Phylogenetic relationships of *Neonectria/Cylindrocarpon* on *Fagus* in North America[1]

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Abstract: The relationship of two species of *Neonectria* associated with beech bark canker in North America was evaluated by comparing isolates of these and additional species of the *Neonectria coccinea* (Pers.:Fr.) Rossman & Samuels group found on *Fagus*. Gene regions in the translation elongation factor 1-alpha (EF1-α), RNA polymerase II second largest subunit (RPB2), and β-tubulin were sequenced and analyzed. Results indicate that the fungus associated with beech bark disease previously known as *Neonectria coccinea* var. *faginata* Lohman et al. (≡ *Nectria coccinea* (Pers.:Fr.) Fr. var. *faginata* Lohman et al.) should be recognized as a separate species, *Neonectria faginata*, distinct from *Neonectria coccinea*. *Neonectria faginata* including its anamorphic state, *Cylindrocarpon faginatum* C. Booth, is known only on *Fagus* in North America. A second species associated with beech bark disease in North America is *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman, which can be distinguished morphologically from *Neonectria faginata* based on ascospore size, conidial size and shape, and colony pigmentation. Morphological and molecular data indicate that *Neonectria ditissima* represents an older name for *Neonectria galligena* Bres. Similarly, the anamorphic state of *Neonectria ditissima* is the older epithet *Cylindrocarpon heteronema* with *Cylindrocarpon willkommii* as a synonym. *Neonectria ditissima* occurs on a variety of hardwood trees in North America and Europe. *Neonectria coccinea* occurs only on *Fagus* in Europe. *Neonectria major* (Wollenw.) Castl. & Rossman is recognized as a species that occurs only on *Alnus* in Canada (British Columbia), France, Norway, and the United States (Washington). The following nomenclatural changes are proposed: ***Neonectria faginata*** comb. and stat. nov., ***Neonectria fuckeliana*** comb. nov., ***Neonectria hederae*** comb. nov., ***Neonectria major*** comb. and stat. nov., and ***Neonectria punicea*** comb. nov.

Key words: beech bark disease, birch bark disease, *Cylindrocarpon*, hardwood cankers, hardwood diseases, *Neonectria*.

Résumé : Les auteurs ont évalué les relations entre deux espèces de *Neonectria* associées au chancre de l'écorce du hêtre en Amérique du Nord, en comparant deux isolats de ceux-ci ainsi que d'autres espèces venant sur *Fagus,* du groupe *Neonectria coccinea* (Pers.:Fr.) Rossman & Samuels. Ils ont séquencé et analysé les régions génétiques du facteur de traduction de l'élongation 1-alpha (EF1-α), de la deuxième plus grande sous-unité de l'ARN polymérase II (RPB2), et de la β-tubuline. Les résultats indiquent que le champignon associé à la maladie de l'écorce du hêtre, connu jusqu'ici comme le *Neonectria coccinea* var. *faginata* Lohman et al. (≡ *Nectria coccinea* Pers.:Fr.) Fr. var. *faginata*) devrait faire l'objet d'une espèce distincte du *Neonectria coccinea,* et être nommée *Neonectria faginata*. Le *Neonectria faginata*, incluant son stade anamorphique, le *Cylindrocarpon faginatum* C. Booth, n'existe que sur les *Fagus* de l'Amérique du Nord. Une deuxième espèce associée à une maladie de l'écorce du hêtre en Amérique du Nord est le *Neonectria ditissima*(Tul. & C. Tul.) Samuels & Rossman, qu'on peut distinguer morphologiquement du *Neonectria faginata* sur la base de la dimension des ascospores, la dimension et la forme des conidies, et la pigmentation des colonies. Les données morphologiques et moléculaires indiquent que le *Neonectria ditisima* est l'ancien nom du *Neonectria galligena* Bres. De la même façon, le stade anamorphe du *Neonectria ditissima* est l'ancien nom du *Cylindrocarpon heteronoma* avec, comme synonyme, le *Cylindrocarpon willkommiii*. Le *Neonectria ditissima* vient sur une variété d'essences à bois francs de l'Amérique du Nord et de l'Europe. Le *Neonectria coccinea* vient seulement sur des *Fagus* d'Europe. On reconnaît le *Neonectria major* (Wollenw.) Castl. & Rossman comme une espèce qui vient seulement sur les *Alnus* au Canada (Colombie-Britannique), en France, en Norvège, et aux États-Unis (Washington). On propose donc les changements de nomenclature suivants : ***Neonectria faginata*** comb. et stat. nov., ***Neonectria fuckelina*** comb. nov., ***Neonectria hederae*** comb. nov., ***Neonectria major*** comb. et stat. nov., et ***Neonectria punicea*** comb. nov.

Mots clés : maladies de l'écorce du hêtre, maladies de l'écorce du bouleau, *Cylindrocarpon*, chancres des essences à bois francs, maladies des essences à bois francs, *Neonectria*.

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Introduction

Although known in Europe since the mid-1800's, the first outbreak of beech bark canker in North America occurred in Nova Scotia about 1920 (Ehrlich 1934). This report of the disease occurred at least three decades after the introduction from Europe of the associated scale insect (*Cryptococcus fagisuga* Lind.). By 1932 the disease had spread south to Maine and was identified as an ascomycete in the Hypocreales belonging to the genus *Nectria* (Fr.) Fr. (Ehrlich 1934), later named *Nectria coccinea* (Pers.:Fr.) Fr. var. *faginata* Lohman et al. (Lohman and Watson 1943) with a *Cylindrocarpon faginatum* C. Booth asexual state (Booth 1966). Spaulding et al. (1936) recognized that more than one species of *Nectria* was causing cankers of beech trees in North America following attack by scale insects. Cotter and Blanchard (1981) were able to distinguish these two species on *Fagus* L., known as *Nectria coccinea* var. *faginata* and *Nectria galligena* Bres., based on differences in ascospore sizes. Since its discovery in North America, the seriousness of beech bark disease has varied and it has continued to spread southward (Houston 1994*a*). In the last few years, it has also spread westward into Ohio and Michigan (O'Brien et al. 2001; MacKenzie and Iskra 2005).

Although for many years any hypocrealean species having superficial, uniloculate perithecia was placed in *Nectria*, this genus is now restricted to those species related to the type species, *Nectria cinnabarina* (Tode:Fr.) Fr., with *Tubercularia* Tode:Fr. anamorphs (Rossman 1989; Rossman et al. 1999). Species having *Nectria*-like perithecia are now divided between genera in two hypocrealean families, Bionectriaceae and Nectriaceae (Rossman et al. 1999). Species related to the fungi causing beech bark canker have recently been transferred to *Neonectria* Wollenw. (Nectriaceae, Hypocreales), all of which have asexual states in *Cylindrocarpon* Wollenw. (Brayford et al. 2004; Mantiri et al. 2001; Rossman et al. 1999). Within *Neonectria*, the two species associated with beech bark canker belong to the *Nectria coccinea* (Pers.:Fr.) Fr. group as defined by Booth (1959), specifically *Neonectria coccinea* (Pers.:Fr.) Rossman & Samuels var. *faginata* Lohman et al. and *Neonectria galligena* (Bres.) Rossman & Samuels (Rossman et al. 1999). The genus *Cylindrocarpon* was divided into four major groups by Booth (1966) and most of the asexual states of the '*Nectria coccinea*' group belong in group 1 of *Cylindrocarpon* for species that produce microconidia but lack mycelial chlamydospores. However, *Cylindrocarpon obtusiusculum* (Sacc.) U. Braun (= *Cylindrocarpon magnusianum* Wollenw, nom. superfl. fide Braun 1993), the anamorph of the type species of *Neonectria*, *Neonectria ramulariae* Wollenw., was placed in group 4 of *Cylindrocarpon* for species having chlamydospores but lacking microconidia (Booth 1966). *Cylindrocarpon cylindroides* Wollenw., the type species of *Cylindrocarpon*, is a member of group 1. The sexual state of *C. cylindroides* has been considered to be *Neonectria neomacrospora* (Booth & Samuels) Mantiri & Samuels [as *Nectria cucurbitula* Tode:Fr. var. *macrospora* (Wollenw.) Booth], in the '*Nectria coccinea*' group (Booth 1959, 1966; Mantiri et al. 2001).

Although two species of *Neonectria* are associated with beech bark canker in North America, most reports have continued to be attributed to *Neonectria coccinea* var. *faginata,* while *Neonectria galligena* has been reported on a variety of hardwood trees (Booth 1959; Sinclair and Lyon 2005). To distinguish and evaluate the relationship of the two species of *Neonectria* associated with beech bark canker in North America, isolates were obtained that represent these and additional species on *Fagus* related to *Neonectria coccinea* (Pers.:Fr.) Rossman & Samuels. Isolates representative of the type species of *Neonectria* and *Cylindrocarpon* and related taxa including *Nectria hederae* C. Booth, *Nectria punicea* Schmidt:Fr., *Neonectria coccinea, Neonectria coccinea* var. *faginata, Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman, *Neonectria galligena*, *Neonectria neomacrospora*, and *Cylindrocarpon* group 1 taxa (*C. album* (Sacc.) Wollenw., *C. candidum* (Link) Wollenw., *C. faginatum, C. heteronema* (Berk. & Broome) Wollenw., and *C. willkommii* (Lindau) Wollenw.) were also included in this study. Regions in the translation elongation factor 1-alpha (EF1-α), RNA polymerase II second largest subunit (RPB2), and β-tubulin genes were sequenced and analyzed. In addition type and additional specimens and cultures were examined to determine if species based on DNA sequence data could be distinguished by morphological characters.

Materials and methods

Isolation, maintenance, and deposition of cultures

Newly sequenced isolates (GenBank accession Nos. DQ789681–DQ789895) are listed in Table 1. Fresh specimens were obtained as air-dried collections. Isolates from these specimens were grown from single ascospores or conidia plated on Difco corn meal agar (CMA) supplemented with 0.2% dextrose and antibiotics (2 mg/mL each neomycin and streptomycin). Germinated spores were transferred to both Difco potato dextrose agar (PDA) and CMA plates for observation. All isolates were maintained on CMA slants at 4 °C. Living cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. The original specimens from which isolates were obtained were deposited in the US National Fungus Collections (BPI) as listed in Table 1. For living cultures obtained from repositories such as ATCC (American Type Culture Collection), CBS, and IMI (International Mycological Institute, now CABI), specimens made from dried cultures were deposited in BPI if the culture sporulated. Type or authentic isolates were included if available as noted in Table 1. Authentic isolates are those examined by the describing author but are not derived from the type specimen. If neither a type or authentic isolate was available, the isolate most similar to the type specimen in both origin and morphology was designated as representative of the taxon as noted in Table 1 and Figs. 1–3. Where available, type specimens were examined as cited in the text.

