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FULL PAPER

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Neonectria amamiensis and *Cylindrocarpon amamiense*, a new nectrioid fungus and its sporodochial anamorph on *Pinus luchuensis* from Japan

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Abstract A new nectrioid fungus with its sporodochial *Cylindrocarpon* anamorph, collected on dead bark of Luchu pines (*Pinus luchuensis*) in the southern part of Japan (Kagoshima and Okinawa), having perithecia slightly constricted just below the papilla and conidia with a strongly hooked and acute apical cell, belongs to the genus *Neonectria* according to the recent concept of the Nectriaceae (Hypocreales). Molecular phylogenetic analysis based on the *tub2* region of β-tubulin genes also supports the morphological consideration. This fungus is described as *Neonectria amamiensis* (anamorph: *Cylindrocarpon amamiense*).

Key words Hypocreales · Nectriaceae · New species · Taxonomy

Introduction

During the survey of pitch canker of Luchu pine *Pinus luchuensis* Mayr, caused by *Fusarium circinatum* Nirenberg & O'Donnell in Amami Island (Kagoshima Prefecture) in 1985, a nectrioid fungus with reddish perithecia and pale yellow sporodochia of its *Cylindrocarpon* anamorph were found by the second author (T.K.) on the bark lesions of pitch canker. Thereafter, this fungus was repeatedly collected from some islands in Okinawa Prefecture (Kobayashi and Muramoto 1989). However, further study on taxonomy of this fungus had not been carried out because negative results on its pathogenicity were obtained in two replicate inoculation experiments using 3-year-old seedlings of *P. luchuensis*, compared with the positive results on *F. circinatum* (Kobayashi et al. 1988; Kobayashi and Muramoto 1989).

During the investigation of Japanese nectrioid fungi based on fresh materials, this fungus inhabiting Luchu pine, tentatively treated as *Nectria* sp. and its *Cylindrocarpon* anamorph (Kobayashi et al. 1988; Kobayashi and Muramoto 1989), was reexamined using many old and fresh materials. Based on the recent concept of the Nectriaceae (Brayford and Samuels 1993; Samuels and Brayford 1994; Rossman et al. 1999), this fungus was suggested to belong to the genus *Neonectria* Wollenw.

Phylogenetic analysis of *Neonectria* with *Cylindrocarpon* anamorphs has been carried out by several authors (Mantiri et al. 2001; Brayford et al. 2004; Halleen et al. 2004). Mantiri et al. (2001) and Brayford et al. (2004) analyzed mitochondrial small subunit ribosomal DNA (mSSU rDNA) sequence data and confirmed the teleomorph–anamorph relationship. In addition, Halleen et al. (2004) partially supported the relationship between *Cylindrocarpon* and *Neonectria* based on mitochondrial large subunit (mLSU) rDNA, β-tubulin genes, and nrDNA internal transcribed space (ITS) sequence data.

In the present study, we carried out taxonomic research of the present fungus based on morphological comparison with the hitherto known species of *Neonectria* and their anamorphs and molecular phylogenetic analysis, and describe it here as a new species of *Neonectria* with a new *Cylindrocarpon* anamorph.

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Table 1. Corresponding numbers of specimens, strains, and DDBJ accession numbers of their DNA sequences for the examined *Neonectria amamiensis* (teleomorph) and *Cylindrocarpon amamiense* (anamorph)

Collected as	Specimen no.	Strain no.	DDBJ no. of *tub2* DNA sequences
Teleomorphic and anamorphic forms: *Neonectria amamiensis* (anamorph: *Cylindrocarpon amamiense*)	TFM FPH-6571[a,b]	MAFF 239818[c] = FFPRI N27[d] (from ascospore) MAFF 239819 = FFPRI N28 (from ascospore)	AB237472
	TFM FPH-6558		
	TFM FPH-6573, 6574, 6576		
	TFM FPH-6648	MAFF 239820 = FFPRI N29 (from ascospore)	AB237473
	TFM FPH-6650, 6651, 6653, 6654, 6659		
	TUA TPP-h106[e]	TUA h106[f] (from ascospore)	AB237470
	TUA TPP-h109	TUA h109 (from ascospore)	AB237469
Anamorphic form: *Cylindrocarpon amamiense*	–	MAFF 239778 (from diseased tissue)	AB237471
	–	MAFF 239779 (from diseased tissue)	AB237483
	–	MAFF 239779 (from diseased tissue)	AB237482
	–	MAFF 239781 (from diseased tissue)	AB237481
	–	MAFF 239782 (from diseased tissue)	AB237480
	–	MAFF 239783 (from diseased tissue)	AB237479
	–	MAFF 239784 (from diseased tissue)	AB237478
	–	MAFF 239785 (from diseased tissue)	AB237477
	–	MAFF 239786 (from diseased tissue)	AB237476
	–	MAFF 239787 (fromdiseased tissue)	AB237475
	–	MAFF 239788 (from diseased tissue)	AB237474

[a] Holotype of *Neonectria amamiensis* (teleomorph) and *Cylindrocarpon amamiense* (anamorph)
[b] TFM FPH, Herbarium of Forest Mycology and Pathology, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan
[c] MAFF, MAFF Genebank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan
[d] FFPRI, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan
[e] TUA TPP, Herbarium of Laboratory of Tropical Plant Protection, Tokyo University of Agriculture, Tokyo, Japan
[f] TUA h, Y. Hirooka, Tokyo University of Agriculture, Tokyo, Japan

Materials and methods

Herbarium specimens and cultures

Herbarium specimens of the teleomorph and anamorph of the present nectrioid fungus and the isolates from an ascospore and/or a conidium kept in the Herbarium of Forest Mycology and Pathology (TFM) of the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Ibaraki, Japan, were reexamined. Additional collections were obtained from Kagoshima and Okinawa Prefectures in 2003 (Table 1). Teleomorph specimens newly collected were deposited at the herbarium of the Laboratory of Tropical Plant Protection, Tokyo University of Agriculture (TUA TPP), Tokyo, Japan, and cultures derived from ascospores and conidia were preserved at MAFF Genebank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan, and TUA (see Table 1). To obtain cultures from the teleomorph, an ascospore suspension in water taken from crushed perithecia was streaked on 2% (w/v) water agar (WA). After 24 h incubation at 25°C, a single germinating ascospore was selected and transferred directly to potato dextrose agar (PDA; Difco, Detroit, MI, USA) slants by a sterilized needle.

