Mycoscience (2008) 49:359–372
DOI 10.1007/s10267-008-0440-6

FULL PAPER

**Izumi Okane · Prasert Srikitikulchai · Kyoko Toyama
Thomas Læssøe · Somsak Sivichai · Nigel Hywel-Jones
Akira Nakagiri · Wanchern Potacharoen
Ken-ichiro Suzuki**

Study of endophytic Xylariaceae in Thailand: diversity and taxonomy inferred from rDNA sequence analyses with saprobes forming fruit bodies in the field

Received: February 8, 2008 / Accepted: July 12, 2008

Abstract A study of the diversity, taxonomy, and ecology of endophytic Xylariaceae (Ascomycota) was carried out. In this study, we obtained isolates of Xylariaceae from healthy, attached leaves and teleomorphic stromata on decayed plant materials in a permanent plot at Khao Yai National Park (Thailand). In addition, strains deposited beforehand were selected in which both endophytic strains isolated from living plant tissues and saprobic strains from fruit bodies were included. Consequently, 405 strains of Xylariaceae (273 endophytic and 132 saprobic strains, including identified strains) were studied to reveal the diversity and taxonomy of endophytes and the relationships between those endophytes and saprobic Xylariaceae in Thailand that have been recorded according to fruit-body formation on decayed plant materials. Analysis of 28S rDNA D1/D2 sequences revealed 21 xylariaceous species inhabiting tropical foliage at the site, and several species that are already known as saprobes appear to be among those isolated from living leaves. Furthermore, several clades that consisted of only endophytic strains were found, and some of these have no known matches in public DNA sequence banks.

Key words Diversity · Endophytic-saprobic relationships · rDNA sequence analysis · Thailand · Xylariaceous endophytes

I. Okane (✉) · K. Toyama · A. Nakagiri · K. Suzuki
NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan
Tel. +81-438-20-5763; Fax +81-438-52-2329
e-mail: okane-izumi@nite.go.jp

P. Srikitikulchai · S. Sivichai · N. Hywel-Jones · W. Potacharoen
BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

T. Læssøe
Department of Biology, University of Copenhagen, Denmark

Introduction

Members of the Xylariaceae are important components of terrestrial ecosystems and play such functional roles as decomposers of angiosperms and gymnosperms, plant pathogens, association with termites, and endophytes (Petrini and Petrini 1985; Rogers et al 2005). Xylariaceous endophytes, i.e., fungi living inside the plant tissue for at least part of their life cycle without causing any disease symptoms in the host (Petrini 1991), have been found in all investigated major groups of plants including conifers, monocots, dicots, ferns, and lycopsids plus liverworts/hepatics (Brunner and Petrini 1992; Davis et al. 2003). Some Xylariaceae are considered as highly important endophytes of palms and other tropical plants (Rodrigues and Samuels 1990; Rodrigues 1994; Lodge et al. 1996; Rodrigues and Petrini 1997; Bayman et al. 1998; Fröhlich et al. 2000). Several hypotheses and reports on lifestyles and life cycles of endophytic and/or saprobic Xylariaceae have been described (Rodrigues et al. 1993; Læssøe and Lodge 1994; Lodge et al. 1996; Whalley 1996; Bayman et al. 1998; Rogers 2000; Collado et al. 2001; Osono 2002; Osono et al. 2004; Promputtha et al. 2007). However, there is still no clear picture of the biological significance of these fungi despite their ubiquitous appearance in isolations based on living plant tissues.

Thailand is considered one of the areas containing a high percentage of unknown taxa of Xylariaceae (Rogers 2000). In Thailand, several studies on endophytic fungi have been documented, namely, from several bamboos (Lumyong et al. 2000), banana (*Musa acuminata*) (Photita et al. 2001), monkeypod/rain tree (*Samanea saman*) (Chareprasert et al. 2006), and teak (*Tectona grandis*) (Mekkamol et al. 1997; Mekkamol 1998; Chareprasert et al. 2006), and several xylariaceous endophytes were also reported from those plants. Thienhirun (1997) provided a preliminary account of teleomorphic stages of Xylariaceae. However, it is uncertain which species among those possess the ability to live as endophytes. In this study, both endophytic strains isolated from foliage and saprobic strains derived from fruit bodies were examined for the 28S rDNA D1/D2 and rDNA internal transcribed spacer (ITS) sequences, to reveal the diversity and taxonomy of endophytes and the relationships between endophytes in foliage and saprobes of Xylariaceae producing fruit bodies on decayed plant materials, and to enhance hypotheses on the ecology of these fungi.

Materials and methods

Strains examined

Strains belonging to the Xylariaceae were isolated from healthy leaves of mainly various tropical woody plants belonging to a range of families and orders that were collected in a permanent plot of Khao Yai National Park in Thailand in September 2005 and January 2006. Plants tested belonged to 24 species of 23 genera in 20 families, including pteridophytes. The leaves collected for isolation were taken back to the mycology laboratory of National Center for Genetic Engineering and Biotechnology (BIOTEC) and processed as follows. The leaves were surface sterilized by immersing in 70% ethanol solution for 1 min, in sodium hypochlorite solution (1% available chlorine) for 2 min, and rinsed in sterile distilled water, then blotted dry in sterile paper towels for 3 h. After drying, the leaves were cut into several segments, and then they were placed on the surface of half-strength malt extract agar in 90-mm-diameter plates. The plates were incubated at 17°C to slow mycelium growth for 2–3 weeks. Mycelia growing from the leaf segments were isolated and cultured on the same medium. In the isolation process, a selective direct-hyphal isolation method was employed: the mycelia that were assumed to be Xylariaceae by linear, nondendritic branching, silky, and white hyphae at the initial growth (Fig. 1) were carefully picked and transferred to fresh media by using a fine tungsten needle. There were 279 successful cultures from 353 isolations. Eight isolates were non-Xylariaceae, and the other 271 isolates of tentative xylariaceous endophytes were obtained and deposited in BIOTEC Culture Collection (BCC). Finally, 224 of the 271 strains were selected for the study, being shown as "newly isolated endophytic strains." An additional 181 strains from the BCC collection, which had been deposited as 49 endophytic strains isolated from teak (Mekkamol 1998) and 132 saprobic strains isolated from fruit bodies mainly on wood samples from several parts on Thailand (Table 1), in which identified strains were included as references, were also selected. In the additional 181 strains, several newly isolated saprobic strains that were derived from ascospores produced on stromata on decomposing wood in the same plot at Khao Yai National Park were also included. Thus, 405 strains in total (273 endophytic and 132 saprobic) were employed for molecular analysis.

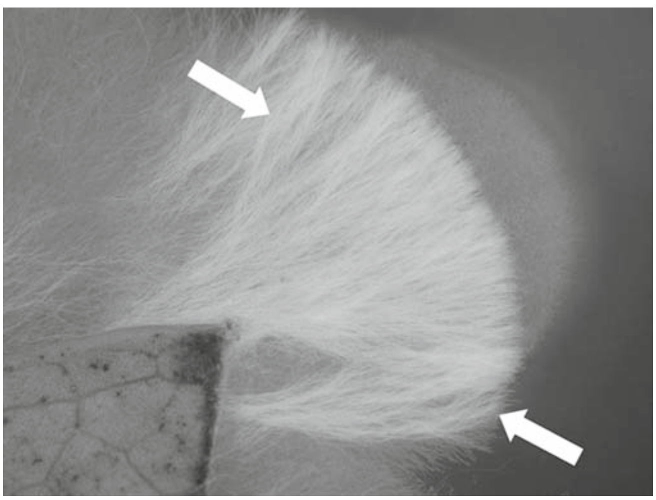


Fig. 1. Mycelium of an expected xylariaceous fungus characterized by linear, nondendritic branching, silky, and white hyphae at the initial growth (*arrows*) appearing from a leaf segment. Such mycelia were isolated under the stereomicroscope using a fine tungsten needle

Phylogenetic analysis

DNA isolation

The strains were incubated for 2 weeks at 25°C on potato dextrose agar (PDA) plates, and their mycelia were harvested and put into 2-ml plastic tubes using a spatula. DNA was extracted using Nucleon PhytoPure DNA extraction kit (GE Healthcare UK, Amersham Place, Little Chalfont, Buckinghamshire, England) or DNeasy Plant Mini Kit (Qiagen, Tokyo) according to the manufacturer's instructions.