Morphological characterization of taxa

For microscopic examination, material was rehydrated and mounted in water or 3% KOH. Observations of microscopic features were made using a Zeiss Axioplan 2 microscope with bright-field and Nomarski illumination. Images were captured with a Nikon DXM1200F digital camera (Nikon, Tokyo), and microscopic features were measured

Table 1. Isolates and specimens examined in this study.

Species	Source	Host	Locality	Specimen	Source of specimen or culture
Cylindrocarpon cylindroides	CBS 189.61	*Abies alba*	France	BPI 871040 (dried culture)	W. Gerlach, W. Gams
C. cylindroides	CBS 198.62	*Abies concolor*	N/A	None	J.A. von Arx, W. Gerlach
C. cylindroides, representative isolate	CBS 324.61	*Abies concolor*	Netherlands	None	W. Gerlach
Neonectria coccinea	CBS 118914	*Fagus sylvatica*	France	BPI 870938	A. Kunca
Neonectria coccinea	CBS 118915	*F. sylvatica*	Romania	BPI 870937	A. Kunca
Neonectria coccinea	CBS 118916	*F. sylvatica*	Romania	BPI 870939	A. Kunca
Neonectria coccinea	CBS 119150	*F. sylvatica*	Slovakia	BPI 870941	A. Kunca
Neonectria coccinea	CBS 119156	*F. sylvatica*	Slovakia	BPI 870940	A. Kunca
Neonectria coccinea	CBS 119157	*Fagus* sp.	Germany	BPI 748289	G.J. Samuels
Neonectria coccinea, representative isolate	CBS 119158	*Fagus* sp.	Germany	BPI 748295	G.J. Samuels
Neonectria coccinea	CBS 119159	*Fagus* sp.	Germany	BPI 748299	G.J. Samuels
Neonectria coccinea	CBS 291.81	*F. sylvatica*	Austria	BPI 871036 (dried culture)	W. Gams
Neonectria coccinea	CBS 394.80	*F. sylvatica*	Netherlands	BPI 871061 (dried culture)	H.A. van der Aa
Neonectria ditissima	CBS 100316	*Malus domestica*	Ireland	BPI 871042 (dried culture)	A. McCracken, S. Langrell
Neonectria ditissima	CBS 100319	*M. domestica*	Canada	None	G. Braun, S. Langrell
Neonectria ditissima	CBS 100320	*Populus grandidentata*	Canada	BPI 871039 (dried culture)	G. Braun, S. Langrell
Neonectria ditissima	CBS 100325	*M. domestica*	United Kingdom	None	S. Langrell
Neonectria ditissima	CBS 118919	*Liriodendron tulipifera*	Tennessee, USA	BPI 864075	G.J. Samuels
Neonectria ditissima	CBS 118921	*Fagus grandifolia*	West Virginia, USA	BPI 870947	M. MacKenzie
Neonectria ditissima	CBS 118920	*F. sylvatica*	Slovakia	BPI 870951	A. Kunca
Neonectria ditissima	CBS 118922	*F. grandifolia*	West Virginia, USA	BPI 870948	M. MacKenzie
Neonectria ditissima	CBS 118923	*F. grandifolia*	Michigan, USA	BPI 870950	J. O'Brien
Neonectria ditissima	CBS 118924	*F. grandifolia*	West Virginia, USA	BPI 870946	M. MacKenzie
Neonectria ditissima	CBS 118925	*F. sylvatica*	France	None	A. Kunca
Neonectria ditissima	CBS 118926	*Betula nigra*	Connecticut, USA	BPI 871033	G.J. Samuels
Neonectria ditissima	CBS 118927	*Acer* sp.	Virginia, USA	BPI 1112879	G.J. Samuels
Neonectria ditissima	CBS 118928	*F. sylvatica*	Slovakia	None	A. Kunca
Neonectria ditissima	CBS 119230	*F. grandifolia*	West Virginia, USA	BPI 870949	M. MacKenzie
Neonectria ditissima	CBS 119151	*Betula lenta*	Connecticut, USA	BPI 871032	R. Marra
Neonectria ditissima	CBS 119152	*B. lenta*	Connecticut, USA	BPI 871031	R. Marra
Neonectria ditissima, authentic isolate of anamorph, *C. willkommii*	CBS 226.31	*F. sylvatica*	Germany	None	H.W. Wollenweber
Neonectria ditissima	CBS 227.31	*Betula* sp.	Norway	None	H.W. Wollenweber
Neonectria ditissima	CBS 379.50	*Quercus borealis*	Connecticut, USA	None	
Neonectria ditissima, representative isolate of *Neonectria galligena*	CBS 835.97	*Salix cinerea*	Belgium	BPI 871047 (dried culture)	W. Gams, H.-J. Schroers
Neonectria ditissima	GJS 94-12	*Populus tremuloides*	Canada	BPI 749320	G.J. Samuels

Table 1 (*continued*).

Species	Source	Host	Locality	Specimen	Source of specimen or culture
Neonectria faginata	CBS 118917	*F. grandifolia*	West Virginia, USA	BPI 870943	M. MacKenzie
Neonectria faginata	CBS 118918	*F. grandifolia*	Michigan, USA	BPI 870945	J. O'Brien
Neonectria faginata	CBS 118938	*F. grandifolia*	Pennsylvania, USA	BPI 870942	M. MacKenzie
Neonectria faginata	CBS 118983	*Fagus* sp.	West Virginia, USA	None	E. Mahoney, M. Milgroom
Neonectria faginata	CBS 119153	*Fagus* sp.	New York, USA	None	E. Mahoney, M. Milgroom
Neonectria faginata	CBS 119154	*Fagus sp.*	New Hampshire, USA	None	E. Mahoney, M. Milgroom
Neonectria faginata	CBS 119155	*Fagus* sp.	Maine, USA	None	E. Mahoney, M. Milgroom
Neonectria faginata	CBS 119160	*F. grandifolia*	Tennessee, USA	BPI 864079	G.J. Samuels
Neonectria faginata	CBS 119231	*F. grandifolia*	Pennsylvania, USA	BPI 870944	M. MacKenzie
Neonectria faginata, type isolate of *Cylindrocarpon faginatum*	CBS 217.67	*F. grandifolia*	New Brunswick, Canada	None	G.L. Stone
Neonectria fuckeliana	CBS 119200	*Picea abies*	Austria	BPI 871034	W. Jaklitsch
Neonectria fuckeliana, representative isolate	CBS 239.29	*Picea sitchensis*	United Kingdom	None	H.W. Wollenweber
Neonectria hederae	CBS 714.97	*Hedera helix*	Netherlands	BPI 871043 (dried culture)	J.W. Veenbaas-Rijks
Neonectria hederae, type isolate	IMI 058770a	*H. helix*	United Kingdom	BPI 871044 (dried culture)	C. Booth
Neonectria major	CBS 118981	*?Alnus* sp.	France	None	A. Kunca
Neonectria major	CBS 118982	*Alnus rubra*	Washington, USA	BPI 870952	C. Cootsona
Neonectria major	CBS 119229	*Alnus* sp.	France	None	A. Kunca
Neonectria major, type isolate	CBS 240.29	*Alnus incana*	Norway	None	H.W. Wollenweber
Neonectria neomacrospora, representative isolate	CBS 118984	*Abies balsamea*	Canada	None	G.J. Samuels
Neonectria neomacrospora	CBS 118985	*Tsuga heterophylla*	Canada	BPI 744533	G.J. Samuels
Neonectria punicea	CBS 119724	*Frangula alnus*	Austria	BPI 871063	W. Jaklitsch
Neonectria punicea, representative isolate	CBS 242.29	*Rhamnus* sp.	Germany	None	H.W. Wollenweber
Neonectria ramulariae as *C. obtusiusculum* (= *C. magnusianum* sensu Wollenw.)	ATCC 16237	Soil	Germany	None	W. Gams
Neonectria ramulariae as *C. obtusiusculum,* authentic isolate	CBS 151.29	*Malus sylvestris*	United Kingdom	None	H.W. Wollenweber
Neonectria ramulariae as *C. obtusiusculum*	CBS 182.36	*M. sylvestris*	Not available	None	H.W. Wollenweber
Neonectria sp. 1	CBS 119525	*Fagus* sp.	Slovakia	None	A. Kunca
Neonectria sp. 1	CBS 119532	*F. sylvatica*	Slovakia	None	A. Kunca
Neonectria sp. 1	CBS 119533	*F. sylvatica*	Slovakia	None	A. Kunca
Neonectria sp. 1	GJS 98–133	Decorticated hardwood	France	BPI 748311	G.J. Samuels
Neonectria sp. 2	CBS 119530	*Acer macrophyllum*	Scotland	BPI 802647	G.J. Samuels
Neonectria sp. 2	CBS 119531	*F. sylvatica*	Slovakia	None	A. Kunca
Neonectria sp. 3	CBS 119529	*F. sylvatica*	Switzerland	BPI 1107108	G.J. Samuels

Table 1 (*concluded*).

Species	Source	Host	Locality	Specimen	Source of specimen or culture
Neonectria sp. 3	CBS 125.24	Not available	Germany	BPI 871038 (dried culture)	H.W. Wollenweber
Neonectria sp. 3	CBS 208.30	*Ulmus* sp.	Germany	BPI 871035 (dried culture)	H.W. Wollenweber
Neonectria sp. 4	CBS 730.87	*Hypocrea pachybasioides* on decayed trunk of *P. abies*	Germany	BPI 871037 (dried culture)	W. Gams
Neonectria sp. 5	CBS 119528	*A. saccharinum*	New York, USA	BPI 802504	G.J. Samuels
Neonectria sp. 6	CBS 119527	*Rhamnus alpina* subsp. *fallax*	Austria	BPI 871062	W. Jaklitsch

using Scion Image software (National Institutes of Health, Bethesda, Maryland).