Morphological examination

Perithecia were picked up from bark samples with a fine needle. A drop of Shear's mounting fluid (Kirk et al. 2001) was applied directly to the perithecia for rehydration. From some dried materials, slides were prepared by hand-sectioning. The morphological characteristics were examined by light microscopy (Olympus BX50; Olympus, Tokyo, Japan). The color reaction test for the perithecia was carried out using 3% KOH and 100% lactic acids (LA) (Rossman et al. 1999). The isolate was grown on PDA in 9-cm plastic Petri dishes at 25°C in the dark to evaluate the growth rates, colony color, and odor. For observation of the *Cylindrocarpon* anamorph, cultures were grown on synthetic low-nutrient agar (SNA; Nirenberg and O'Donnell 1998). SNA plates were incubated for 2 weeks at 25°C in complete darkness and for 2 weeks at 20°C in either complete darkness or under continuous black light (BLB; Nirenberg 1990). In each case, 50 conidia randomly selected from individual isolates were measured. Color of the colony on PDA from the top and reverse views was described according to Kornerup and Wanscher (1978).

Phylogenetic analysis

Mycelia grown on PDA at 25°C were harvested after 1–2 weeks. Genomic DNA was extracted from lyophilized hyphae based on the method of O'Donnell et al. (1997) with some modification, or with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The *tub2* region of β-tubulin genes was amplified with the primer pairs T1 and T224 (O'Donnell and Cigelnik 1997). Polymerase chain reaction amplification of *tub2* genes was performed with the TaKaRa ExTaq system (TaKaRa Bio, Otsu, Japan), with a first denaturation for 2 min at 95°C followed by 40 cycles of incubation for 35 s at 94°C, 55 s at 52°C, and 2 min at 72°C. Sequencing was conducted with the ABI-Prism 377 DNA sequencing system (Applied Biosystems, Foster City, CA, USA) and DNA sequencing kit (Perkin-Elmer, Foster City, CA, USA) following the ABI protocol, by using the same primer pairs as described above.

Phylogenetic analysis used a segment of about 600 nucleotides including three introns and exons in *tub2* region (β-tubulin gene). The taxon matrix consisted of 23 *Neonectria*/*Cylindrocarpon* and 9 *Fusarium* and their teleomorphs in-group strains having similar hooked conidia or typical conidial morphology for the anamorphic genera (Tables 1, 2). The length differences were compensated in the alignments by gaps. The sequence alignment and homology analysis were carried out using AssemblyLIGNTM 1.0.9c (Accelrys, San Diego, CA, USA) and the CLUSTAL W package with Mac Vector 6.5.3 (Accelrys) (Thompson et al. 1994). The aligned sequences (TreeBASE SN2634) were analyzed by the neighbor-joining method (Saitou and Nei 1987), using PAUP 4.0b (Swofford 1998). The distance matrix was calculated using DNADIST with Kimura's two-parameter method (Kimura 1980), and the topology was tested with 1000 bootstrap trials.

Results

Taxonomy

Neonectria amamiensis Hirooka & Tak. Kobay., sp. nov. Figs. 1–5, 13a–c

Peritheciis supra stromatia in cortice emortuis formantibus, 5–20 aggregatis vel dispersis, obpyriformibus, 210–440 µm altis, 180–300 µm diametro, rubris, apice discoidalibus, 80–160 µm altis, 100–180 µm diametro; stromatibus, "textura epidermoidea" vel "textura angularis"; ascis octosporis, cylindricis vel leviter clavatis, 72–92 × 10–20 µm, apparatu apicali nullo; ascosporis mono-vel distichis, hyalinis, ellipsoideis, 16–20 × 5–8.5 µm, 1-septatis, verruculosis.

Holotypus: TFM FPH-6571, on dead bark of *Pinus luchuensis* Mayr (Japanese name: Ryukyu-matsu), Tatsugou-cho, Ohshima, Kagoshima Prefecture (Amami-Ohshima Island), March 17, 1986, T. Kobayashi.

Etymology: *amami* + *-ensis*; indicates the collection place of the type material.

Anamorph: ***Cylindrocarpon amamiense*** Hirooka & Tak. Kobay., anam. nov. Figs. 6–12, 13d–h

Sporodochiais in cortice emortuis, albis vel flavidis, 30 mm diametro erumpentibus, "textura intricata" vel "textura porrecta"; conidiophoris densis, simplicibus; macroconidiis cylindricis vel leviter fusiformibus, ad apicem argute aculeatis, ad basim truncatis vel obtusis, 3–7-septatis, eis 3-septatis 42.5–60 × 5–7.5 µm, eis 4-septatis 47.5–77.5 × 5–7.5 µm, eis 5-septatis 57.5–80 × 5–7.5 µm, eis 6-septatis 62.5–80 × 7.5–10 µm, eis 7-septatis 62.5–82.5 × 7.5–10 µm; microconidiis et chlamydosporis nullis.