Sequence analysis of 28S rDNA D1/D2 and rDNA ITS regions

The 5′-end of the 28S ribosomal DNA (rDNA) including D1 and D2 was amplified by polymerase chain reaction (PCR) using TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan) or Blend Taq Plus (Toyobo, Tsuruga, Fukui, Japan) as a single fragment with the standard primer pairs NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993). Amplification of the desired fragment was performed with a GenAmp PCR System 7000 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following program: 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, extension for 2 min at 72°C, incubation for 5 min at 72°C, and soaking at 4°C. Amplified DNA was sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a thermal cycler employing the following ramp: 25 cycles of 15 s at 96°C and 4 min at 55°C, followed by a 4°C soak. Nucleotide sequences were determined in both directions using the primers NL1 and NL4. Sequences were analyzed with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007) to generate the evolutionary distances [the K_{nuc} value (Kimura 1980)] and the similarity values, and to perform the neighbor-joining (NJ)

Table 1. List of strains examined

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 1001	*Xylaria schweinitzii*	Saprobic	Wood	Doi Inthanon National Park	–	AB376678	–
BCC 1002	*Xylaria grammica*	Saprobic	Wood	Klong Lan National Park	16	AB376679	–
BCC 1005	*Xylaria mellisii*	Saprobic	Twig	Kaeng Tana National Park	18	AB376680	–
BCC 1007	*Xylaria curta*	Saprobic	Wood	Doi Inthanon National Park	–	AB376681	–
BCC 1013	*Xylaria schweinitzii*	Saprobic	Wood	Doi Inthanon National Park	16	AB376682	–
BCC 1027 (= NBRC 104653)	*Xylaria cubensis*	Saprobic	Wood	Khao Laem National Park	1	AB376683	–
BCC 1036	*Xylaria multiplex*	Saprobic	No data	No data	–	AB376684	–
BCC 1053	*Xylaria obovata*	Saprobic	No data	No data	16	AB376685	–
BCC 1065	*Xylaria phyllocharis*	Saprobic	No data	No data	–	AB376686	–
BCC 1083	*Xylaria juruensis*	Saprobic	No data	No data	–	AB376687	–
BCC 1085	*Xylaria coccophora*	Saprobic	No data	No data	17	AB376688	–
BCC 1086	*Xylaria juruensis* var. *microspora*	Saprobic	No data	No data	–	AB376689	–
BCC 1100	*Xylaria obovata*	Saprobic	Wood	Doi Inthanon National Park	–	AB376690	–
BCC 1101 (= NBRC 104654)	*Biscogniauxia nummularia* var. *exutans*	Saprobic	Bark	Nam Nao National Park	20	AB376691	–
BCC 1103	*Daldinia bambusicola*	Saprobic	Bamboo	Klong Lan National Park	–	AB376692	–
BCC 1106	*Hypoxylon fendleri*	Saprobic	Wood	Klong Lan National Park	–	AB376693	–
BCC 1109	*Hypoxylon rubiginosum*	Saprobic	No data	No data	–	AB376694	–
BCC 1114	*Hypoxylon crocopeplum*	Saprobic	No data	No data	–	AB376695	–
BCC 1115	*Xylaria feejeensis*	Saprobic	No data	No data	–	AB376696	–
BCC 1119	*Pestalotiopsis* sp. (as *Xylaria aristata*)	Saprobic	No data	No data	–	AB376697	–
BCC 1127	*Xylaria psidii*	Saprobic	No data	No data	–	AB376698	–
BCC 1133	*Xylaria* sp.	Saprobic	Twig	Doi Inthanon National Park	–	AB376699	–
BCC 1136	*Xylaria apiculata*	Saprobic	Twig	Khao Laem National Park	–	AB376700	–
BCC 1144 (= NBRC 104655)	*Xylaria cubensis*	Saprobic	Wood	Khao Laem National Park	1	AB376701	–
BCC 1151	*Xylaria curta*	Saprobic	Wood	Doi Inthanon National Park	–	AB376702	–
BCC 1156	*Xylaria arbuscula*	Saprobic	Twig	Khao Laem National Park	18	AB376703	–
BCC 1170	*Xylaria grammica*	Saprobic	Wood	Doi Inthanon National Park	16	AB376704	–
BCC 1171 (= NBRC 104656)	*Xylaria badia*	Saprobic	Bamboo	Doi Inthanon National Park	–	AB376705	–
BCC 1176 (= NBRC 104657)	*Annulohypoxylon moriforme* (as *Hypoxylon moriforme*)	Saprobic	Bark	Nam Nao National Park	20	AB376706	–
BCC 1177	*Xylaria multiplex*	Saprobic	Wood	Nam Nao National Park	18	AB376707	–
BCC 1181 (= NBRC 104658)	*Xylaria* sp.	Saprobic	Wood	Klong Lan National Park	19	AB376708	–
BCC 1182 (= NBRC 104659)	*Xylaria laevis*	Saprobic	Wood	Klong Lan National Park	5	AB376709	–
BCC 1186	Xylaria mellisii	Saprobic	Wood	Kaeng Tana National Park	–	AB376710	–
BCC 1190 (= NBRC 104660)	*Xylaria badia*	Saprobic	No data	No data	–	AB376711	–
BCC 1198	*Biscogniauxia mediterranea* var. *microspora*	Saprobic	No data	No data	–	AB376712	–

Table 1. *Continued*

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 1199	*Xylaria psidii*	Saprobic	No data	No data	–	AB376713	–
BCC 1213	*Biscogniauxia nummularia* var. *merrillii*	Saprobic	No data	No data	–	AB376714	–
BCC 1219 (= NBRC 104661)	*Xylaria cubensis*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	1	AB376715	–
BCC 1229 (= NBRC 104662)	*Xylaria aristata*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376716	–
BCC 1232 (= NBRC 104663)	*Xylaria juruensis* var. *microspora*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	18	AB376717	–
BCC 1233 (= NBRC 104664)	*Xylaria juruensis* var. *microspora*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376718	–
BCC 1234 (= NBRC 104665)	*Xylaria juruensis* var. *microspora*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376719	–
BCC 1242 (= NBRC 104666)	*Daldinia eschscholzii*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376720	–
BCC 1252	*Hypoxylon haematostroma*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376721	–
BCC 1260 (= NBRC 104667)	*Xylaria aristata*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376722	–
BCC 1263	*Xylaria juruensis* var. *microspora*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376723	–
BCC 1288 (= NBRC 104668)	*Xylaria* sp.	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	2	AB376724	–
BCC 1303 (= NBRC 104669)	*Xylaria cubensis*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	1	AB376725	–
BCC 1308	*Hypoxylon* sp.	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	21	AB376726	–
BCC 1313 (= NBRC 104670)	*Daldinia eschscholzii*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376727	–
BCC 1314	*Hypoxylon haematostroma*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	–	AB376728	–
BCC 1321	*Xylaria cubensis*	Endophytic	Leaf of *Tectona grandis* (Verbenaceae)	No data	16	AB376729	–
BCC 1340	*Xylaria allantoidea*	Saprobic	Wood	Klong Lan National Park	–	AB376730	–
BCC 1352	*Xylaria phyllocharis*	Saprobic	No data	No data	–	AB376731	–
BCC 17352	*Xylaria anisopleura*	Saprobic	Wood	No data	–	AB376732	–
BCC 18196	*Xylaria consociata*	Saprobic	Wood	No data	–	AB376733	–
BCC 18212	*Kretzschmaria* cf. *lucidula*	Saprobic	Wood	No data	–	AB376734	–
BCC 18288	*Hypoxylon perforatum*	Saprobic	Wood	No data	–	AB376735	–
BCC 18361	*Xylaria tuberoides*	Saprobic	Wood	No data	–	AB376736	–
BCC 18718	*Xylaria* cf. *obovata*	Saprobic	Wood	No data	–	AB376737	–
BCC 18731 (= NBRC 104671)	Xylariaceae	Endophytic	Leaf of *Eugenia* sp. (Myrtaceae)	Khao Yai National Park	11	AB376738	AB440075
BCC 18734 (= NBRC 104672)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	11	AB376739	AB440076
BCC 18737 (= NBRC 104673)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	5	AB376740	AB440077