Growth trials were performed to determine the growth characteristics on PDA and synthetic low-nutrient agar (SNA, Nirenberg 1976). For each isolate two plates of PDA for cultural characterization and one plate of SNA with sterilized filter paper (Whatman Int., Maidstone, Kent, UK) placed on the agar surface to stimulate sporulation were inoculated. Cultures were placed in an incubator with a 12 h cycle between blacklight (near UV) and cool white fluorescent light at 23 °C for 7, 14, and 21 d. After 14 d, the mycelial radius was measured, and the colony colors from above and reverse of the plates were recorded along with the mycelial characteristics. Colors are based on Raynor (1970).

Nucleic acid extraction and PCR amplification

Mycelia for DNA extractions were grown on PDA plates, scraped from the plates with a sterile scalpel and placed into a microcentrifuge tube containing Lysing Matrix A (Qbiogene, Irvine, California) and lysed in a FastPrep Instrument (Qbiogene, Irvine, California). DNA was extracted with the PureGene DNA Extraction Kit (Gentra Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. Individual genes were amplified in a 50 μL reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California) or I-Cycler (Bio-Rad, Hercules, California) using the following primers: EF1728F (Carbone and Kohn 1999) and EF1-1567R (Rehner 2001); RPB2-5F and RPB2-7CR (Liu et al. 1998); BTUB-T1 and BTUB-T2 or BTUB-T22 (O'Donnell and Cigelnik 1997).

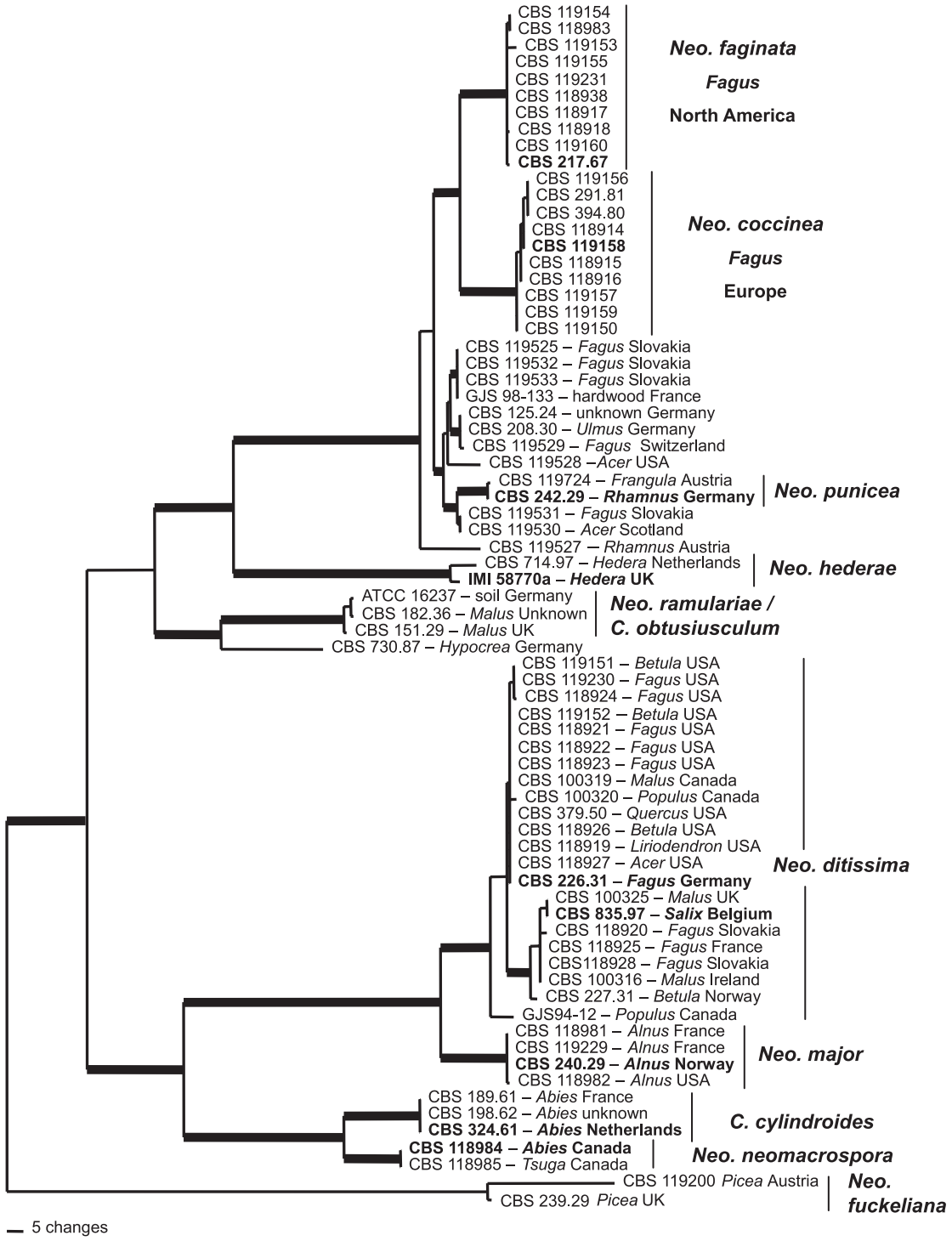
Standard reaction conditions consisting of 10–15 ng of genomic DNA, 200 μmol/L each dNTP, 2.5 units Amplitaq Gold (Applied Biosystems, Foster City, California), 25 pmol of each primer and the supplied 10× PCR buffer with 15 mmol/L $MgCl_2$ were used. The thermal cycler program was as follows: 10 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, with a final extension period of 10 min at 72 °C. For difficult to amplify isolates using the EF1-α primers, the following parameters were used: (1) 10 min at 95 °C; (2) 35 s denaturation at 94 °C, 55 s annealing at 66 °C, 1.5 min extension at 72 °C for 9 cycles decreasing the annealing temperature by 1 °C each cycle; (3) 35 s at 94 °C, 55 s at 56 °C, 1.5 min at 72 °C for 35 cycles; and (4) final extension 10 min 72 °C. PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio) according to the manufacturer's instructions. Amplified products were sequenced with the BigDye version 3.1 dye terminator kit (Applied Biosystems) on an ABI 3100 automated DNA sequencer. Forward PCR primers were used as forward sequencing primers for all the genes. For β-tubulin the region between the T1 and T2 primers (O'Donnell and Cigelnik 1997) was sequenced with the T2 primer used as the reverse sequencing primer. For RPB2 the entire gene region amplified was sequenced. For EF1-α, only the variable intron region between the EF1-728F and EF1-986R primers (Carbone and Kohn 1999) was sequenced for all isolates. Owing to a base pair change at the third position from the 3′ end of the EF1-986R primer sequence in these isolates, the EF1-986RN (5′-TACTTGAAGGAACCCTTGCC-3′) primer was designed as a reverse sequencing primer. The entire amplified region for EF1-α was sequenced only for selected representatives of each taxon. The following nested sequencing primers were designed for RPB2 and β-tubulin and used as necessary: RPB2intF (5′-AGTACGAGGTGTCGCTGGTC-3′); RPB2intR (5′-TGCCTCTGTTGATCATG-3′); BTUBintF (5′-GTCTACTTCAACGAG-GTTCGTG-3′); and BTUBintR (5′-CACGAACCTCGTTGAAGTAGAC-3′).

Sequence analysis

Raw sequences were edited using Sequencher version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, Michigan). Alignments were manually adjusted using GeneDoc 2.6.001 (http://www.psc.edu/biomed/genedoc/). The three genes were aligned individually and then concatenated into a single alignment. Each gene was analyzed separately through the use of data partitions and a combined three-gene analysis was performed for all available taxa. In addition, because the EF1-α gene region analyzed in this study consisted entirely of an intron that required the deletion of ambiguously aligned positions in the combined analysis of all taxa, separate analyses were performed using subgroups of the most closely related taxa with all available data included.

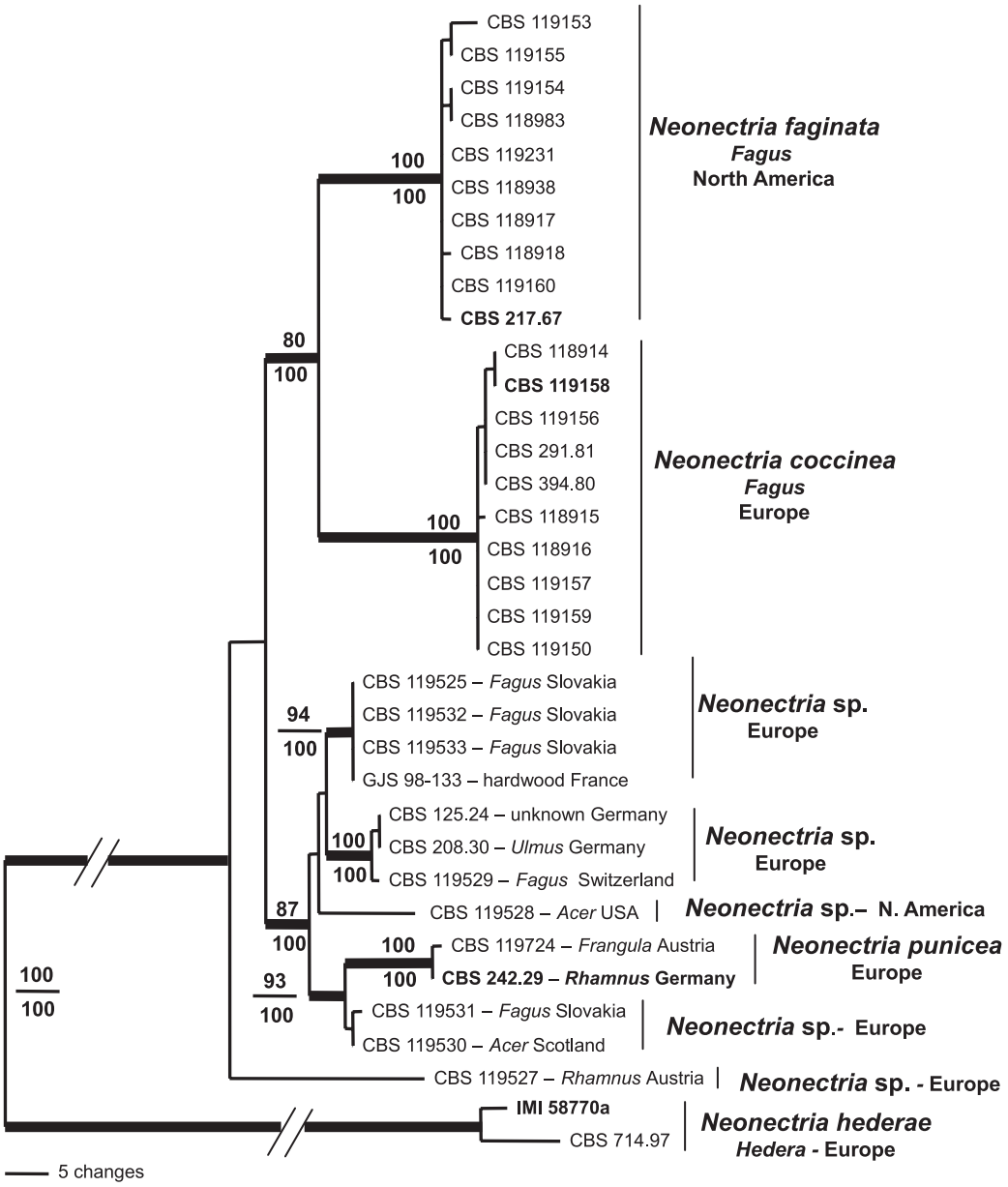
Trees were inferred by the neighbor-joining (NJ) method (Kimura 2-parameter distance calculation) and by maximum parsimony (MP) using the heuristic search option with 1000 random taxon additions and the branch swapping (tree bisection-reconnection) option of PAUP* 4.0b10 (Swofford