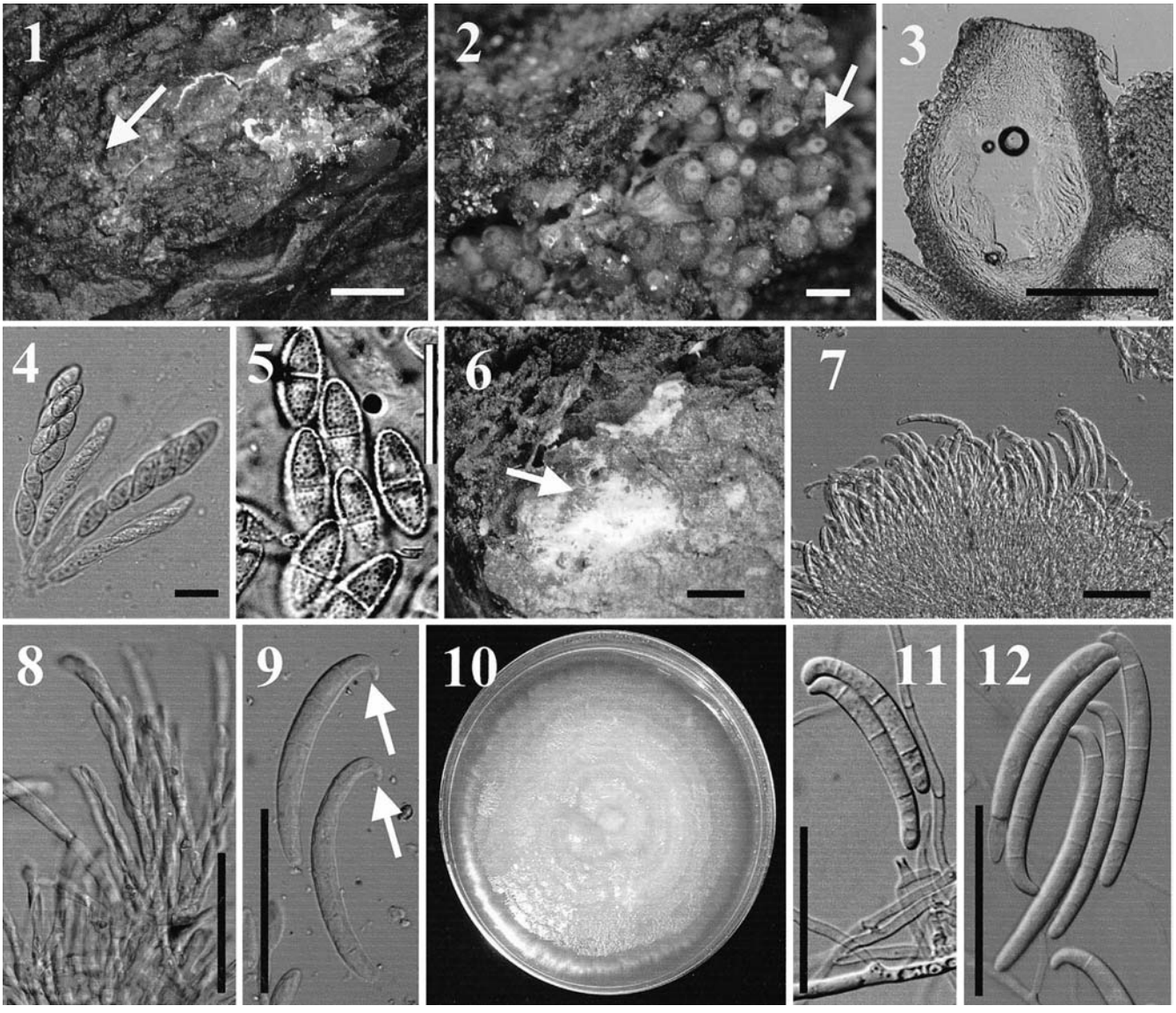
Holotypus: TFM FPH-6571, on dead bark of *Pinus luchuensis* Mayr (Japanese name: Ryukyu-matsu), Tatsugou-cho, Ohshima, Kagoshima Prefecture (Amami-Ohshima Island), March 17, 1986, T. Kobayashi.

Etymology: *amami* + *-ense*; indicates the collection place of the type material.

Stromata formed in the epidermal layer of outer bark of the host plant, erumpent through the epiderm, with structures of textura intricata to angularis. Perithecia solitary or gregarious in groups of 5–20, subglobose, pyriform to elongated flask-shaped, narrowly constricted just below the papillate protrusion, 210–440 µm in height and 180–300 µm in diameter, collapsing laterally when dry, yellowish-red to red with orange papillae, uniformly stained violet to dark red in 3% KOH and yellow in LA, smooth to scaly; papillary flat, with a ostiolar canal (100–180 µm diameter and 80–160 µm high). Perithecial base wall 30–50 µm thick, composed of two layers. Outer layer 22–35 µm or to 5 cells thick; cells angular to globose, usually 10–18 µm diameter. Inner layer 10–15 µm thick; cells oblong to fusiform, ca. 17 × 4 µm, becoming increasingly flattened and compressed toward the perithecial locule. Perithecial apex wall formed by fusiform to subcircular or angular cells, becoming progressively narrower toward the ostiolar canal and merging in the periphysis. Asci clavate, 72–92 × 10–20 µm, without special structure at the apex, contained 8 spores in two rows. Ascospores fusiform, equally 2-celled, 16–20 × 5–8.5 µm, hyaline to pale yellow, tuberculate.

Sporodochia produced on a slit of bark of the host, white to pale yellow, sticky, up to 30 mm diameter, composed of textura intricata to porrecta. Conidiophores densely packed, loosely branched. Conidiogenous cells monophialidic, 12.5–32.5 × 3.7–5 µm, cylindrical, with an indistinct collarette. Conidia on sporodochia cylindrical to slightly fusiform with a strongly hooked and acute apical cell and indistinct basal foot cell, usually broadest in the upper half to third, slightly curved, 3–7-septate, 42.5–60 × 5–7.5 µm (3-septate), 47.5–77.5 × 5–7.5 µm (4-septate), 57.5–80 × 5–7.5 µm (5-septate), 62.5–80 × 7.5–10 µm (6-septate), 62.5–82.5 × 7.5–10 µm (7-septate). Microconidia and chlamydospores absent.

