from hosts residing in different plant families. These include *Quercus*, *Castanopsis*, *Pyrus*, *Eucalyptus*, and *Betula*. In addition to these, isolates labeled as *Cryphonectria nitschkei* from *Castanea* and *Rhus* also grouped in this clade. Other hosts previously reported for *Cryphonectria nitschkei* (Kobayashi 1970) but not included in this study are *Carpinus tschonoskii* Maxim. (*Cupuliferae*), *Larix leptolepis* (Siebold & Zucc.) Gordon (*Pinaceae*), and a *Prunus* sp. (*Rosaceae*). Isolates from all of these hosts form part of the clade representing *Cryphonectria nitschkei*, indicating that this fungus has a diverse host range.

Specimen TFM:FPH 633 was covered with two types of fruiting structures that produced ascospores of different size (Table 3). One type of fruiting structure had ascospores similar in size to specimens of *Cryphonectria nitschkei* but the other type had smaller ascospores (Table 3). An isolate from *Eucalyptus globulus* (CMW 10910) linked to this specimen, grouped with other isolates of *Cryphonectria nitschkei* in the phylogenetic analyses. We believe that isolate CMW 10910 is connected to the fungus producing a fruiting structure of the first type with ascospores similar to those of *Cryphonectria nitschkei*. Specimens of the second type of fruiting structure were old and in poor condition and we are uncertain whether these represent a distinct species. There are also no isolates that can be linked to the fungus producing these fruiting structures. Isolations from *Eucalyptus* spp. in Japan would be of great interest and should acknowledge that more than one *Cryphonectria* sp. might occur on a single *Eucalyptus* tree.

Several species of *Cryphonectria* other than *Cryphonectria nitschkei* and *Cryphonectria havanensis* occur on *Eucalyptus* spp. The best known of these is *Cryphonectria cubensis*, which occurs in tropical and subtropical areas (Conradie et al. 1990; Hodges 1980; Wingfield 2003), and *Cryphonectria eucalypti*, which has been reported from Australia and South Africa (Gryzenhout et al. 2003; Old et al. 1986; Van der Westhuizen et al. 1993; Walker et al. 1985). Of the species treated in this study that are mainly known to occur on Fagaceae, *Cryphonectria parasitica* has been reported to occur on *Eucalyptus* spp. in Japan (Old and Kobayashi 1988). The same study also noted the presence of an unidentified *Cryphonectria* sp. and unidentified *Endothiella* anamorphs other than those of *Cryphonectria parasitica* on specimens that have been collected from disease symptoms on *Eucalyptus* in Japan. The identification of *Cryphonectria parasitica* was based on spore size from fruiting structures on bark specimens, but identifications of the other fungi on the specimens were inconclusive (Old and Kobayashi 1988). Results of this study and those of Old and Kobayashi (1988) suggest that more than one species of *Cryphonectria* occurs on *Eucalyptus* spp. in Japan and surveys to collect these fungi from *Eucalyptus* spp. and characterize them based on the DNA sequence data presented in this study would be valuable.

Hoegger et al. (2002) showed that *Cryphonectria radicalis* occurs sympatrically with *Cryphonectria parasitica*. Our results support this finding. Both our data and those of others (Hoegger et al. 2002; Sotirovski et al. 2004) show that *Cryphonectria radicalis* continues to exist in Asia, Europe, and the USA, even though it is apparently not common. This species can be distinguished from *Cryphonectria parasitica* based on ascospore length and width, although in the absence of a teleomorph, it will be difficult to distinguish between the two species because conidial dimensions of *Cryphonectria parasitica* and *Cryphonectria radicalis* overlap. *Cryphonectria parasitica* also produces mycelial fans in the wood and these are not present in the case of *Cryphonectria radicalis* (Roane 1986*a*; Shear et al. 1917). Another important distinguishing characteristic between the two species is that *Cryphonectria radicalis* colours growth medium purple because of the production of a pigment known as endothine red, while *Cryphonectria parasitica* does not produce this pigment (Hoegger et al. 2002; Roane and Stipes 1978; Shear et al. 1917). Myburg et al. (1999) showed that PCR-RFLP banding patterns distinguish *Cryphonectria parasitica* from *Cryphonectria cubensis* and *E. gyrosa*, and these can be used to identify *Cryphonectria parasitica* (Liu et al. 2003; Sotirovski et al. 2004). However, further studies will be needed to make this PCR-RFLP based identification applicable to all of the *Cryphonectria* and *Endothia* spp. used in this study.