Fig. 1. One randomly chosen maximum parsimony phylogenetic tree of 178 equally parsimonious trees based on a combined analysis of EF1-α, β-tubulin, and RPB2 gene regions for all available taxa in the *Neonectria coccinea* group and *Cylindrocarpon* group 1 (length = 866, CI = 0.760, RI = 0.969, RC = 0.736). Thirty ambiguously aligned positions in the EF1-α region were excluded. Thickened branches indicate support of ≥70% for maximum parsimony bootstraps and ≥95% posterior probabilities from four pooled Bayesian analyses. Isolates in bold are type, authentic, or representative cultures for the taxa in the tree as listed in Table 1.



2002). For MP analyses, a limit of 10 trees per random addition sequence was enforced with a MAXTREE limit of 10 000. For both types of analyses, ambiguously aligned positions were excluded. All characters were unordered and given equal weight during the analysis. Gaps were treated as missing data in the MP and NJ analyses. Missing or ambiguous sites were ignored for affected pairwise comparisons. Relative support for branches was estimated with 1000 bootstrap replications (Felsenstein 1985) with MULTREES off and 10 random sequence additions per bootstrap

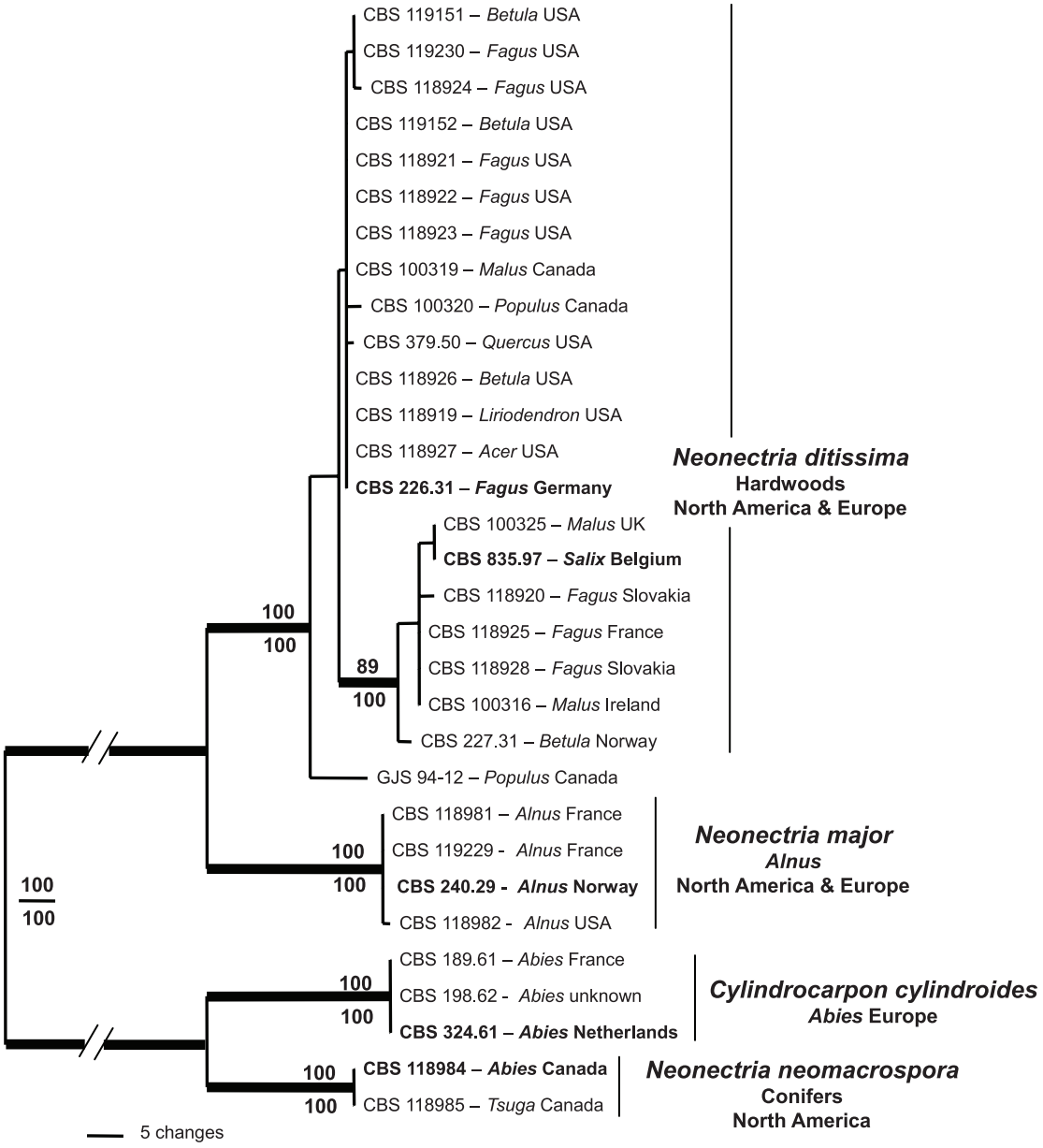
Fig. 2. One randomly chosen maximum parsimony phylogenetic tree of 6260 equally parsimonious trees based on a combined analysis of EF1-α, β-tubulin, and RPB2 gene regions for taxa in a subgroup containing *Neonectria coccinea* and *Neonectria faginata* (length = 251, CI = 0.924, RI = 0.970, RC = 0.897). All positions in the alignment were included. Thickened branches indicate support of ≥70% for maximum parsimony bootstraps and ≥95% posterior probabilities from four pooled Bayesian analyses. Maximum parsimony bootstrap supports are listed above the branches with Bayesian posterior probabilities below the branches. Isolates in bold are type, authentic, or representative cultures for the taxa in the tree as listed in Table 1.



replicate for the MP bootstrap analysis. The partition homogeneity test with 1000 replicates as implemented in PAUP* 4.0b10 was used to determine if significant incongruence among genes existed. Data were not partitioned by codon position for exon regions. In addition, a reciprocal 70% bootstrap criterion as in Reeb et al. (2004) was used to assess topological incongruence among the genes (Mason-Gamer and Kellogg 1996). Bootstraps were generated using a NJ bootstrap of 1000 replicates with likelihood model assumptions as determined in MrModeltest 2.2 as detailed below (Posada and Crandall 1998; Nylander 2004).

Phylogenetic trees were also inferred for using Bayesian inference as implemented in MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). Likelihood model assumptions were as determined with MrModeltest 2.2 (Posada and Crandall 1998; Nylander 2004) for each gene. A general time reversible (GTR) model was selected for all partitions, including a proportion of invariable sites parameter for the EF1-α gene region, a gamma distribution parameter for β-tubulin, and a gamma distribution parameter and a proportion of invariable sites for RPB2. Four independent analyses, each starting from a random tree, were run under the same conditions for the combined gene alignment with 2 000 000 generations to allow each run to reach stationarity with sampling every

Fig. 3. One of two equally parsimonious maximum parsimony phylogenetic trees based on a combined analysis of EF1-α, β-tubulin, and RPB2 gene regions for taxa in a subgroup containing *Neonectria ditissima* and *Neonectria neomacrospora* (length = 245, CI = 0.935, RI = 0.982, RC = 0.918). All positions in the alignment were included. Thickened branches indicate support of ≥70% for maximum parsimony bootstraps and ≥95% posterior probabilities from four pooled Bayesian analyses. maximum parsimony bootstrap supports are listed above the branches with Bayesian posterior probabilities below the branches. Isolates in bold are type, authentic, or representative cultures for the taxa in the tree as listed in Table 1.



100 generations. Four chains were run simultaneously with the "heat" set to 0.4. A burnin of 5000 (500 000 generations) trees was used.

Results

Molecular results

The combined alignment, excluding primer binding site regions, consisted of EF1-α (296 bp), β-tubulin (598 bp), and RPB2 (1110 bp) sequences for 70 isolates of *Neonectria* and two isolates of *Nectria fuckeliana* C. Booth as outgroups with 2004 total characters. Thirty-five ambiguously aligned positions (95–129) were excluded from the EF1-α gene region. Of the remaining 1969 characters, 481 were parsimony informative, 1413 were constant, and 75 were variable but not parsimony-informative. Although contained within a larger group of species encompassing the genus *Neonectria, Nectria fuckeliana* was determined to be the most appropriate outgroup taxon through a neighbor-joining analysis of partial β-tubulin sequences from species of *Neonectria* available in GenBank (tree not shown) as well as a midpointed rooted MP tree resulting from analysis of the combined alignment generated in this study. This analysis indicated that longest branch is that between *Nectria fuckeliana*

and a clade containing the rest of the isolates in this study. The partition homogeneity test as implemented in PAUP* 4.0b10 indicated no significant conflict among the data partitions (P = 0.12). No incongruence was detected among genes at the 70% bootstrap level for species-level clades.