Colonies on PDA 18–20 mm diameter after 7 days at 25°C, with less aerial mycelia, white, pale yellow to yellow; reverse white, grayish-orange, to yellow; odor slightly acidic; sclerotia absent. Sporulation on SNA in the dark or under BLB starting within 2 weeks in aerial mycelium or conidiophores arising directly from the agar surface,



Figs. 1–5. *Neonectria amamiensis* (TFM FPH-6571). **1** Clustered perithecia (*arrow*) on resinous bark of *Pinus luchuensis*. **2** Close-up of clustered and laterally collapsed perithecia (*arrow*). **3** Median section of perithecium. **4** Asci with eight ascospores arranged in irregularly biseriate rows. **5** Ascospores showing tuberculate surface

Figs. 6–9. *Cylindrocarpon amamiense* on bark of *P. luchuensis* (TFM FPH-6571). **6** White to creamy-yellow sporodochia (*arrow*) on bark of *P. luchuensis* partly with white aerial hyphae. **7** Cross section of sporodochium fruiting; many conidia on its surface. **8** Branched conidiophores and conidiogenous cells producing conidia. **9** Conidia with strongly hooked and acute apical cells (*arrows*)

Figs. 10–12. *Cylindrocarpon amamiense* (MAFF 239818). **10** Colony on potato dextrose agar (PDA) at 25°C for 1 month. **11** Conidiophores and conidiogenous cells. **12** Conidia with slightly hooked and round apical cells. *Bars* **1**, **6** 2 mm; **2, 3** 200 µm; **4, 5** 20 µm; **7–9, 11, 12** 50 µm

but not produced richly. Sporodochia not formed in culture. Conidia mostly formed singly on the tips of conidiophores. Aerial conidiophores unbranched at first, becoming loosely to densely branched, sometimes verticillate, often proliferating sympodially. Conidiogenous cells of scattered aerial conidiophores monophialidic, 16–44 × 3–5 µm, cylindrical, with broadly collared. Conidia on SNA cylindrical to slightly fusiform with a rounded but slightly hooked apical cell and rounded to truncate basal cell, usually broadest in their upper half to third, slightly curved, 3–6-septate, 50–57 × 5–7.5 µm (3-septate), 60–65 × 6.25–7.5 µm (4-septate), 62.5–80 × 7.5 µm (5-septate), 65–80 × 7.5 µm (6-septate). Microconidia and chlamydospores absent.

No mature perithecia were formed through preliminary pairing experiments by using ten selected strains in all possible combinations, performed on sterilized branches of poplar placed on PDA.

Additional specimens examined: teleomorph and anamorph, TFM FPH-6558, 6573, 6574, 6576, on *P. luchuensis*, Tatsugou-cho, Ohshima, Kagoshima Prefecture (Amami-Ohshima Island), March 17, 1986, T. Kobayashi;