Table 1. *Continued*

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 18738 (= NBRC 104674)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	11	AB376741	AB440078
BCC 18739 (= NBRC 104675)	Xylariaceae	Endophytic	Leaf of *Schizostachyum longispiculatum* (Gramineae)	Khao Yai National Park	15	AB376742	AB440079
BCC 18741 (= NBRC 104676)	Xylariaceae	Endophytic	Leaf of *Schizostachyum longispiculatum* (Gramineae)	Khao Yai National Park	12	AB376743	AB440080
BCC 18743 (= NBRC 104677)	Xylariaceae	Endophytic	Leaf of *Licuala* sp. (Palmae)	Khao Yai National Park	17	AB376744	AB440081
BCC 18747 (= NBRC 104678)	Xylariaceae	Endophytic	Leaf of *Licuala* sp. (Palmae)	Khao Yai National Park	12	AB376745	AB440082
BCC 18752 (= NBRC 104679)	Xylariaceae	Endophytic	Leaf of *Aglaia elaeagnoidea* (Meliaceae)	Khao Yai National Park	11	AB376746	AB440083
BCC 18756 (= NBRC 104680)	Xylariaceae	Endophytic	Leaf	Khao Yai National Park	16	AB376747	AB440084
BCC 18768 (= NBRC 104681)	Xylariaceae	Endophytic	Leaf of *Canthium* sp. (Rubiaceae)	Khao Yai National Park	1	AB376748	AB440085
BCC 18771 (= NBRC 104682)	Xylariaceae	Endophytic	Leaf of *Canthium* sp. (Rubiaceae)	Khao Yai National Park	6	AB376749	AB440086
BCC 18772 (= NBRC 104683)	Xylariaceae	Endophytic	Leaf of *Canthium* sp. (Rubiaceae)	Khao Yai National Park	13	AB376750	AB440087
BCC 18777 (= NBRC 104684)	Xylariaceae	Endophytic	Leaf of *Areca triandra* (Palmae)	Khao Yai National Park	12	AB376751	AB440088
BCC 18778	*Pestalotiopsis* sp.	Endophytic	Leaf of *Areca triandra* (Palmae)	Khao Yai National Park	–	AB376752	AB440089
BCC 18786 (= NBRC 104685)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	2	AB376753	AB440090
BCC 18793 (= NBRC 104686)	Xylariaceae	Endophytic	Leaf of *Polyalthia* sp. (Annonaceae)	Khao Yai National Park	9	AB376754	AB440091
BCC 18796 (= NBRC 104687)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	11	AB376755	AB440092
BCC 18797 (= NBRC 104688)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	16	AB376756	AB440093
BCC 18798	*Pestalotiopsis* sp.	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	–	AB376757	AB440094
BCC 18853 (= NBRC 104689)	Xylariaceae	Endophytic	Leaf of *Daemonorops* sp. (Palmae)	Khao Yai National Park	8	AB376758	AB440095
BCC 18861 (= NBRC 104690)	Xylariaceae	Endophytic	Leaf of *Melicope pteleifolia* (Rutaceae)	Khao Yai National Park	10	AB376759	AB440096
BCC 18862 (= NBRC 104691)	Xylariaceae	Endophytic	Leaf of fern	Khao Yai National Park	5	AB376760	AB440097
BCC 18863 (= NBRC 104692)	Xylariaceae	Endophytic	Leaf of fern	Khao Yai National Park	8	AB376761	AB440098
BCC 18865 (= NBRC 104693)	Xylariaceae	Endophytic	Leaf of fern	Khao Yai National Park	1	AB376762	AB440099
BCC 18869 (= NBRC 104694)	Xylariaceae	Endophytic	Leaf of *Saprosma longifolium* (Rubiaceae)	Khao Yai National Park	17	AB376763	AB440100
BCC 18870 (= NBRC 104695)	Xylariaceae	Endophytic	Leaf of *Saprosma longifolium* (Rubiaceae)	Khao Yai National Park	2	AB376764	AB440101
BCC 18873 (= NBRC 104696)	Xylariaceae	Endophytic	Leaf of *Melicope pteleifolia* (Rutaceae)	Khao Yai National Park	15	AB376765	AB440102
BCC 18886 (= NBRC 104697)	Xylariaceae	Endophytic	Leaf of *Saprosma longifolium* (Rubiaceae)	Khao Yai National Park	16	AB376766	AB440103

Table 1. *Continued*

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 18902 (= NBRC 104698)	Xylariaceae	Endophytic	Leaf of *Aphanamixis polystachya* (Meliaceae)	Khao Yai National Park	7	AB376767	AB440104
BCC 18905 (= NBRC 104699)	Xylariaceae	Endophytic	Leaf of *Cyathostemma micranthum* (Annonaceae)	Khao Yai National Park	7	AB376768	AB440105
BCC 20324 (= NBRC 104700)	*Hypoxylon* cf. *symphyon*	Saprobic	Wood	No data	–	AB376769	–
BCC 20327 (= NBRC 104701)	*Nemania* cf. *bipapillata*	Saprobic	Wood	No data	–	AB376770	–
BCC 20377 (= NBRC 104702)	*Xylaria* cf. *amphithele*	Saprobic	Palm frond	No data	–	AB376771	–
BCC 20656	*Xylaria atrosphaerica*	Saprobic	Wood	No data	–	AB376772	–
BCC 20667	*Xylaria mellisii*	Saprobic	Palm frond	No data	18	AB376773	–
BCC 20842 (= NBRC 104703)	Xylariaceae	Endophytic	Leaf of *Licuala* sp. (Palmae)	Khao Yai National Park	8	AB376774	AB440106
BCC 20844 (= NBRC 104704)	Xylariaceae	Endophytic	Leaf of *Licuala* sp. (Palmae)	Khao Yai National Park	20	AB376775	AB440107
BCC 20845 (= NBRC 104705)	Xylariaceae	Endophytic	Leaf of *Aglaia elaeagnoidea* (Meliaceae)	Khao Yai National Park	17	AB376776	AB440108
BCC 20849 (= NBRC 104706)	Xylariaceae	Endophytic	Leaf of *Aglaia elaeagnoidea* (Meliaceae)	Khao Yai National Park	18	AB376777	AB440109
BCC 20850 (= NBRC 104707)	Xylariaceae	Endophytic	Leaf of *Aglaia elaeagnoidea* (Meliaceae)	Khao Yai National Park	13	AB376778	AB440110
BCC 20930 (= NBRC 104708)	Xylariaceae	Endophytic	Leaf of *Gonocaryum lobbianum* (Icacinaceae)	Khao Yai National Park	14	AB376779	AB440111
BCC 20932 (= NBRC 104709)	Xylariaceae	Endophytic	Leaf of *Eugenia* sp. (Myrtaceae)	Khao Yai National Park	16	AB376780	AB440112
BCC 20933 (= NBRC 104710)	Xylariaceae	Endophytic	Leaf of *Eugenia* sp. (Myrtaceae)	Khao Yai National Park	4	AB376781	AB440113
BCC 20936 (= NBRC 104711)	Xylariaceae	Endophytic	Leaf of *Eugenia* sp. (Myrtaceae)	Khao Yai National Park	16	AB376782	AB440114
BCC 20940 (= NBRC 104712)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	21	AB376783	AB440115
BCC 20943 (= NBRC 104713)	Xylariaceae	Endophytic	Leaf of *Schizostachyum longispiculatum* (Gramineae)	Khao Yai National Park	8	AB376784	AB440116
BCC 20950 (= NBRC 104714)	Xylariaceae	Endophytic	Leaf of *Selaginella inaequalifolia* (Selaginellaceae)	Khao Yai National Park	15	AB376785	AB440117
BCC 20951 (= NBRC 104715)	Xylariaceae	Endophytic	Leaf of *Selaginella inaequalifolia* (Selaginellaceae)	Khao Yai National Park	3	AB376786	AB440118
BCC 20955 (= NBRC 104716)	Xylariaceae	Endophytic	Leaf of *Tectaria impressa* (Dryopteridaceae)	Khao Yai National Park	7	AB376787	AB440119
BCC 20960 (= NBRC 104717)	Xylariaceae	Endophytic	Leaf of *Tectaria impressa* (Dryopteridaceae)	Khao Yai National Park	8	AB376788	AB440120
BCC 20971 (= NBRC 104718)	Xylariaceae	Endophytic	Leaf of *Knema elegans* (Myristicaceae)	Khao Yai National Park	17	AB376789	AB440121
BCC 20973 (= NBRC 104719)	Xylariaceae	Endophytic	Leaf of *Canthium* sp. (Rubiaceae)	Khao Yai National Park	17	AB376790	AB440122
BCC 20986 (= NBRC 104720)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	19	AB376791	AB440123