MP phylogenetic analysis of the combined alignment resulted in 178 equally parsimonious trees (length = 866, consistency index (CI) = 0.760, retention index (RI) = 0.969, rescaled consistency index (RC) = 0.736) differing only in the arrangement of isolates with the terminal clades (trees not shown). Figure 1 shows one randomly chosen MP tree generated for the combined alignment. Thickened lines represent branches with MP bootstrap support of 70% or greater and posterior probabilities at 95% or greater. Combined analysis of the three gene regions distinguished nine previously described taxa (Fig. 1). These taxa could be divided into two subgroups (Figs. 2–3). *Cylindrocarpon obtusiusculum* (teleomorph *Neonectria ramulariae*) and an undescribed species closely related to *C. obtusiusculum* were not supported as more closely related to one subgroup than the other.

The first subgroup (Fig. 2), which consisted of eight species, of which four are named, included *Neonectria coccinea* and its anamorph *C. candidum* on *Fagus* from Austria, France, Germany, Netherlands, Slovakia, and Romania. Although no living material of the type specimen exists, the sequenced specimens agree morphologically with the description and type material (isolectotype BPI 738862) of *Neonectria coccinea* originally described from *Fagus*. None of the isolates from North American *Fagus* included in this study grouped within this species and we concluded that *Neonectria coccinea* does not occur in North America. The RPB2 gene region did not vary within this species, the EF1-α intron region varied at two base positions, and the β-tubulin region varied at one position. This represents 99.7% sequence identity for EF1-α and β-tubulin combined.

A second species within the first subgroup that is sister to *Neonectria coccinea* consisted of isolates exclusively from North American beech bark cankers, including the type strain of *Cylindrocarpon faginatum* from Canada and isolates derived from ascospores in ascomata associated with beech bark cankers in Maine, Michigan, New Hampshire, New York, Pennsylvania, Tennessee, and West Virginia. This species is known only on *Fagus grandifolia* Ehrh. from North America and is herein recognized as ***Neonectria faginata*** (Lohman et al.) Castl. & Rossman comb. and stat. nov. (Basionym: *Neonectria coccinea* (Pers.: Fr.) Samuels & Rossman var. *faginata* Lohman, Watson & Ayers, Lloydia 6: 100, 1943, holotype BPI 551558) having the asexual state *C. faginatum*. The RPB2 gene region did not vary within this species, the EF1-α intron region differed at four gapped positions, and the β-tubulin region differed at seven positions. This represents 98.8% sequence identity for EF1-α and β-tubulin combined including the gapped positions.

Also in this first subgroup are many isolates previously regarded as *Neonectria coccinea* or *C. candidum*, which are phylogenetically distinct from *Neonectria coccinea* sensu stricto and *Neonectria faginata*. These isolates appear to constitute at least six species that occur on *Fagus* and other hardwood hosts. This group also includes ***Neonectria hederae*** (C. Booth) Castl. & Rossman comb. nov. (Basionym: *Nectria hederae* C. Booth, Mycol. Pap. 73: 59, 1959), anamorph *Cylindrocarpon hederae* C. Booth, on *Hedera helix* L. (type strain IMI 058770a). Based on the results of the all-taxa combined alignment and analyses, a phylogenetic analysis of this subgroup using *Neonectria hederae* as the outgroup taxon provided increased support for distinction of these species (Fig. 2). Two additional species in this first group occur on *Frangula alnus* P. Mill. and species of *Rhamnus* L. in Europe. ***Neonectria punicea*** (Schmidt:Fr.) Castl. & Rossman comb. nov. (Basionym: *Sphaeria punicea* Schmidt in P. Kunze & Schmidt:Fr., Myk. Hefte 1: 61, 1817: System. Mycol. 2: 415, 1823; anamorph *C. album*) is represented by isolates CBS 242.29 and CBS 119724. An undescribed species of *Neonectria* is represented by a single isolate (CBS 119527). Three additional undescribed species occurring on *Fagus* in Europe and a single representative of an unidentified species on *Acer* L. from New York were also found within this subgroup of species. Increased sampling will be required to definitively determine morphological and molecular species limits of these undescribed species.

The second subgroup of species in this study includes the other *Neonectria* on *Fagus* in North America, here shown to be *Neonectria ditissima* (type host *Fagus,* isotype BPI 551714) but previously referred to as *Neonectria galligena* (type host *Salix* L., isotype BPI 552356), cause of a serious canker disease of apples and pears (Figs. 1, 3). This large and well-supported group includes isolates from *Fagus* as well as from *Acer, Betula* L., *Liriodendron* L., *Malus* P. Mill., *Populus* L., and *Quercus* L. in Europe and North America. These results indicate that *Neonectria galligena* is a synonym of *Neonectria ditissima.* The inclusion of an isolate from *Liriodendron* within this species indicates that *Nectria magnoliae* M.L. Lohman & Hepting (holotype BPI 552527) is also a synonym of *Neonectria ditissima*. Examination of the respective type materials referenced above also supports these conclusions. *Neonectria ditissima* isolates differed at 14 base positions and one gapped position in the EF1-α intron, 17 base positions and one gapped position in β-tubulin, and seven base positions in RPB2 (98% sequence identity across all genes).

Three additional species were distinguished in the second subgroup (Figs. 1, 3). The first is closely related to *Neonectria ditissima* and occurs on *Alnus* P. Mill. in France, Norway and western North America. This species was previously identified as *Nectria ditissima* Tul & C. Tul. var. *major* Wollenw. by Zeller (1935) and is used in the biocontrol of *Alnus rubra* Bong. in western North America (Dorworth 1995, Dorworth et al. 1996). It is recognized herein as ***Neonectria major*** (Wollenw.) Castl. & Rossman comb. and stat. nov. (Basionym: *Nectria ditissima* Tul. & C. Tul. var. *major* Wollenw., Angew. Bot. 8: 189, 1926). A second well-supported species in this subgroup is *Neonectria neomacrospora* on dwarf hemlock mistletoe-infected *Abies balsamea* (L.) P. Mill. and *Tsuga heterophylla* (Raf.) Sarg. from Canada. Although considered the anamorph of *Neonectria neomacrospora*, the third species is shown to be closely related but distinct as *C. cylindroides* on *Abies* P. Mill. in Europe.

Table 2. Morphological and cultural characteristics of *Neonectria coccinea, Neonectria ditissima, Neonectria faginata* and *Neonectria major* and their asexual states.

	Neonectria coccinea / *Cylindrocarpon candidum*	*Neonectria ditissima* / *Cylindrocarpon heteronema*	*Neonectria faginata* / *Cylindrocarpon faginatum*	*Neonectria major* / *Cylindrocarpon* sp.
Geographic distribution	Europe	Europe and North America	North America	France, Norway, and United States (Washington), probably panboreal
Host	*Fagus*	Hardwoods, including *Acer, Betula, Fagus, Liriodendron, Malus, Populus, Quercus,* and *Salix*	*Fagus*	*Alnus*
Ascomata	Ascomata forming superficially on thinly developed stroma, within or erumpent through cracks in bark, often aggregated in groups up to 30, rarely solitary, 200–350 µm diam., subglobose to globose, slightly roughened, red to dark red, KOH^+ purple, with small darkened ostiole, often laterally collapsed, collapsed cupulate, or not collapsed	Ascomata forming superficially on well-developed stroma, erumpent through bark, aggregated in groups up to 30, rarely solitary, 250–400 µm diam., subglobose to globose, smooth, shiny to slightly roughened, red to dark red, KOH^+ purple, with slightly darkened ostiolar region, usually not collapsed but occasionally laterally collapsed, rarely collapsed cupulate	Ascomata forming superficially on well-developed stroma, erumpent through bark, aggregated, few to a stroma, rarely in groups up to 60, 250–400 µm diam., subglobose to globose, smooth, rarely slightly roughened, red to dark red, KOH^+ purple, with slightly darkened ostiolar region, occasionally slightly pointed or shiny around ostiole, usually not collapsed but occasionally laterally collapsed	Ascomata forming superficially on a thinly developed stroma, erumpent through bark, solitary or in groups up to five, 200–300 µm diam., red to dark red, KOH^+ purple, shiny with distinct darkened, slightly papillate ostiolar region, not collapsed
Asci	Asci (71–) 76–93 (–98) µm × (7–) 8–10 (–11) µm, avg. 84 µm × 9.3 µm, narrowly clavate, stipitate, with an apical ring, eight-spored, with obliquely uniseriate ascospores that are occasionally obliquely biseriate near apex	Asci (77–) 88–116 (–130) µm × (11–) 12–17 (–20) µm, avg. 102 µm × 15 µm, narrowly clavate, stipitate, without apical ring, eight-spored, with obliquely biseriate ascospores except near base	Asci (78–) 79–105 (–128) µm × (7–) 9–12 (–14) µm, avg. 91 µm × 10 µm, cylindrical, stipitate, without apical ring, eight-spored, with uniseriate ascospores	Asci (101–) 108–123 (–143) × 12–16 µm, avg. 120 µm × 14 µm, narrowly clavate, stipitate, without apical ring, eight-spored, with obliquely biseriate ascospores except near base
Ascospores	Ascospores (10.5–) 12.4–14.4 × 5.1–6.5 (–8.5) µm (avg. 13.4 µm × 5.8 µm), ellipsoid to broadly ellipsoid with narrowly rounded ends, hyaline, 1-septate, not constricted, finely spinulose	Ascospores (12.2–) 14.9–18.9 (–24.3) × (5.5–) 6.5–8.3 (–10.2) µm (avg. 16.9 µm × 7.4 µm), ellipsoid to fusiform with narrowly rounded ends, hyaline, 1-septate, often slightly constricted at septum, smooth to very finely spinulose	Ascospores (9.6–) 10.4–12.0 (–13.3) × (4.4–) 5.2–6.4 (–6.5) µm, (avg. 11.0 µm × 5.5 µm), ellipsoid to broadly ellipsoid with broadly rounded apices, hyaline, 1-septate, often constricted at septum, surface covered with small, regularly scattered spines	Ascospores (16.9–) 18.0–20.4 (–25.0) × (5.4–) 7.6–8.2 (–8.6) µm (avg. 19.7 µm × 7.4 µm), fusiform with rounded ends, hyaline, one-septate, not constricted at septum, smooth, or very finely spinulose
Micro- and macroconidia	Microconidia 0–1-septate, cylindrical with rounded ends, straight, hyaline. Macroconidia primarily 5-septate (58.7–) 67.1–78.5 (–86.8) µm × (5.8–) 6.3–7.7 (–8.6) µm, avg. 72.8 µm × 8.0 µm, less commonly 3-septate, (36.3–) 40.4–54.8 µm × (4.7–) 5.3–6.5 (–6.7) µm, avg. 47.6 µm × 5.9 µm, and 4-septate (33.3–) 55.1–70.1 (–75.3) µm × (5.8–) 6.4–7.6 (–8.0) µm, avg. 62.6 µm × 7.0 µm, rarely 6-septate, cylindric with rounded ends, straight to slightly curved, hyaline	Microconidia (0–) 1-septate, cylindrical with rounded ends, straight, hyaline. Macroconidia (3–) 5–6 (–7)-septate, primarily 5–6-septate, 5-septate (48.8–) 58.7–74.9 (–86.3) µm × (4.9–) 6.0–7.8 (–9.3) µm, (avg. 66.8 µm × 6.9 µm), 6-septate (58.9–) 67.7–84.1 (–93.5) µm × (4.9–) 6.2–8.0 (–9.3) µm, (avg. 75.9 µm × 7.1 µm), cylindric with rounded ends, straight to slightly curved, hyaline	Microconidia primarily 0–1– (3-) septate, ellipsoid with rounded ends, hyaline. Macroconidia (4–) 5–6 (–8)-septate, 5-septate (62.6–) 82.0–100.4 (–104.2) µm × (5.8–) 6.3–7.9 (–9.2) µm (avg. 91.2 µm × 7.1 µm), 6-septate (73.4–) 91.7–110.1 (–120.3) µm × (5.1–) 6.4–8.2 (–9.6) µm, (avg. 100.9 µm × 7.3 µm), cylindrical with rounded ends, hyaline, often strongly curved, crooked or sigmoid, occasionally straight, hyaline	Microconidia rare, 1–3-septate, cylindrical, hyaline. Macroconidia (3–) 5–6 (–8)-septate, 5-septate conidia (71.3–) 82.0–99.0 (–106.7) µm × (3.8–) 4.7–5.9 (–6.3) µm, (avg. 90.5 µm × 5.3 µm); 6-septate conidia (80.0–) 87.7–103.5 (–105.0) µm × (5.1–) 5.2–6.6 (–7.8) µm, (avg. 96.1 µm × 5.9 µm, cylindrical with rounded ends, straight or very slightly curved, hyaline