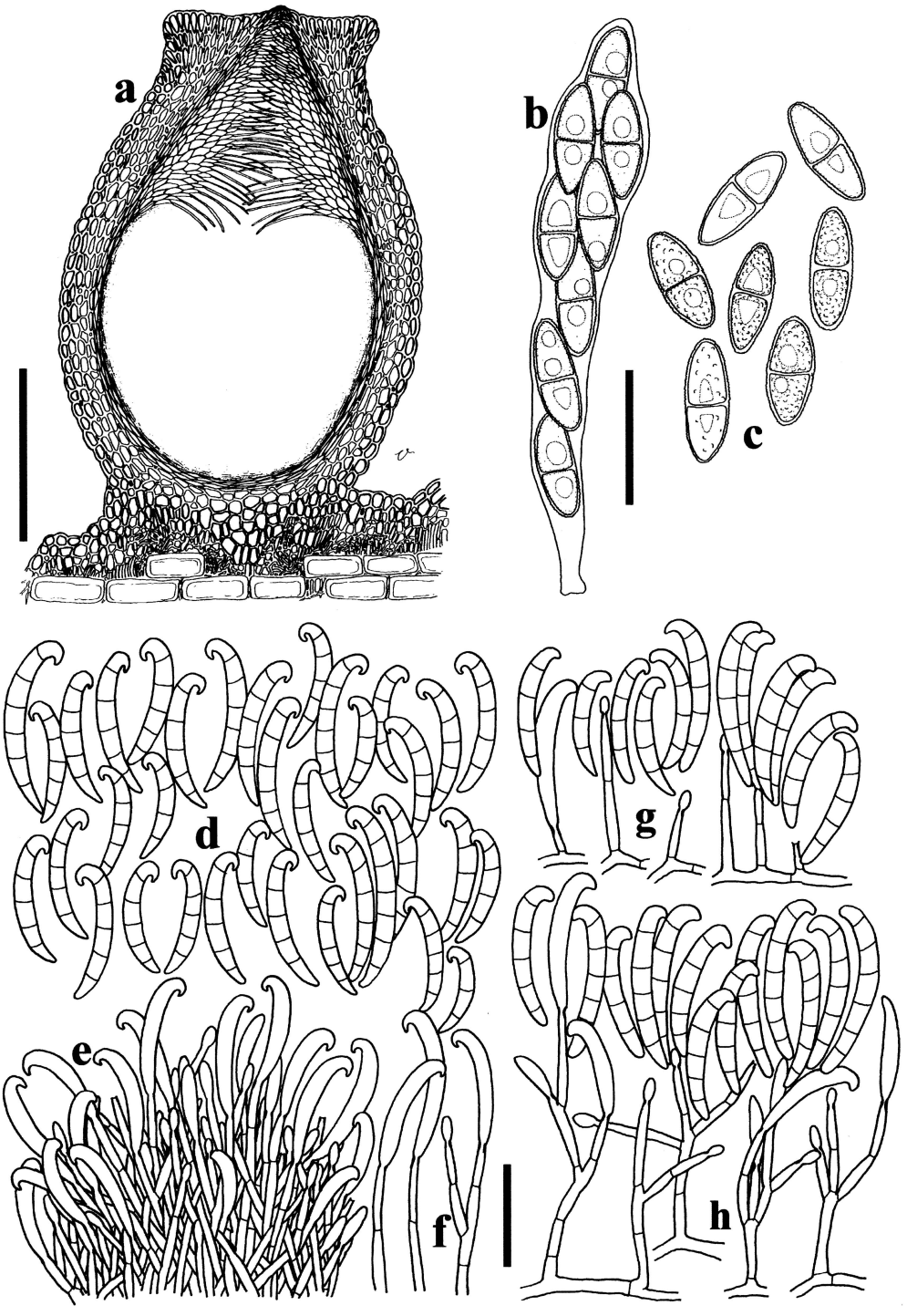


Fig. 13. *Neonectria amamiensis* (anamorph: *Cylindrocarpon amamiense*) on resinous bark of *Pinus luchuensis* (**a–f,** TFM FPH-6571) and synthetic low-nutrient agar (SNA) in the dark (**g, h,** MAFF 239818). **a** Median section of perithecium. **b** Ascus with eight ascospores arranged in irregularly biseriate rows. **c** Ascospores with or without tuberculate surface. **d** Conidia with a strongly hooked and acute apical cell. **e** A part of sporodochia composed of branched conidiophores, conidiogenous cells, and conidia. **f** Conidiogenous cells. **g** Conidia with slightly hooked and round apical cells on unbranched aerial conidiophores on SNA. **h** Conidia with slightly hooked and round apical cells on loosely branched aerial conidiophores on SNA. *Bars* **a** 100 μm; **b, c** 20 μm; **d–h** 50 μm

TFM FPH-6648, 6650, 6651, 6653, 6654, 6659, on *P. luchuensis*, Shimajiri, Okinawa Prefecture (Kume Island), April 1987, M. Gushiken; TUA TPP-h106, on *P. luchuensis*, Yomitan-son, Nakagami, Okinawa Prefecture (Okinawa Island), January 22, 2003, Y. Hirooka; TUA TPP-h109, on *P. luchuensis*, Onna-son, Kunigami, Okinawa Prefecture (Okinawa Island), January 22, 2003, Y. Hirooka.

Isolates examined: from ascospore, MAFF 239818 (= FFPRI N27), ex holotype (TFM FPH-6571); MAFF 239819 (= FFPRI N28), ex TFM FPH-6558; MAFF 239820 (= FFPRI N29), ex TFM FPH-6648; TUA h106, ex TUA TPP-h106; TUA h109, ex TUA TPP-h109. From diseased tissue of *P. luchuemsis,* MAFF 239778, Uken-son, Ohshima, Kagoshima Prefecture (Amami-Ohshima Island), November 13, 2003, T. Aoki; MAFF 239779, MAFF 239780, Miyazato, Nago, Okinawa Prefecture (Okinawa Island), December 8, 2003, T. Aoki; MAFF 239781, MAFF 239782, Hedo, Kunigami-son, Kunigami, Okinawa Prefecture