Table 1. *Continued*

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 20987 (= NBRC 104721)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	16	AB376792	AB440124
BCC 20988 (= NBRC 104722)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	1	AB376793	AB440125
BCC 20989 (= NBRC 104723)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	21	AB376794	AB440126
BCC 20991 (= NBRC 104724)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	1	AB376795	AB440127
BCC 20992 (= NBRC 104725)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	21	AB376796	AB440128
BCC 20993 (= NBRC 104726)	Xylariaceae	Endophytic	Leaf of *Polyalthia* sp. (Annonaceae)	Khao Yai National Park	15	AB376797	AB440129
BCC 20995 (= NBRC 104727)	Xylariaceae	Endophytic	Leaf of *Polyalthia* sp. (Annonaceae)	Khao Yai National Park	16	AB376798	AB440130
BCC 20996 (= NBRC 104728)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	15	AB376799	AB440131
BCC 20997 (= NBRC 104729)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	19	AB376800	AB440132
BCC 20998 (= NBRC 104730)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	16	AB376801	AB440133
BCC 20999 (= NBRC 104731)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	16	AB376802	AB440134
BCC 21000 (= NBRC 104732)	Xylariaceae	Endophytic	Leaf of *Ardisia nervosa* (Myrsinaceae)	Khao Yai National Park	16	AB376803	AB440135
BCC 21001 (= NBRC 104733)	Xylariaceae	Endophytic	Leaf of *Miliusa lineata* (Annonaceae)	Khao Yai National Park	15	AB376804	AB440136
BCC 21002 (= NBRC 104734)	Xylariaceae	Endophytic	Leaf of *Miliusa lineata* (Annonaceae)	Khao Yai National Park	21	AB376805	AB440137
BCC 21041	*Daldinia sabahense*	Saprobic	Wood	Khao Sok National Park	–	AB376806	–
BCC 21050	*Hypoxylon* cf. *fendleri*	Saprobic	Wood	Khao Pu – Khao Ya National Park	–	AB376807	–
BCC 21238	*Whalleya* sp. aff. *microplaca*	Saprobic	Wood	No data	–	AB376808	–
BCC 22739	*Xylaria bambusicola*	Saprobic	Bamboo	Nam Nao National Park	–	AB376809	–
BCC 22760	*Kretzschmaria* cf. *macrosperma*	Saprobic	Wood	Phu Hin Rong Kla National Park	–	AB376810	–
BCC 22966	*Xylaria papulis*	Saprobic	Wood	Khao Chong Wildlife Development and Conservation Promotion Station	–	AB376811	–
BCC 22968	*Xylaria hypoerythra*	Saprobic	Wood	Khao Chong Wildlife Development and Conservation Promotion Station	–	AB376812	–
BCC 22982	*Hypoxylon dieckmannii*	Saprobic	Wood	Phu Hin Rong Kla National Park	–	AB376813	–

Table 1. *Continued*

Strain no.[a]	Name	Category[b]	Source (plant family)	Locality	Clade	DDBJ/EMBL/GenBank accession no. (left, 28S; right, ITS)	
BCC 22987	*Xylaria* cf. *piperiformis*	Saprobic	Soil	Khlong Lan National Park	–	AB376814	–
BCC 22993	*Hypoxylon duranii*	Saprobic	Wood	Nam Nao National Park	–	AB376815	–
BCC 22995	*Hypoxylon* cf. *subgilvum*	Saprobic	Wood	Nam Nao National Park	–	AB376816	–
BCC 23010	*Hypoxylon* cf. *trugodes*	Saprobic	Wood	Namtok Samlan National Park	–	AB376817	–
BCC 23279 (= NBRC 104735)	*Xylaria escharoidea*	Saprobic	Soil	Thung Salaeng Luang National Park	–	AB376818	–
BCC 23565	*Hypoxylon nitens*	Saprobic	Wood	Thung Salaeng Luang National Park	–	AB376819	–
BCC 23627	*Xylaria* cf. *bambusicola*	Saprobic	Bamboo	No data	–	AB376820	–
BCC 23628	*Xylaria* cf. *bambusicola*	Saprobic	Bamboo	No data	–	AB376821	–
BCC 23634 (= NBRC 104736)	*Xylaria escharoidea*	Saprobic	Soil	Khao Yai National Park	–	AB376822	–
BCC 23642	*Hypoxylon* sp.	Saprobic	Wood	Phu Hin Rong Kla National Park	7	AB376823	–
BCC 23653	*Hypoxylon macrocarpum*	Saprobic	Wood	Phu Hin Rong Kla National Park	–	AB376824	–
BCC 23659	*Xylaria bambusicola*	Saprobic	Bamboo	No data	–	AB376825	–
BCC 23666 (= NBRC 104737)	*Nemania diffusa*	Saprobic	Wood	Phu Hin Rong Kla National Park	9	AB376826	–
BCC 23674	*Hypoxylon monticulosum*	Saprobic	Wood	Phu Hin Rong Kla National Park	–	AB376827	–