Table 2 (*concluded*).

	Neonectria coccinea / *Cylindrocarpon candidum*	*Neonectria ditissima* / *Cylindrocarpon heteronema*	*Neonectria faginata* / *Cylindrocarpon faginatum*	*Neonectria major* / *Cylindrocarpon* sp.
Cultural characteristics	Cultures on PDA after 14 d 2.2–3.1 cm diam.; aerial mycelium even to slightly, irregularly or unevenly tufted, honey, buff to rosey buff; reverse amber, sienna to umber, umber near center of reverse; margin even; no pigment in media	Cultures on PDA after 14 d 4.2–7.4 cm diam.; aerial mycelium even to slightly, irregularly or unevenly tufted, white to pale buff; reverse rosy buff at margin, becoming cinnamon near center; margin even; no pigment in media	Colonies on PDA after 14 d 5.2–7.4 cm diam.; surface regularly tufted; aerial mycelium honey near margin becoming brick to cinnamon toward center with dark brick elements at center; reverse sienna at margin, mostly dark brick to sepia in outer regions; margin smooth; some cultures turning agar sienna	Cultures on PDA after 14 d 3.7–4.3 cm diam.; aerial mycelium low, even, regularly tufted, white to honey, reverse pale luteous to saffron, becoming saffron near center, margin even; no pigment in media

Morphological characteristics of taxa

The salient features of *Neonectria coccinea, Neonectria ditissima, Neonectria faginata*, and *Neonectria major* are presented in Table 2 and illustrated as Figs. 4–19. Of the two species of *Neonectria* that occur on *Fagus* in North America, *Neonectria faginata* was determined to be morphologically distinct from *Neonectria ditissima* based on ascomata, asci, ascospores, macroconidial size and shape, and colony morphology. Ascomata of *Neonectria faginata* are generally numerous and aggregated on a stroma within cracks of the bark while those of *Neonectria ditissima* are generally fewer in number on a stroma and often scattered on the substratum. Asci of *Neonectria faginata* are narrowly clavate with the ascospores arranged uniseriately while those of *Neonectria ditissima* are clavate, wider, and the ascospores are arranged biseriately near the apex. Ascospores of *Neonectria ditissima* are ellipsoid to fusiform and smooth to very finely spinulose (Figs. 8–9) while those of *Neonectria faginata* are ellipsoid to broadly ellipsoid and ornamented with regularly scattered warts (Figs. 12–14). In addition the ascospores of *Neonectria faginata* are considerably shorter (10.4–12.0 × 5.2–6.4 μm, avg. 11.0 × 5.5 μm) than those of *Neonectria ditissima* (14.9–18.9 × 6.5–8.3 μm, avg. 16.9 × 7.4 μm). Macroconidia of the asexual state of *Neonectria faginata*, *C. faginatum*, are very long, up to 120 μm, and often strongly curved (Fig. 15) while those of *Neonectria ditissima*, *C. heteronema*, are straight, rarely slightly curved, and generally shorter (Fig. 10) than those of *C. faginatum*. Cultures of *Neonectria faginata* become dark reddish brown after 14 d on PDA while cultures of *Neonectria ditissima* are yellow to pale orange, and generally less colorful than *Neonectria faginata*.

Neonectria coccinea is macroscopically distinct in having ascomata that are always slightly roughened and usually collapse upon drying. In addition, an apical ring is present in each ascus (Fig. 4). *Neonectria coccinea* has relatively small ascospores (12.4–14.4 μm × 5.1–6.5 μm, avg. 13.4 μm × 5.8 μm) with regularly scattered warts that are similar to those of *Neonectria faginata* (Figs. 5–6). Macroconidia of *Neonectria coccinea* are 3- to 5-septate (Fig. 7). Cultures of *Neonectria coccinea* are the slowest growing of the species studied. Finally, *Neonectria major* is known only on *Alnus* and is distinct in having smooth ascospores that are even longer than those of *Neonectria ditissima*. Ascospores of *Neonectria major* are 18.0–20.4 μm × 7.6–8.2 μm, avg. 19.7 μm × 7.4 μm (Figs. 16–17). Macroconidia of the anamorph of *Neonectria major* are very long similar to those of *Neonectria faginata* but tend to be straight rather than curved (Fig. 19). In addition, cultures of *Neonectria major* are white, pale luteous to honey, never becoming deeply pigmented. Cultures maintained for more than two years often did not sporulate and tended to produce less aerial mycelium and fewer pigments in culture.

Discussion

The genus *Neonectria* (Hypocreales, Nectriaceae) was described by Wollenweber (1917) based on *Neonectria ramulariae* but was essentially ignored until Rossman et al. (1999) recognized this genus for species segregated from *Nectria* having a specific ascomatal wall structure and *Cy-*

lindrocarpon asexual states. Similar to most members of the Nectriaceae, the ascomata are characterized by orange to bright- or dark-red, superficial, uniloculate perithecia that become darker, usually purple, in 3% KOH and yellow in 100% lactic acid. Few to numerous ascomata develop on more or less well-developed, pseudoparenchymatous stromata often in cracks or cankers in the bark of hardwood or conifer trees (Rossman et al. 1999; Brayford et al. 2004). Other members of this genus including *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels and related species are associated with plant roots and are commonly isolated from soil (Seifert et al. 2003; Halleen et al. 2004). The ascomatal wall of *Neonectria* is relatively thick, more than 50 μm, and consists of two or three regions. A ring may or may not be present in the ascal apex (Rossman et al. 1999). Most ascospores of species of *Neonectria* are one-septate, although a few species with multiseptate ascospores are known (Samuels and Brayford 1993).

Other members of the Nectriaceae appear superficially similar to *Neonectria* species on *Fagus*. The common species *Nectria cinnabarina* has dark red perithecia that turn purple in KOH similar to those of *Neonectria* but the perithecia of *Nectria cinnabarina* are warted and the anamorph is a *Tubercularia*, which forms coral-colored, cushion-shaped sporodochia. This species causes a disease of hardwood trees especially those in a weakened condition that is known as coral spot (Sinclair and Lyon 2005). *Cosmospora episphaeria* (Tode:Fr.) Rossman & Samuels is a hyperparasite on stromatic pyrenomycetes, which produces small, less than 250 μm diameter, smooth, gelatinous perithecia that collapse laterally. The ascospores are often yellow to pale brown and ornamented. The anamorph is a slow-growing *Fusarium*-like fungus (Rossman et al. 1999). Additional species of *Nectria*-like fungi on *Fagus* include members of the Bionectriaceae that have non-red, KOH-negative perithecia such as *Bionectria ochroleuca* (Schwein.) Schroers & Samuels, *Bionectria pityrodes* (Mont.) Schroers, and *Hydropisphaera peziza* (Tode: Fr.) Dumort (Rossman et al. 1999).