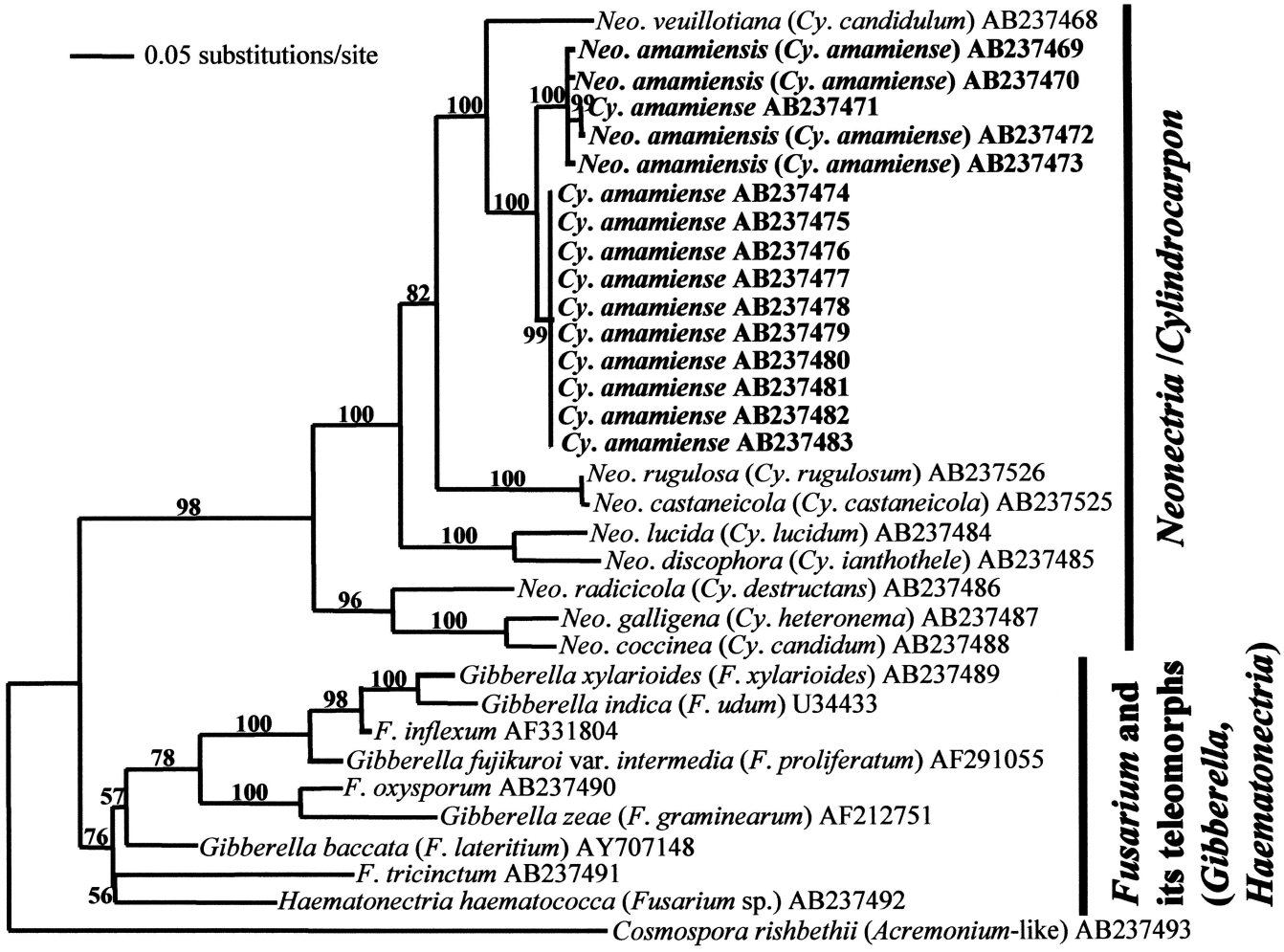


Fig. 14. Phylogenetic tree for *Neonectria amamiensis* (anamorph: *Cylindrocarpon amamiense*) by neighbor-joining analysis of the *tub2* (exons and introns) sequences rooted with *Cosmospora rishbethii* as an outgroup. Anamorphs are shown in *parentheses* followed by the retrieved DNA sequence numbers. *Numbers above nodes* represent bootstrap values from 1000 replications. Note the taxon matrix consisted of two groups: *Neonectria* species with *Cylindrocarpon* anamorphs and *Fusarium* species or nectrioid species with *Fusarium* anamorphs

(Okinawa Island), December 9, 2003, T. Aoki; MAFF 239783, MAFF 239784, Kunigami-son, Kunigami, Okinawa Prefecture (Okinawa Island), December 9, 2003, T. Aoki; MAFF 239785, MAFF 239786, Aha, Kunigami-son, Kunigami, Okinawa Prefecture (Okinawa Island), December 9, 2003, T. Aoki; MAFF 239787, Taira, Higashi-son, Kunigami, Okinawa Prefecture (Okinawa Island), December 9, 2003, T. Aoki; MAFF 239788, Moromizato, Okinawa, Okinawa Prefecture (Okinawa Island), December 11, 2003, T. Aoki.

Molecular phylogenetic analysis

Neonectria (*Neo.*) *amamiensis* (*Cylindrocarpon amamiense*) were nested in the clade of the genus *Neonectria* with *Cylindrocarpon* anamorphs with the support of high bootstrap values (Fig. 14). In contrast, *Neo. amamiensis* was not grouped with *Fusarium inflexum* R. Schneid., *F. udum* (Berk.) Wollenw. (teleomorph: *Gibberella indica* B. Rai & R.S. Upadhyay), or *F. xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* R. Heim & Saccas).

Discussion

Based on the positive KOH and LA reaction, *Neo. amamiensis* is classified in the Nectriaceae (Hypocreales). According to the recent classification of the Nectriaceae (Rossman et al. 1999), the main criteria to distinguish the related genera are the morphology of perithecia and the anamorphic states.

Cylindrocarpon amamiense produces conidia with a strongly hooked and acute apical cell and an indistinct basal foot cell on the pine (Figs. 9, 13d–f), similar to *Fusarium*. However, conidia on SNA in the dark usually have slightly hooked but rounded apical cells and a rounded to trun-

Table 2. Additional nectrioid species compared in molecular analysis

State		GenBank no.	Strain no.[a]	Locality	Collector	Collection date
Teleomorph	Anamorph					
Neonectria veuillotiana	*Cylindrocarpon candidulum*	AB237468	TUA h224	Okutama-cho, Tokyo, Japan	Y. Hirooka	Nov. 2003
Neonectria castaneicola	*Cylindrocarpon castaneicola*	AB237525	MAFF 235731	Shinto-mura, Gunma Prefecture, Japan	T. Kobayashi	Aug. 1990
Neonectria rugulosa	*Cylindrocarpon rugulosum*	AB237526	TUA h32	Yokohama, Kanagawa Prefecture, Japan	Y. Hirooka	Aug. 2002
Neonectria lucida	*Cylindrocarpon lucidum*	AB237484	GJS 89-71	Guyana	G.J. Samuels	July 1989
Neonectria discophora	*Cylindrocarpon ianothele*	AB237485	TUA h174-1	Tosa-cho, Kochi Prefecture, Japan	Y. Hirooka	July 2003
Neonectria radicicola	*Cylindrocarpon destructans*	AB237486	TUA h163	Takao, Tokyo, Japan	S. Inaba	June 2003
Neonectria galligena	*Cylindrocarpon heteronemum*	AB237487	TUA h291	Akiyu-tyo, Sendai, Miyagi Prefecture, Japan	Y. Hirooka	Aug. 2004
Neonectria coccinea	*Cylindrocarpon candidum*	AB237488	TUA h176	Tosa-cho, Kochi Prefecture, Japan	Y. Hirooka	Aug. 2003
Cosmospora rishbethii	*Acremonium*-like	AB237493	TUA h76	Tamagusukuson, Okinawa Prefecture, Japan	Y. Hirooka	June 2003
Haematonectria haematococca	*Fusarium* sp.	AB237492	TUA h67	Shimoda, Shizuoka Prefecture, Japan	Y. Hirooka	Oct. 2002
Gibberella xylarioides	*Fusarium xylarioides*	AB237489	TUA h60	Utsunomiya, Tochigi Prefecture, Japan	Y. Hirooka	Sept. 2002
Unknown	*Fusarium oxysporum*	AB237490	TUA h533	Tateyama, Chiba Prefecture, Japan	S. Yebihara	June 2001
Unknown	*Fusarium tricinctum*	AB237491	TUA h419	Ashikaga, Tochigi Prefecture, Japan	T. Kobayashi	Dec. 2004
Gibberella fujikuroi var. *intermedia*	*Fusarium proliferatum*	AF291055	NRRL 31071	Utah, USA		
Gibberella baccata	*Fusarium lateritium*	AY707148	FRC L-112	Papua New Guinea		
Gibberella indica	*Fusarium udum*	U34433	NRRL 22949	Germany		
Gibberella zeae	*Fusarium graminearum*	AF212730	NRRL 29169	Kansas, USA		
Unknown	*Fusarium inflexum*	AF331804	FRC O-1244			