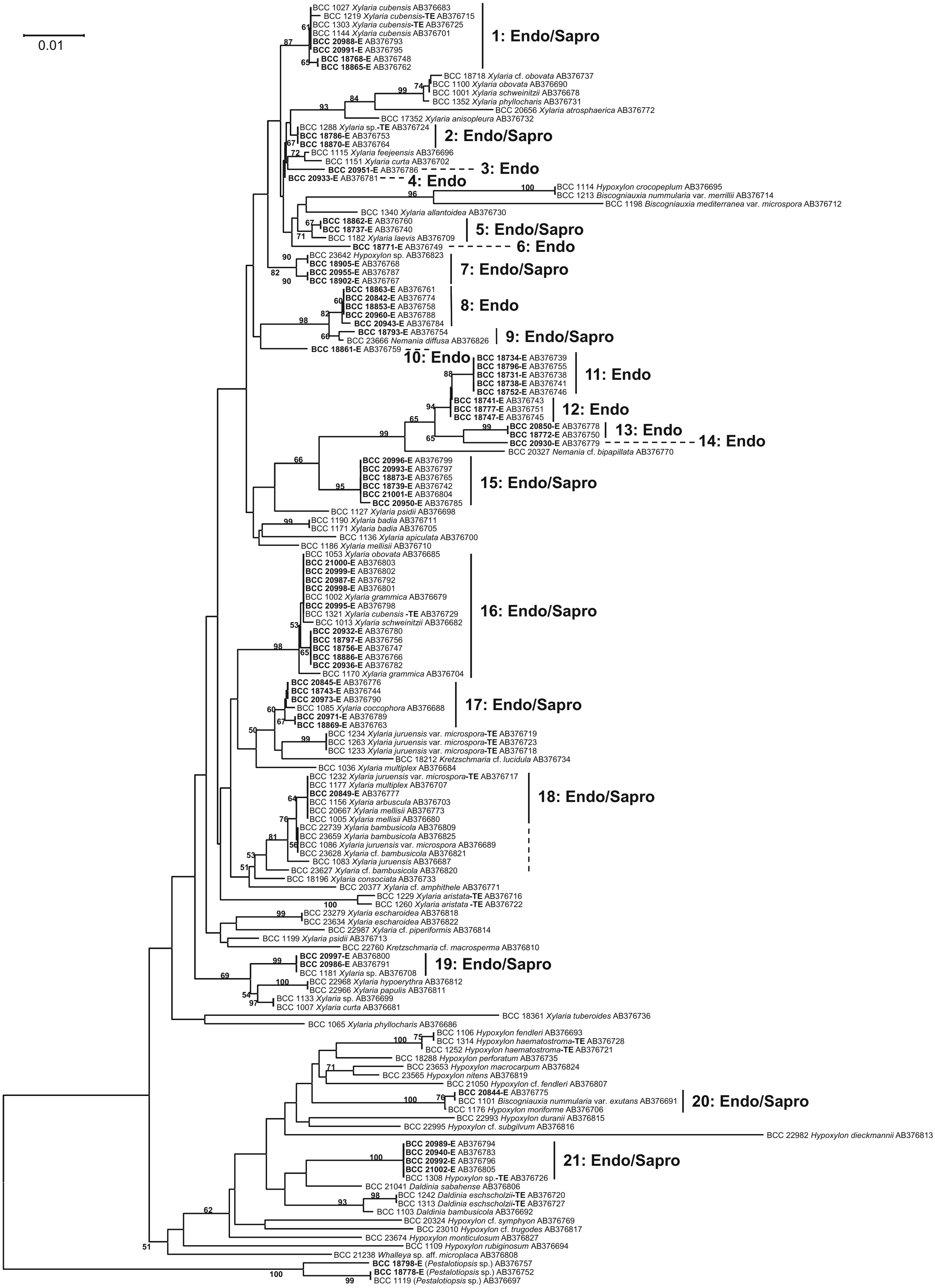
[a]BCC, BIOTEC Culture Collection; NBRC, NITE Biological Resource Center
[b]Saprobic, strains isolated from fruit bodies on decayed plant materials; endophytic, strains from living leaves

analysis (Saitou and Nei 1987) from K_{nuc} values and the bootstrap resampling method (Felsenstein 1985) with 1000 replicates for evaluation of the topology of the phylogenetic tree. The NJ plot (Perrière and Gouy 1996) was used for plotting the phylogenetic tree.

The ITS regions of rDNA were amplified by polymerase chain reaction (PCR) using TaKaRa Ex Taq (TaKaRa Bio) or Blend Taq Plus (Toyobo) as a single fragment with the standard primer pairs ITS5 (5′-GGAAGTAAAAGTC GTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) (White et al. 1990). Amplification of the desired fragment was performed in the same manner as the 28S rDNA analysis. Amplified DNA was sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in the same manner as the 28S rDNA D1/D2 analysis. Nucleotide sequences were determined in both directions using the primers ITS2 (5′-GCTGC GTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGAT GAAGAACGGAGC-3′), ITS4, and ITS5 (White et al. 1990). The analyses after this were similar to the aforementioned methods in the 28S rDNA analysis.

Among the 405 Thailand-derived strains that we have been studying under this project, we selected 150 representative strains (78 endophytic and 72 saprobic), in which 3 outgroup strains were included (Table 1), by omitting taxonomically uncertain saprobic strains and some endophytic strains included in the same cluster. Sequence data of 28S rDNA D1/D2 regions of the 150 strains and ITS regions of 63 newly isolated endophytic strains were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases. Each accession number is shown in Table 1. The alignments were deposited in TreeBASE (http://www.treebase.org/treebase/index.html) under the study number of S2167 . In this study, the more comprehensive phylogenetic analyses using the data of 28S rDNA D1/D2 and rDNA ITS regions sequences retrieved from the DDBJ/EMBL/GenBank nucleotide sequence databases and the BLAST search on the sequence databases were performed to presume species closely relating to endophytic Xylariaceae. Evolutionary clades shown in phylogenetic trees were evaluated for species-level definition according to the above-mentioned sequence analyses.

Fig. 2. Neighbor-joining tree generated from the alignment of the 28S rDNA D1/D2 regions sequences of 150 BCC strains of xylariaceous fungi. Strains marked "*E*" are newly isolated endophytic strains under the project, "*TE*" are endophytes from teak, and the remainder are saprobic strains derived from fruit bodies. *Arabic numerals* are used to number individual clades; "*Endo/Sapro*" and "*Endo*" indicate clades including both endophytic and saprobic strains and endophytic strains, respectively. Three strains identified as *Pestalotiopsis* (Amphisphaeriaceae, Xylariales) in this study (BCC 1119, BCC 18778, and BCC 18798) were specified as the outgroup. Bootstrap values > 50% are shown above branches. *Bar* 0.01 K_{nuc} in nucleotide sequences