Results of the present study and those of Myburg et al. (2004) show the presence of two groups within the fungus known as *Cryphonectria radicalis* in Europe. These groups were defined independently based on DNA sequence data and morphology. It is, however, difficult to resolve whether the two groups emerging from analysis of DNA sequence data, correspond with the two groups distinguished based on morphology. This is because isolates used in the phylogenetic analyses were not linked to specimens in the morphological comparisons. It is, furthermore, difficult to deduce from previous studies which of the morphological groups in Europe correspond most closely with published data on *Cryphonectria radicalis*. Shear et al. (1917) made comparisons of various key specimens that included European and North American material. They obtained ascospore dimensions of 6–10 µm long and 3–4.5 µm wide for *Cryphonectria radicalis*. This encompasses the two morphological groups identified in the present study from Europe. Shear et al. (1917) also observed that *Cryphonectria radicalis* had highly variable ascospores. It is thus possible that they were treating the two different species, which we distinguish using DNA sequence data, as a single species. Ascospore dimensions given for the isolates of Hoegger et al. (2002) correspond with measurements of the one group of *Cryphonectria radicalis* specimens from Europe and Japan that had ascospores (6–)7–8.5 µm long. These isolates resided in the phylogenetic group that includes *Cryphonectria radicalis* isolates from Greece, Italy, and Switzerland. Unfortunately, no morphological data are available for the isolates in the

second phylogenetic clade, incorporating CMW 10436 from Portugal and CMW 10484 from Italy.

The presence of two groups labeled as *Cryphonectria radicalis* in Europe, makes it unclear which of these represent the true *Cryphonectria radicalis* in Europe. The type specimen of *Cryphonectria radicalis* has a North American origin (Fries 1828). The specimens from the USA examined in this study, which included the type specimen, had ascospore ranges that corresponded most closely with those of the group of *Cryphonectria radicalis* specimens from Europe with smaller ascospores. Furthermore, North American specimens also had pale luteous linings to the conidial locules, similar to those of the European specimens with smaller ascospores. Based on ascospore sizes, the group of *Cryphonectria radicalis* specimens with smaller ascospores ((6–)7–8.5 × (2–)2.5–3 µm) corresponded most closely with the type specimen of *Cryphonectria radicalis*. We suggest that this group represents *Cryphonectria radicalis* in Europe, although confirmation of this fact must await additional collections and comparisons based on DNA sequence data.

Resolution of the identity of *Cryphonectria radicalis* in Europe will require additional studies including those on North American *Cryphonectria radicalis* specimens. This is particularly necessary since Shear et al. (1917) mentioned a second form of *Cryphonectria radicalis*, named *Endothia radicalis mississippiensis* Shear and N.E. Stevens and existing in North America. It is thus possible that *Cryphonectria radicalis* in North America represents a number of different fungi. Unfortunately neither the type specimen of *Cryphonectria radicalis* nor other collections of this fungus from the USA are linked to living cultures. Numerous enquiries have lead us to believe that these isolates do not exist and new collections will be needed to resolve the identity of *Cryphonectria radicalis* in the USA.

A number of questions relating to *Cryphonectria radicalis* remain. We have no knowledge regarding the phylogenetic relatedness of isolates known as *Cryphonectria radicalis* from Europe and those from North America. A *Cryphonectria radicalis* isolate from *Fagus japonica* in Japan, grouped close to the phylogenetic clade including isolates from Italy, Greece, and Switzerland, and thus, it appears that this phylogenetic group of *Cryphonectria radicalis* isolates, has a wide Eurasian distribution. It would be interesting to determine the relationships of the different continental groups currently referred to as *Cryphonectria radicalis*. This would show whether humans have moved *Cryphonectria radicalis* sensu lato around the world, as is case for *Cryphonectria parasitica*. Alternatively it might indicate that *Cryphonectria radicalis* has the widest geographical distribution of all the species currently residing in *Cryphonectria*. Data presented in this study should aid future researchers in answering these questions and making correct identifications of *Cryphonectria* species in Eurasia. This is particularly so given the difficulty of making firm identifications of these fungi based solely on morphology. We recommend comparisons of DNA sequences in addition to analysis of morphological criteria to characterize new collections of these fungi.

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