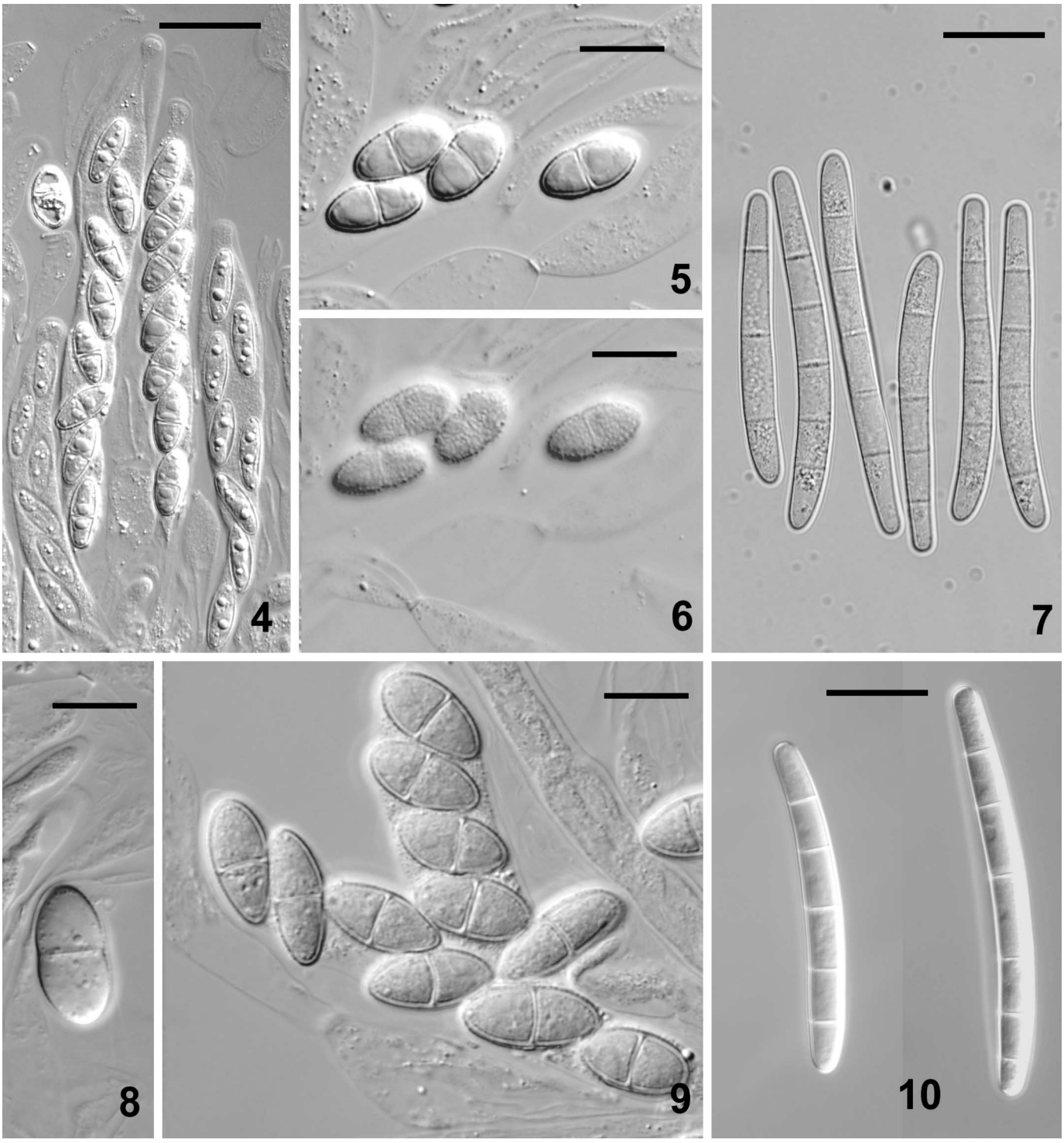
Species of *Neonectria* can be divided into three to five major groups as outlined by Booth (1959), Mantiri et al. (2001), Brayford et al. (2004), and Halleen et al. (2004). Species of *Neonectria/Cylindrocarpon* on *Fagus* and other hardwood and conifer trees studied herein belong to the '*Nectria coccinea*' group (Booth 1959), which is equivalent to *Neonectria* group I including *Neonectria galligena* and *Neonectria neomacrospora* according to Mantiri et al. (2001) and Brayford et al. (2004), and the group labeled '*Cylindrocarpon cylindroides* and other species' by Halleen et al. (2004). Although representatives of the other groups of *Neonectria* and *Cylindrocarpon* were not included, the data presented here place the type species of *Neonectria*, *Neonectria ramulariae,* as its asexual state, *C. obtusiusculum,* among this group of species.

Brayford et al. (2004) reported that *Nectria fuckeliana* did not group within *Neonectria* and was more closely related to the genera *Nectria* and *Calonectria* based on the comparison of mitochondrial small subunit rDNA sequences. A BLAST search of GenBank with the sequence of *Nectria fuckeliana* used in Brayford et al. (2004) reveals this sequence to be 98% identical to *Fusarium solani* (Mart.) Sacc., the asexual state of *Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg. Other studies (Seifert et al. 2003; Halleen et al. 2004) place *Nectria fuckeliana* within *Neonectria* although in varying positions. Although outside the taxa of interest in this study, *Nectria fuckeliana* belongs within the genus *Neonectria* as evidenced by its *Cylindrocarpon* anamorphic state and by analyses including representatives from the other groups of *Neonectria* not included in this study (Seifert et al. 2003; Halleen et al. 2004; this study, tree not shown) and is herein transferred to that genus as follows: ***Neonectria fuckeliana*** (C. Booth) Castl. & Rossman comb. nov. (Basionym: *Nectria fuckeliana* C. Booth, Mycol. Pap. 73: 56, 1959).

All anamorphs of *Neonectria* are species of *Cylindrocarpon*, a connection supported by DNA sequence analysis (Rehner and Samuels 1995, Schoch et al. 2000). The genus *Cylindrocarpon* was described by Wollenweber (1913) based on *C. cylindroides.* Booth (1966) and Mantiri et al. (2001) considered *C. cylindroides* to be the anamorph of *Neonectria neomacrospora* (as *Nectria fuckeliana* var. *macrospora*). Although closely related, our data do not support the conspecificity of *Neonectria neomacrospora,* a species described from conifers in western North America, with *C. cylindroides* described from *Abies* in Europe. Species of *Cylindrocarpon* are characterized by the production of slimy, 1- to multiseptate macroconidia that are straight to curved, cylindrical to fusiform with rounded ends on simple phialides, often borne on penicillately branched conidiophores in white to colored cultures. Some species also produce 0- to 1- or more septate microconidia and chlamydospores singly or in chains (Booth 1966). The only comprehensive account of *Cylindrocarpon* (Booth 1966) divides the genus into four groups based on the presence or absence of microconidia and mycelial chlamydospores. For the relatively few species studied, the three groups of *Neonectria* recognized by Mantiri et al. (2001) generally correlate with the first three groups of *Cylindrocarpon* recognized by Booth (1966). All of the taxa studied herein belong to *Cylindrocarpon* Group 1 having micro- and macroconidia but lacking chlamydospores except for *C. obtusiusculum* with chlamydospores and lacking microconidia (Booth 1966). Our data confirm that *Neonectria ramulariae/C. obtusiusculum,* type of the genus *Neonectria*, is allied with species of *Neonectria/Cylindrocarpon* in Booth's Group 1 including *C. cylindroides*, type of the genus *Cylindrocarpon*.

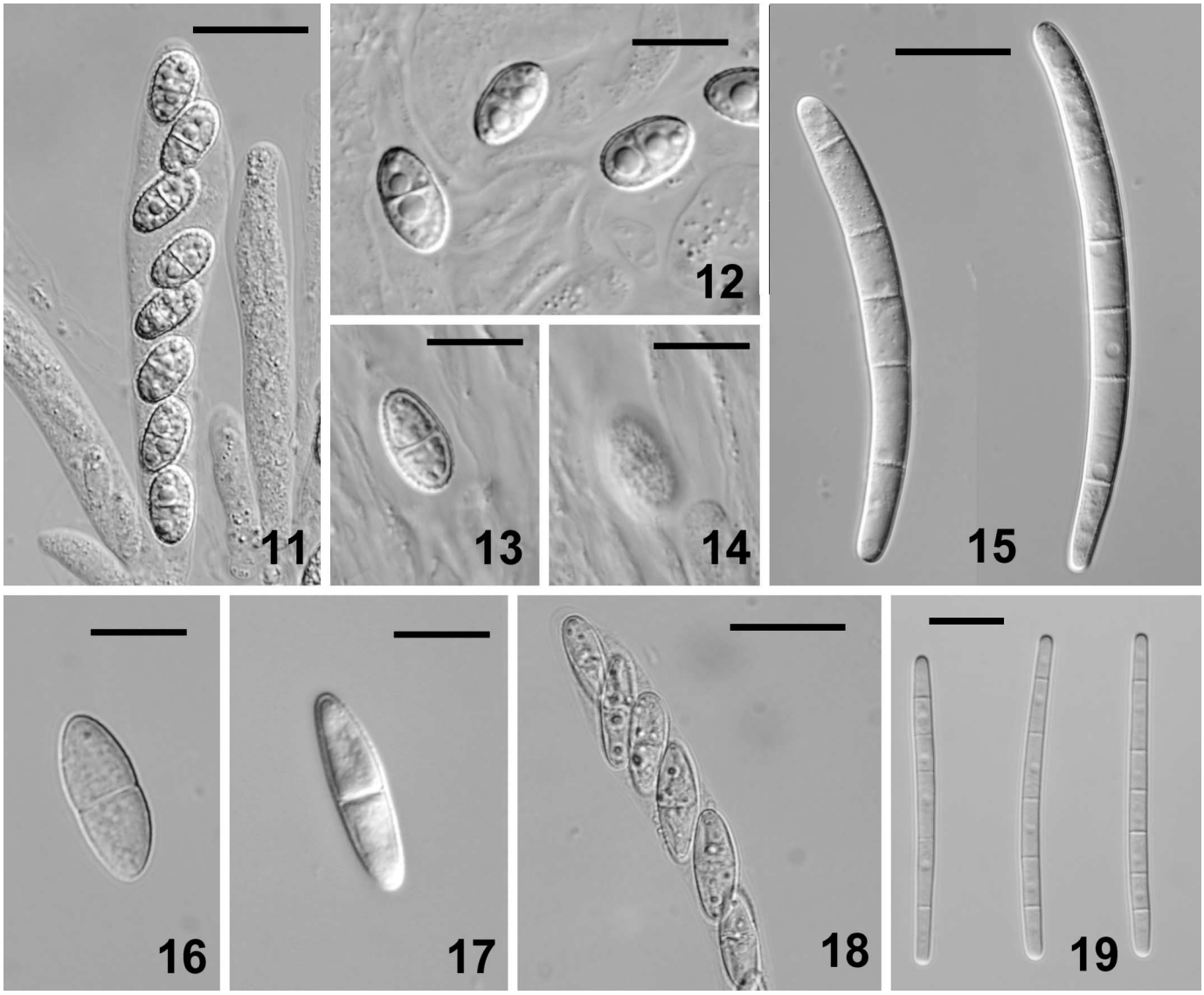
Two species of *Neonectria* are associated with beech canker in North America as initially reported by Spaulding et al. (1936) and later by Cotter and Blanchard (1981) and Houston (1994*a*, 1994*b*). A useful method for distinguishing them was reported by Cotter and Blanchard (1981) based on Lohman and Watson (1943) in which *Neonectria faginata* (as *Nectria coccinea* var. *faginata*) could be differentiated from *Neonectria ditissima* (as *N. galligena*) based on ascospore sizes. The differences in ascospore size as well as shape of the asci, arrangement of the ascomata, and differences in cultural characteristics are still relatively easy, nonmolecular ways to differentiate these taxa. We found that the differences in ascospore morphology that Cotter and Blanchard (1981) provided are still valid and useful for distinguishing these species as defined by DNA sequence data.