0.01
BCC 1027 Xylaria cubensis AB376683
BCC 1219 Xylaria cubensis-TE AB376715
BCC 1303 Xylaria cubensis-TE AB376725
BCC 1144 Xylaria cubensis AB376701
BCC 20988-E AB376793
BCC 20991-E AB376795
BCC 18768-E AB376748
BCC 18865-E AB376762
1: Endo/Sapro
BCC 18718 Xylaria cf. obovata AB376737
BCC 1100 Xylaria obovata AB376690
BCC 1001 Xylaria schweinitzii AB376678
BCC 1352 Xylaria phyllocharis AB376731
BCC 20656 Xylaria atrosphaerica AB376772
BCC 17352 Xylaria anisopleura AB376732
BCC 1288 Xylaria sp.-TE AB376724
BCC 18786-E AB376753
BCC 18870-E AB376764
2: Endo/Sapro
BCC 1115 Xylaria feejeensis AB376696
BCC 1151 Xylaria curta AB376702
BCC 20951-E AB376786
3: Endo
BCC 20933-E AB376781
4: Endo
BCC 1114 Hypoxylon crocopeplum AB376695
BCC 1213 Biscogniauxia nummularia var. merrillii AB376714
BCC 1198 Biscogniauxia mediterranea var. microspora AB376712
BCC 1340 Xylaria allantoidea AB376730
BCC 18862-E AB376760
BCC 18737-E AB376740
BCC 1182 Xylaria laevis AB376709
5: Endo/Sapro
BCC 18771-E AB376749
6: Endo
BCC 23642 Hypoxylon sp. AB376823
BCC 18905-E AB376768
BCC 20955-E AB376787
BCC 18902-E AB376767
7: Endo/Sapro
BCC 18863-E AB376761
BCC 20842-E AB376774
BCC 18853-E AB376758
BCC 20960-E AB376788
BCC 20943-E AB376784
8: Endo
BCC 18793-E AB376754
BCC 23666 Nemania diffusa AB376826
9: Endo/Sapro
BCC 18861-E AB376759
10: Endo
BCC 18734-E AB376739
BCC 18796-E AB376755
BCC 18731-E AB376738
BCC 18738-E AB376741
BCC 18752-E AB376746
11: Endo
BCC 18741-E AB376743
BCC 18777-E AB376751
BCC 18747-E AB376745
12: Endo
BCC 20850-E AB376778
BCC 18772-E AB376750
13: Endo
BCC 20930-E AB376779
14: Endo
BCC 20327 Nemania cf. bipapillata AB376770
BCC 20996-E AB376799
BCC 20993-E AB376797
BCC 18873-E AB376765
BCC 18739-E AB376742
BCC 21001-E AB376804
BCC 20950-E AB376785
15: Endo/Sapro
BCC 1127 Xylaria psidii AB376698
BCC 1190 Xylaria badia AB376711
BCC 1171 Xylaria badia AB376705
BCC 1136 Xylaria apiculata AB376700
BCC 1186 Xylaria mellisii AB376710
BCC 1053 Xylaria obovata AB376685
BCC 21000-E AB376803
BCC 20999-E AB376802
BCC 20987-E AB376792
BCC 20998-E AB376801
BCC 1002 Xylaria grammica AB376679
BCC 20995-E AB376798
BCC 1321 Xylaria cubensis -TE AB376729
BCC 1013 Xylaria schweinitzii AB376682
BCC 20932-E AB376780
BCC 18797-E AB376756
BCC 18756-E AB376747
BCC 18886-E AB376766
BCC 20936-E AB376782
BCC 1170 Xylaria grammica AB376704
16: Endo/Sapro
BCC 20845-E AB376776
BCC 18743-E AB376744
BCC 20973-E AB376790
BCC 1085 Xylaria coccophora AB376688
BCC 20971-E AB376789
BCC 18869-E AB376763
17: Endo/Sapro
BCC 1234 Xylaria juruensis var. microspora-TE AB376719
BCC 1263 Xylaria juruensis var. microspora-TE AB376723
BCC 1233 Xylaria juruensis var. microspora-TE AB376718
BCC 18212 Kretzschmaria cf. lucidula AB376734
BCC 1036 Xylaria multiplex AB376684
BCC 1232 Xylaria juruensis var. microspora-TE AB376717
BCC 1177 Xylaria multiplex AB376707
BCC 20849-E AB376777
BCC 1156 Xylaria arbuscula AB376703
BCC 20667 Xylaria mellisii AB376773
BCC 1005 Xylaria mellisii AB376680
18: Endo/Sapro
BCC 22739 Xylaria bambusicola AB376809
BCC 23659 Xylaria bambusicola AB376825
BCC 1086 Xylaria juruensis var. microspora AB376689
BCC 23628 Xylaria cf. bambusicola AB376821
BCC 1083 Xylaria juruensis AB376687
BCC 23627 Xylaria cf. bambusicola AB376820
BCC 18196 Xylaria consociata AB376733
BCC 20377 Xylaria cf. amphithele AB376771
BCC 1229 Xylaria aristata-TE AB376716
BCC 1260 Xylaria aristata -TE AB376722
BCC 23279 Xylaria escharoidea AB376818
BCC 23634 Xylaria escharoidea AB376822
BCC 22987 Xylaria cf. piperiformis AB376814
BCC 1199 Xylaria psidii AB376713
BCC 22760 Kretzschmaria cf. macrosperma AB376810
BCC 20997-E AB376800
BCC 20986-E AB376791
BCC 1181 Xylaria sp. AB376708
19: Endo/Sapro
BCC 22968 Xylaria hypoerythra AB376812
BCC 22966 Xylaria papulis AB376811
BCC 1133 Xylaria sp. AB376699
BCC 1007 Xylaria curta AB376681
BCC 18361 Xylaria tuberoides AB376736
BCC 1065 Xylaria phyllocharis AB376686
BCC 1106 Hypoxylon fendleri AB376693
BCC 1314 Hypoxylon haematostroma-TE AB376728
BCC 1252 Hypoxylon haematostroma-TE AB376721
BCC 18288 Hypoxylon perforatum AB376735
BCC 23653 Hypoxylon macrocarpum AB376824
BCC 23565 Hypoxylon nitens AB376819
BCC 21050 Hypoxylon cf. fendleri AB376807
BCC 20844-E AB376775
BCC 1101 Biscogniauxia nummularia var. exutans AB376691
BCC 1176 Hypoxylon moriforme AB376706
20: Endo/Sapro
BCC 22993 Hypoxylon duranii AB376815
BCC 22995 Hypoxylon cf. subgilvum AB376816
BCC 22982 Hypoxylon dieckmannii AB376813
BCC 20989-E AB376794
BCC 20940-E AB376783
BCC 20992-E AB376796
BCC 21002-E AB376805
BCC 1308 Hypoxylon sp.-TE AB376726
21: Endo/Sapro
BCC 21041 Daldinia sabahense AB376806
BCC 1242 Daldinia eschscholzii-TE AB376720
BCC 1313 Daldinia eschscholzii-TE AB376727
BCC 1103 Daldinia bambusicola AB376692
BCC 20324 Hypoxylon cf. symphyon AB376769
BCC 23010 Hypoxylon cf. trugodes AB376817
BCC 23674 Hypoxylon monticulosum AB376827
BCC 1109 Hypoxylon rubiginosum AB376694
BCC 21238 Whalleya sp. aff. microplaca AB376808
BCC 18798-E (Pestalotiopsis sp.) AB376757
BCC 18778-E (Pestalotiopsis sp.) AB376752
BCC 1119 (Pestalotiopsis sp.) AB376697

Results and discussion

Diversity of xylariaceous endophytes inferred from rDNA sequence analyses

Of the 224 newly isolated endophytic strains, 218 were confirmed as Xylariaceae by the sequence analyses. The other 6 were revealed to belong to other families than Xylariaceae. Identified saprobic strains were distributed throughout various groups of Xylariaceae in the phylogenetic tree based on 28S rDNA D1/D2 region sequences (Fig. 2). Newly isolated endophytic strains (marked "E" in the tree) were also segregated into various taxonomic groups of the family. We could recognize 21 clades in the phylogenetic tree (Fig. 2). In Khao Yai National Park, more than 40 species of Xylariaceae have been recorded based on the occurrence of teleomorphic stromata to date (P. Srikitikulchai, unpublished data). Seven of these teleomorphic species are likely to be present in living foliage in the park according to the result of the analysis using identified BCC strains collected in Thailand, namely, *Xylaria cubensis* (Mont.) Fr. in clade 1, *X. laevis* Lloyd in clade 5, both *X. grammica* (Mont.) Mont. and *X. obovata* (Berk.) Berk. in clade 16, both *X. bambusicola* Y.M. Ju & J.D. Rogers and *X. multiplex* (Kunze) Fr. in clade 18, and *Annulohypoxylon moriforme* (Henn.) Y.M. Ju & J.D. Rogers (as *Hypoxylon moriforme*) in clade 20. The more comprehensive analyses using the sequence data of 28S rDNA D1/D2 and rDNA ITS regions retrieved from the sequence databases indicated that the 21 clades shown in Fig. 2 were mostly retained, harboring the same strains. The comprehensive analyses showed possibilities that clades 11–14 were combined into a single cluster; each of clades 1, 16, and 17 was separated into two groups depending on the combination of operational taxonomic units (OTUs). As one of the phylogenetic analyses based on ITS sequences, an NJ tree generated by the ITS sequences of the same 63 endophytic strains used in 28S rDNA analysis is shown in Fig. 2, and the sequences retrieved from the sequence databases are shown in Fig. 3. In this tree, clade 1 seemed to separate into two groups, and clades 11 and 12 were integrated in a single cluster, while the other clades were retained as well as those in Fig. 2. As an overall result based on the sequence analyses, about 20 species of Xylariaceae were found as foliar endophytes in the Khao Yai site.