[a] TUA h, Y. Hirooka, Tokyo University of Agriculture, Tokyo, Japan; MAFF, MAFF Genebank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan; GJS, Gary J. Samuels, Systematic Botany and Mycology, USDA/ARS, USA; NRRL, Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USA; FRC, *Fusarium* Research Center, Pennsylvania State University, USA

cate basal cell (Figs. 11, 12, 13g,h). In addition, the conidiogenous cells are typically broadly collared (Figs. 11, 13g). These features supported the conclusion that the anamorph of the present nectrioid fungus belongs to *Cylindrocarpon*.

Because of the morphological difference of conidia between that on luchu pine and on SNA, we performed phylogenetic analysis of *Neo. amamiensis* together with selected species of *Fusarium* and *Cylindrocarpon* having similarly hooked conidia or typical conidia for these genera (see Table 2). Recently, phylogenetic analyses have been used to support the taxonomy of the genus *Neonectria* with *Cylindrocarpon* anamorphs (Mantiri et al. 2001; Brayford et al. 2004; Halleen et al. 2004). Halleen et al. (2004) classified *Neonectria* in detail based on the phylogenetic analysis of mLSU rDNA, β-tubulin genes, and nrDNA ITS sequence data. In the present study, all the isolates of *Neo. amamiensis* and their anamorph were grouped in a clade of *Neonectria* together with other species having *Cylindrocarpon* anamorphs (see Fig. 14) but were not grouped with genus *Fusarium* with strongly hooked apical cells such as *F. inflexum*, *F. udum* (teleomorph: *Gibberella indica*), or *F. xylarioides* (teleomorph: *Gibberella xylarioides*) (Booth 1971; Gerlach and Nirenberg 1982). The result of molecular phylogenetic analysis indicates the present anamorph should be assigned to the genus *Cylindrocarpon* but not to *Fusarium*.

Perithecia of *Neo. amamiensis* share some morphological similarities with those of *Cosmospora* and *Nectria* (Table 3). Perithecia of *Neo. amamiensis* differ from those of *Cosmospora* in having a two-layered and perithecial wall more than 30 μm thick. In dry conditions, the perithecial form of *Neo. amamiensis* differs from those of *Nectria* (Table 3). *Neonectria amamiensis* is also clearly distinguished from *Cosmospora* and *Nectria* by its anamorphs (*Fusarium* sect. *Eupionnotes*, *Chaetopsina* Rambelli, *Cylindrocladiella* Boesew., *Stilbella* Lindau, and *Volutella* Fr. in *Cosmospora* and *Gyrostroma* Naumov, *Tubercularia* Tode, and *Zythiostroma* Höhn. ex Falck in *Nectria*). The only teleomorph hitherto described for the *Cylindrocarpon*

Table 3. Comparison of the diagnostic morphological features among *Neonectria*, *Cosmospora*, and *Nectria*

	Neonectria amamiensis (present nectrioid fungus)	*Neonectria*[a]	*Cosmospora*[a]	*Nectria*[a]
Perithecium in dry condition	Collapsing laterally	Collapsing laterally or not collapsing	Usually collapsing laterally	Collapsing cupulate
Surface of perithecial wall	Smooth to scaly	Smooth to warty	Usually smooth	Sometimes warty
Perithecial wall cell	Subcircular	Circular to angular	Subcircular	Circular to angular
Number of perithecial layers	2	2–3	1	2–3
Thickness of perithecial wall	30–50 µm	50 µm or more	Less than 20 µm	More than 25 µm
Surface of ascospore	Tuberculate	Usually smooth	Usually spinulate to tuberculate	Smooth to striate
Anamorph	*Cylindrocarpon amamiense*	*Cylindrocarpon*	*Fusarium* (section *Eupionnotes*), *Chaetopsina*, *Cylindrocladiella, Stilbella*, *Volutella*	*Gyrostroma*, *Tubercularia*, *Zythiostroma*

[a] Rossman et al. (1999)

Table 4. Comparison of the morphological characteristics between *Neonectria amamiensis* and two related *Neonectria* species

	Neonectria amamiensis/ *Cylindrocarpon amamiense* (present nectrioid fungus)	*Neo. veuillotiana*/ *Cy. candidulum*[a]	*Neo. lucida*/ *Cy. lucidum*[b]
Teleomorph			
Size of perithecium (µm)	210–440 × 180–300	280–400 × 250–360	430–480 diameter
Dry condition of perithecium	Collapsing laterally	Not collapsing	Not collapsing
Surface of perithecial wall	Smooth to scaly	Smooth to scaly	Smooth
Cell at surface of perithecial wall	Subcircular	Circular to angular	Hyphal
Size of ascospore (µm)	16–20 × 5–8.5	(12.4–)14.7–19.3(–25) × (5–)6.5–7.8 (–9.3)	(10–)12–15(–17) × (5–)5.5–6.5(–7)
Color of ascospore	Colorless, becoming pale yellow	Colorless, becoming pale brown	Pale brown
Anamorph			
Color of colony on PDA	White, orange to pale yellow	Tan to brown	Buff
Number of septa in macroconidium	3–6	(3–)4–5(–6)	3–8
Size of macroconidium (µm)	50–80 × 5–7.5	46–87 × 5–8	Group 1: 60–70 × 5–6[c] Group 2: 70–90 × 6–7[c] Group 3: 70–80 × 9.5–10[c]
Hooked apical cells of the conidium	Present	Absent	Absent

Cy., Cylindrocarpon; Neo., Neonectria; PDA, potato dextrose agar
[a] Brayford and Samuels (1993)
[b] Brayford et al. (2004)
[c] Brayford et al. (2004) recognized three different intraspecific morphological groups within *Neo. lucida* (*Cy. lucidum*)

anamorph is *Neonectria*. Morphological comparisons with allied genera in the Nectriaceae with special reference to their anamorphs confirm the taxonomy of the teleomorph of the present fungus.