Figs. 4–10. *Neonectria coccinea* and *Neonectria ditissima*. Fig. 4. Asci of *Neonectria coccinea* (CBS 118916). Fig. 5. Ascospores of *Neonectria coccinea* in optical cross-section (CBS 118916). Fig. 6. Ascospores of *Neonectria coccinea* in surface view to illustrate ornamentation (CBS 118916). Fig. 7. Macroconidia of *Neonectria coccinea*, anam. *C. candidum* (CBS 291.81). Figs. 8–9. Ascospores of *Neonectria ditissima* (CBS 118920). Fig. 10. Macroconidia of *Neonectria ditissima*, anamorph *C. heteronema* (CBS 118923). Scale bars = 10 μm for Figs. 5–6, 8–9 and 20 μm for Figs. 4, 7, 10.



Cotter and Blanchard (1981) reported that the mean ascospore length of *Neonectria faginata* ranged from 10–13 μm while those of *Neonectria ditissima* ranged from 14–18.5 μm, which agrees with our results. Lohman and Watson (1943) reported a wider range of ascospore sizes for specimens on hosts other than beech and may have been examining specimens of *Neonectria ditissima* or a mixture of both species. The asexual states of *Neonectria faginata* and *Neonectria ditissima* are also clearly distinguished with the macroconidia of *Neonectria faginata* that are pri-

Figs. 11–19. *Neonectria faginata* and *Neonectria major*. Fig. 11. Asci of *Neonectria faginata* (CBS 119160). Figs. 12–13. Ascospores of *Neonectria faginata* in optical cross-section (Fig. 12, CBS 118917; Fig. 13, CBS 119231). Fig. 14. Ascospores of *Neonectria faginata* in surface view to illustrate ornamentation (CBS 119231). Fig. 15. Macroconidia of *Neonectria faginata*, anamorph *C. faginatum* (CBS 119160). Figs. 16–17. Ascospores of *Neonectria major* (CBS 118982). Fig. 18. Ascus of *Neonectria major* (CBS 118982). Fig. 19. Macroconidia of *Neonectria major*, anamorph *Cylindrocarpon* sp. (CBS 118982). Scale bars = 10 μm for Figs. 12–14, 16–17 and 20 μm for Figs. 11, 15, 18–19.



marily 5- to 6-septate, 82.0–110.1 μm long, cylindrical with rounded ends, often strongly curved, crooked or sigmoid while those of *Neonectria ditissima* are shorter, 58.7–84.1 μm, cylindric with rounded ends, straight to slightly curved. In addition, cultures on PDA after 14 d of *Neonectria faginata* often produce a distinctive dark-brick pigment while those of *Neonectria ditissima* tend to be paler, only honey- to cinnamon-colored.

Neonectria ditissima is known from both North America and Europe on a variety of hardwood trees. Beech canker in Europe was attributed initially to *Nectria ditissima* and this species was reported to cause cankers of yellow birch, tulip tree and other hardwoods in North America as early as 1897 (Galloway and Woods 1897; Spaulding et al. 1936; Lohman and Watson 1943). Considerable confusion has existed about the identity of *Neonectria ditissima, Neonectria coccinea* and *Neonectria galligena*. Weese (1911) considered *Neonectria ditissima* to be a synonym of *Neonectria coccinea.* Wollenweber (1913) identified *Neonectria galligena* as the cause of canker on apple in Massachusetts. Since that time, cankers on hardwoods in North America other than those caused by *Neonectria ditissima* on *Alnus* and *Neonectria faginata* have been attributed to *Neonectria galligena*. In Europe, the name *Neonectria galligena* has been applied to cankers on apples and pears and other hardwoods including *Fagus* (Langrell 2002) although Booth (1959) did not list any on *Fagus* among the specimens examined for *Neonectria galligena. Neonectria ditis-*

sima as defined herein continues to be reported on *Fagus* in Europe, distinct from *Neonectria coccinea* (Metzler et al. 2002).

Our morphological and molecular results including examination of their respective type specimens indicate that *Neonectria ditissima* and *Neonectria galligena* are synonyms. Reference cultures of these taxa isolated from the same hosts as the type specimens together with isolates from *Acer, Betula, Fagus, Liriodendron, Malus, Populus* and *Quercus* in North America and Europe form a single phylogenetic species based on the combined analysis of three gene regions (Figs. 1, 3). The type of the asexual state of *Neonectria galligena, C. heteronema,* was described from *Pyrus* in England while the anamorph of *Neonectria ditissima* is regarded as *C. willkommii*, described from *Fagus* in Germany (Booth 1959, 1966). The *Cylindrocarpon* states of *Neonectria ditissima* and *Neonectria galligena* agree with their type descriptions and the connections between the sexual and asexual states are well established. Considering *Neonectria ditissima* and *Neonectria galligena* as synonyms, their asexual states are synonyms as well. Thus, the correct name for the asexual state of *Neonectria ditissima* is *C. heteronema* because this name has priority.

Within *Neonectria ditissima*, there appear to be two populations, one of primarily North American isolates, although including a German isolate from *Fagus* authentic for *C. willkommii,* and an exclusively European population consisting of isolates from *Fagus, Malus,* and *Salix* corresponding to *C. heteronema,* with a Canadian *Populus* isolate intermediate between the two. Seven base substitutions in the EF1-α intron region separate the two groups with the Canadian *Populus* isolate sharing certain substitutions found in each group. RPB2 and β-tubulin do not support the separation of these groups. Results from Mahoney et al. (1999) suggested that there was greater genetic diversity among *Neonectria ditissima* (as *Neonectria galligena)* than *Neonectria faginata.* Plante et al. (2002) investigated the genetic variability of *Neonectria ditissima (*as *Neonectria galligena)* and *Neonectria faginata* (as *N. coccinea* var. *faginata*) with RAPD markers and using isolates representing an expanded host and geographic range for *Neonectria ditissima*. They determined that the genetic diversity of *Neonectria ditissima* on diverse hardwood hosts in North America was higher than that of *Neonectria faginata*. This agrees with the results presented here. Most studies have indicated that *Neonectria ditissima* (as *Neonectria galligena*) is likely native to North America due to the large amount of genetic variation present in North American isolates. However, without a similar comparison of the genetic variation of European populations, it is not possible to draw conclusions concerning the origin of *Neonectria ditissima.* At present it is not clear where *Neonectria ditissima* originated and additional sampling in Europe and North America will be required to determine if a geographic population structure exists in this species.

Both *Neonectria coccinea* and its asexual state *C. candidum* were originally described on *Fagus* in Europe, yet the concept of *Neonectria coccinea* has been extremely broad and confused. *Neonectria coccinea* has been reported on a diversity of hardwood trees as well as conifers throughout the world (Farr et al. 2005). Results from our sampling of *Neonectria coccinea sensu lato* suggest that this group includes at least five species occurring on *Fagus* and other hardwood hosts in Europe with one species on *Acer* in North America. Given that numerous, ill-defined species of *Neonectria* occur on Fagus in Europe, it is not surprising that opinions vary as to the identity of these taxa (Spaulding et al.1936). For many years the fungus causing beech bark disease in North America has been recognized as *Neonectria coccinea* var. *faginata*. Our data indicate that *Neonectria faginata* should be recognized as a distinct species from *Neonectria coccinea*. This has been the case for the asexual states of these species for some time with the anamorph of *Neonectria faginata* described as *C. faginatum* and the anamorph of *Neonectria coccinea* recognized as *C. candidum* (Booth 1966). At present *Neonectria faginata* is known only on *Fagus* in North America and *Neonectria coccinea sensu stricto* is known only on *Fagus* in Europe.

The hypothesis that *Neonectria faginata* was introduced from Europe, as was the scale insect preceding development of the disease, has been generally accepted. At present *Neonectria faginata* is known only from North America and has not been reported from Europe or elsewhere. Mahoney et al. (1999) used RFLPs of rDNA, mtDNA and nDNA to address the question of whether *Neonectria faginata* was native or introduced into North America. They compared isolates of *Neonectria faginata* (as *N. coccinea* var. *faginata*) to European isolates of *Neonectria coccinea*. Based on the lack of genetic diversity in *Neonectria faginata* compared with *Neonectria coccinea* in Europe and *Neonectria ditissima* in North America, Mahoney et al. (1999) concluded that *Neonectria faginata* was recently introduced from Europe. It is now clear that isolates referred to as *Neonectria coccinea* var. *coccinea* from Europe in Mahoney et al. (1999) included more than one taxon and the genetic diversity of *Neonectria coccinea* var. *coccinea* was probably overestimated. In this study, sequence divergence within each of the two narrowly defined species, *Neonectria coccinea* and *Neonectria faginata*, was roughly equivalent and less than found in *Neonectria ditissima*, which appears to be a broadly distributed and variable species.

Neonectria coccinea and *Neonectria faginata* are closely related sister species, suggesting that they are fairly recently diverged from one another. However, there is no evidence in our study that the genetic diversity in either species is significantly greater than in the other, or that *Neonectria faginata* has originated from within *Neonectria coccinea*. Additional sampling from non-*Fagus* hosts in North America will be required to determine how many taxa may occur in this group and their relationships to *Neonectria faginata* or *Neonectria coccinea*. Although not all species in this complex have been accurately defined, based on our sampling *Neonectria faginata* occurs only on *Fagus* in North America and *Neonectria coccinea* occurs only on *Fagus* in Europe. Forest pathologists should be wary of the possibility of inadvertently introducing the North American species, *Neonectria faginata,* into Europe, or the European species, *Neonectria coccinea,* into North America.

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