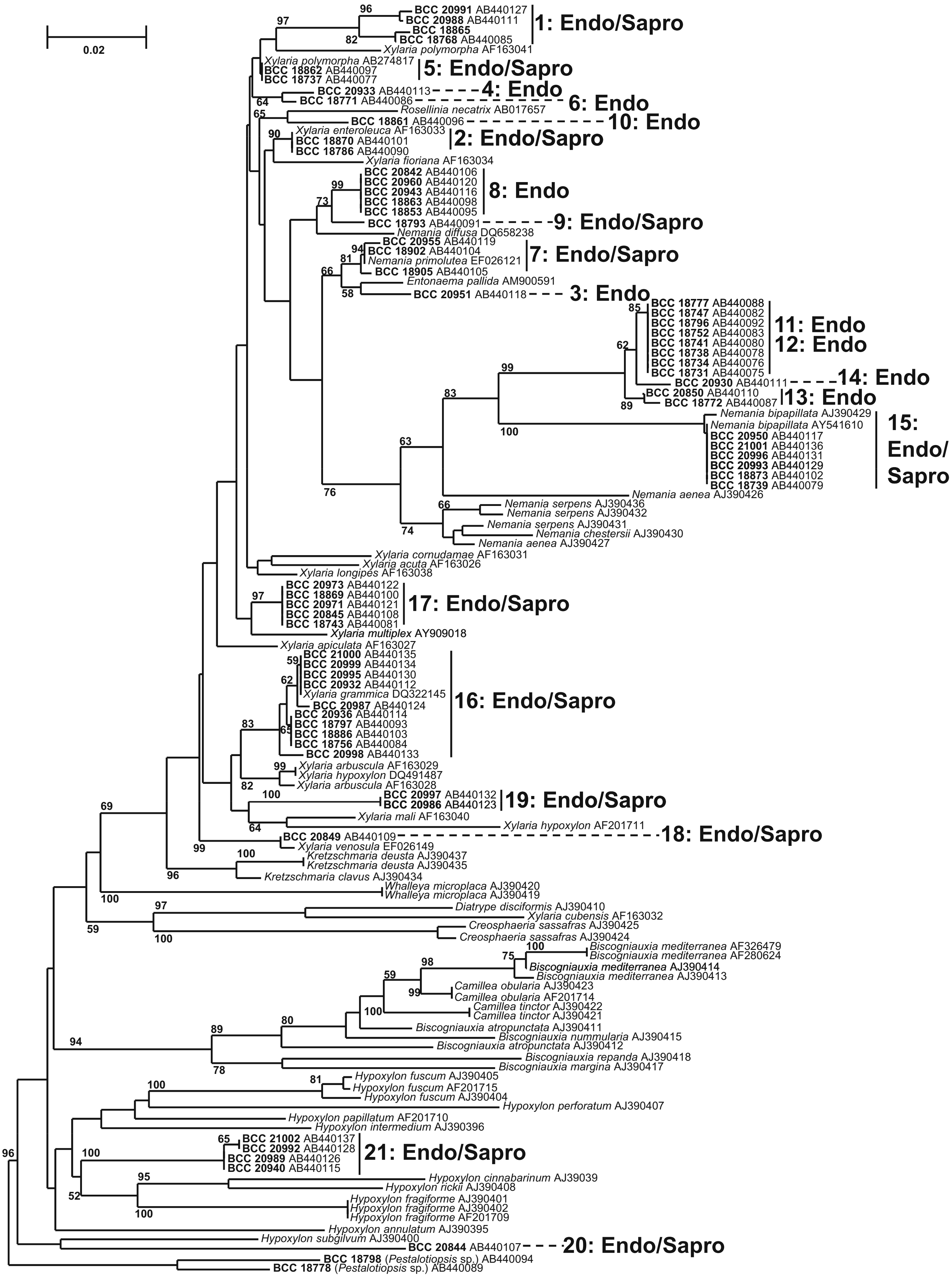
Relatives of *Xylaria* and *Nemania* as major endophytic genera

The phylogenetic analyses and the BLAST search indicated that 19 relative species of the genus *Xylaria* and *Nemania* having *Geniculosporium*-like anamorphs are likely to be the most commonly isolated xylariaceous endophytes at the site, although the definition of those generic taxa is probably still not clear, especially *Xylaria*, being mentioned as being nonmonophyletic (Lee et al. 2000; Davis et al. 2003). Conversely, species of *Hypoxylon* and related fungi seem to be less frequently encountered. Only clades 20 and 21 include such strains, namely, BCC 1176 *A. moriforme* (as *H. moriforme*) and BCC 1308 *Hypoxylon* sp. isolated as an endophyte of teak, respectively. According to the BLAST search on the sequence databases, the clade 21 probably belongs to the closely related *Daldinia* Ces. & De Not. A similar trend of fungal populations was also reported on palms, *Euterpe oleracea* in Brazil (Rodrigues 1994; Rodrigues and Petrini 1997) and *Licuala* sp. (Fröhlich et al. 2000). In the endophytic fungal assemblages of teak leaves studied by Mekkamol (1998), the isolation frequency and species number of *Hypoxylon* spp. were apparently lower than those of *Xylaria* spp. Chareprasert et al. (2006) reported 11 species of *Xylaria* and *Daldinia eschscholzii* (Ehrenb.) Rehm from teak leaves, but not *Hypoxylon* spp.

Clades including saprobes forming fruit body

Some of the strains from living leaves clustered with identified and/or unidentified saprobic strains in 12 clades (clades 1, 2, 5, 7, 9, 15–21; Figs. 2, 3). This observation indicates that some saprobic Xylariaceae also live inside living foliage, or at least are viable upon cultivation. According to sequence analyses based on the 28S rDNA and rDNA ITS regions including the data deposited in the sequence databases, 4 of the 12 clades, clades 1 and 19–21, did not include any already deposited sequence data of 28S rDNA and/or ITS regions. On the other hand, the other 8 clades seemed to closely relate with the already deposited data (see Fig. 3 as an example of the ITS analyses), although the picture was somewhat unstable depending on the combination of OTUs: *Xylaria enteroleuca* (Speg.) P.M.D. Martin AF163033 with clade 2; *X. polymorpha* (Pers.) Grev. AB274817 with clade 5; *Entonaema pallida* G.W. Martin AM900591, *Nemania primolutea* Y.M. Ju, H.M. Hsieh & J.D. Rogers EF026121 with clade 7; *N. diffusa* (Sowerby) Gray DQ840076 with clade 9; *Nemania bipapillata* (Berk. & M.A. Curtis) Pouzar AJ390429 and AY541610 with clade 15; *X. arbuscula* Sacc. AF163028, *X. hypoxylon* (L.) Grev. DQ491487, *X. grammica* DQ322145, *N. diffusa* DQ658238 with clade 16, *X. multiplex* AY909018, and *Halorosellinia oceanica* (S. Schatz) Whalley, E.B.G. Jones, K.D. Hyde & Læssøe AY083822 with clade 17; *X. venosula* Speg. EF026149 with clade 18. Although some might be already have been isolated from living tissues as endophytes, this is the first report to

Fig. 3. Neighbor-joining tree generated from the alignment of the rDNA ITS regions sequences of 63 BCC strains and 63 sequences retrieved from the databases. *Arabic numerals* are used to number individual clades; "*Endo/Sapro*" and "*Endo*" indicate clades including both endophytic and saprobic strains and endophytic strains, respectively. Two strains identified as *Pestalotiopsis* (Amphisphaeriaceae, Xylariales) in this study (BCC 18778 and BCC 18798) were specified as the outgroup. Bootstrap values > 50% are shown above branches. *Bar* 0.02 K_{nuc} in nucleotide sequences