Among more than 40 species of *Neonectria* (Booth 1959, 1966; Samuels 1988; Samuels and Brayford 1990, 1993, 1994; Brayford and Samuels 1993; Rossman et al. 1999; Mantiri et al. 2001; Brayford et al. 2004; Halleen et al. 2004), *Neo. veuillotiana* (Sacc. & Roum.) Mantiri & Samuels [anamorph: *Cy. candidulum* (Sacc.) Wollenw.] and *Neo. lucida* (Höhn.) Samuels & Brayford (anamorph: *Cy. lucidum* C. Booth) are somewhat similar to *Neo. amamiensis* (*Cy. amamiense*) in morphology (Table 4). All of them have constricted perithecia just below the papilla and lack microconidia and chlamydospores (see Figs. 2, 3, 13). However, *Neo. amamiensis* differs from *Neo. lucida* in the dry condition of the perithecium, the cell structure at the surface of the perithecial wall, and the size and color of ascospores, and from *Neo. veuillotiana* in the dry condition of perithecium, the cell structure at the surface of the perithecial wall, and the size of the ascospores (see Table 4). In the anamorphic states observed in culture, *Neo. lucida* (*Cy. lucidum*) has slightly hooked conidia, but the apical cell of conidia is not hooked (Brayford and Samuels 1993), whereas *Neo. veuillotiana* (*Cy. candidulum*) has no hooked conidia nor apical cell (Mantiri et al. 2001). On the other hand, *Neo. amamiensis* (*Cy. amamiense*) bears a clearly hooked apical cell in the conidia (see Figs. 11, 12, 13g,h). The majority of conidia in *Cy. amamiense* bear 3–6 septa, while these are less varied in *Cy. candidulum* (4–6-septate) and more varied in *Cy. lucidum* (3–8-septate). In addition, the whitish to pale yellow colony color of *Cy. amamiense* differs from those of *Cy. candidulum* (tan to brown) and *Cy. lucidum* (buff) (see Table 4). The results of molecular phylogenetic analysis indicate that these three species are distinct from each other. However, all anamorphic isolates of *Neo. amamiensis* were nested in a clade with *Neo. veuillotiana* (*Cy. candidulum*) as the sister taxon. These sister clades were well supported by 100% bootstrap values (see Fig. 14).

From the foregoing analysis, our fungus differed from any of the taxa in *Neonectria* hitherto described as a holomorph; hence, a new taxon was established.

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References

Booth C (1959) Studies of Pyrenomycetes *Nectria* (part 1). Mycol Pap 73:1–115

Booth C (1966) The genus *Cylindrocarpon*. Mycol Pap 104:1–58

Booth C (1971) The genus *Fusarium*. Commonwealth Mycological Institute, Kew

Brayford D, Samuels GJ (1993) Some didymosporous species of *Nectria* with non-microcondial *Cylindrocarpon* anamorphs. Mycologia 85:612–637

Brayford D, Honda BM, Mantiri FR, Samuels GJ (2004) *Neonectria* and *Cylindrocarpon*: the *Nectria mammoidea* group and species lacking microconidia. Mycologia 96:572–597

Gerlach W, Nirenberg H (1982) The genus *Fusarium:* a pictorial atlas. Mitt Biol Bundesanst Land- Forstwirtsch Berl-Dahl 209:1–406

Halleen F, Schroers HJ, Groenewald JZ, Crous PW (2004) Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). Stud Mycol 50:431–455

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J Mol Evol 16:111–120

Kirk PM, Cannon PF, David JC, Stalper JA (eds) (2001) Ainsworth & Bisby's dictionary of the fungi, 9th edn. CAB International, Wallingford

Kobayashi T, Muramoto M (1989) Pitch canker of *Pinus luchuensis*, a new disease in Japanese forests (in Japanese). For Pests 38:169–173

Kobayashi T, Kubono T, Tabata M, Ito S (1988) Fungi associated with resinous canker of *Pinus luchuensis* and their pathogenicity (in Japanese). In: 99th Transactions of the Japanese Forestry Society, Niigata, Japan, April 4–5, pp 515–516

Kornerup A, Wanscher JH (1978) Methuen handbook of colour, 3rd edn. Methuen, London

Mantiri F, Samuels GJ, Rahe JE, Honda B (2001) Phylogenetic relationships in *Neonectria* species having *Cylindrocarpon* anamorphs inferred from mitochondrial ribosomal DNA sequences. Can J Bot 79:334–340

Nirenberg HI (1990) Recent advances in the taxonomy of *Fusarium*. Stud Mycol 32:91–101

Nirenberg HI, O'Donnell K (1998) New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia 90:434–458

O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7:103–116

O'Donnell K, Cigelnik E, Weber NJ, Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89:48–65

Rossman AY, Samuels GJ, Rogerson CT, Lowen R (1999) Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Mycol 42:1–248

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenic trees. Mycol Biol Evol 4:406–425

Samuels GJ (1988) Species of *Nectria* (Ascomycetes, Hypocreales) having orange perithecia and colorless, striate ascospores. Brittonia 40:306–331

Samuels GJ, Brayford D (1990) Variation in *Nectria radicicola* and its anamorph *Cylindrocarpon destructans*. Mycol Res 94:433–442

Samuels GJ, Brayford D (1993) Phragmosporous *Nectria* species with *Cylindrocarpon* anamorphs. Sydowia 45:55–80

Samuels GJ, Brayford D (1994) Species of *Nectria* (*sensu lato*) with red perithecia and striate ascospores. Sydowia 46:75–161

Swofford DL (1998) PAUP*: phylogenetic analysis using parsimony, version 4. Sinauer Associates, Sunderland, MA

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680