0.02
BCC 20991 AB440127
BCC 20988 AB440111
BCC 18865
BCC 18768 AB440085
Xylaria polymorpha AF163041
1: Endo/Sapro
Xylaria polymorpha AB274817
BCC 18862 AB440097
BCC 18737 AB440077
5: Endo/Sapro
BCC 20933 AB440113
4: Endo
BCC 18771 AB440086
6: Endo
Rosellinia necatrix AB017657
BCC 18861 AB440096
10: Endo
Xylaria enteroleuca AF163033
BCC 18870 AB440101
BCC 18786 AB440090
2: Endo/Sapro
Xylaria fioriana AF163034
BCC 20842 AB440106
BCC 20960 AB440120
BCC 20943 AB440116
BCC 18863 AB440098
BCC 18853 AB440095
8: Endo
BCC 18793 AB440091
9: Endo/Sapro
Nemania diffusa DQ658238
BCC 20955 AB440119
BCC 18902 AB440104
Nemania primolutea EF026121
BCC 18905 AB440105
7: Endo/Sapro
Entonaema pallida AM900591
BCC 20951 AB440118
3: Endo
BCC 18777 AB440088
BCC 18747 AB440082
BCC 18796 AB440092
BCC 18752 AB440083
BCC 18741 AB440080
BCC 18738 AB440078
BCC 18734 AB440076
BCC 18731 AB440075
11: Endo
12: Endo
BCC 20930 AB440111
14: Endo
BCC 20850 AB440110
BCC 18772 AB440087
13: Endo
Nemania bipapillata AJ390429
Nemania bipapillata AY541610
BCC 20950 AB440117
BCC 21001 AB440136
BCC 20996 AB440131
BCC 20993 AB440129
BCC 18873 AB440102
BCC 18739 AB440079
15: Endo/ Sapro
Nemania aenea AJ390426
Nemania serpens AJ390436
Nemania serpens AJ390432
Nemania serpens AJ390431
Nemania chestersii AJ390430
Nemania aenea AJ390427
Xylaria cornudamae AF163031
Xylaria acuta AF163026
Xylaria longipes AF163038
BCC 20973 AB440122
BCC 18869 AB440100
BCC 20971 AB440121
BCC 20845 AB440108
BCC 18743 AB440081
17: Endo/Sapro
Xylaria multiplex AY909018
Xylaria apiculata AF163027
BCC 21000 AB440135
BCC 20999 AB440134
BCC 20995 AB440130
BCC 20932 AB440112
Xylaria grammica DQ322145
BCC 20987 AB440124
BCC 20936 AB440114
BCC 18797 AB440093
BCC 18886 AB440103
BCC 18756 AB440084
BCC 20998 AB440133
16: Endo/Sapro
Xylaria arbuscula AF163029
Xylaria hypoxylon DQ491487
Xylaria arbuscula AF163028
BCC 20997 AB440132
BCC 20986 AB440123
19: Endo/Sapro
Xylaria mali AF163040
Xylaria hypoxylon AF201711
BCC 20849 AB440109
18: Endo/Sapro
Xylaria venosula EF026149
Kretzschmaria deusta AJ390437
Kretzschmaria deusta AJ390435
Kretzschmaria clavus AJ390434
Whalleya microplaca AJ390420
Whalleya microplaca AJ390419
Diatrype disciformis AJ390410
Xylaria cubensis AF163032
Creosphaeria sassafras AJ390425
Creosphaeria sassafras AJ390424
Biscogniauxia mediterranea AF326479
Biscogniauxia mediterranea AF280624
Biscogniauxia mediterranea AJ390414
Biscogniauxia mediterranea AJ390413
Camillea obularia AJ390423
Camillea obularia AF201714
Camillea tinctor AJ390422
Camillea tinctor AJ390421
Biscogniauxia atropunctata AJ390411
Biscogniauxia nummularia AJ390415
Biscogniauxia atropunctata AJ390412
Biscogniauxia repanda AJ390418
Biscogniauxia margina AJ390417
Hypoxylon fuscum AJ390405
Hypoxylon fuscum AF201715
Hypoxylon fuscum AJ390404
Hypoxylon perforatum AJ390407
Hypoxylon papillatum AF201710
Hypoxylon intermedium AJ390396
BCC 21002 AB440137
BCC 20992 AB440128
BCC 20989 AB440126
BCC 20940 AB440115
21: Endo/Sapro
Hypoxylon cinnabarinum AJ39039
Hypoxylon rickii AJ390408
Hypoxylon fragiforme AJ390401
Hypoxylon fragiforme AJ390402
Hypoxylon fragiforme AF201709
Hypoxylon annulatum AJ390395
Hypoxylon subgilvum AJ390400
BCC 20844 AB440107
20: Endo/Sapro
BCC 18798 (Pestalotiopsis sp.) AB440094
BCC 18778 (Pestalotiopsis sp.) AB440089

demonstrate that *E. pallida*, *H. oceanica*, *N. bipapillata*, *N. diffusa*, *N. primolutea*, *X. bambusicola*, *X. grammica*, and *X. venosula* are clustered with endophytic isolates (Petrini and Petrini 1985; Rodrigues et al. 1993; Læssøe and Lodge 1994; Rodrigues 1994; Lodge et al. 1996; Bayman et al. 1998; Mekkamol 1998; Charaprasert et al. 2006). It should be noted, however, that some of the names attached to the deposited sequence data might be incorrect: clade 1, that includes several Thai isolates identified as *X. cubensis*, and clade 9 are presumed to be *X. cubensis* and *N. diffusa*, respectively. It was found that clade 15 includes not only endophytic strains from Thailand, but also strains isolated from 12 subtropical plants including orchids in Okinawa Prefecture, Japan (I. Okane, unpublished data). ITS sequence analysis indicated that this clade is close to *N. bipapillata* (see Fig. 3), a presumed cosmopolitan (subtropical-tropical) species (Ju and Rogers 1999). The strains from Thailand and Okinawa were similar in their cultural characters. Such cultural resemblances were found between endophytic and saprobic strains clustered in the same clades. However, the other clades are still unclear in their species-level status, because, for example, different named strains were clustered in the same clade, especially in clades 16 and 18.

Xylaria cubensis, one of the most common *Xylaria* species found as teleomorph and in its independently sporulating coremioid anamorph on decomposing wood (Thienhirun 1997), is also one of the most frequently isolated species from living leaves. Although *X. cubensis* has been reported as one of the major endophytes of palms (Rodrigues 1994; Rodrigues and Petrini 1997; Fröhlich et al 2000), this fungus probably inhabits foliage of other tropical plants as well. Further investigations of its "host" plants in relation to both endophytic and saprobic phases are required to clarify the ecology of this fungus, e.g., niche and specific hosts for production of its teleomorph, that is, "expression specificity" as proposed by Petrini (1996). Strain-level studies may reveal possible physiological differentiation corresponding to ecological adaptation within a species, not only for this fungus but also for other proposed endophytic-saprobic species.

Although the endophytic lifestyles of some Xylariaceae are considered to just be evolutionary dead ends (Bayman et al. 1998), that is, nonfunctional or accidental infections, so far no fruiting bodies of *X. cubensis* have been reported on any kind of dead leaves in the litter layer in tropical forests, despite its occurrence in the living leaves. A similar phenomenon was found in endophytic fungi of *Manilkara bidentata* (Bayman et al. 1998). Several hypotheses on lifestyles and life cycles of xylariaceous endophytes have been offered (Rodrigues et al. 1993; Læssøe and Lodge 1994; Lodge et al. 1996; Whalley 1996; Bayman et al. 1998; Rogers 2000; Collado et al. 2001; Osono 2002; Osono et al. 2004; Promputtha et al. 2007). Carroll (1999) proposed "foraging ascomycetes" for endophytic fungi having a host-switching strategy that permits dispersal and persistence when a primary host is unavailable, or when the fungus alternates between host taxa: one within which it exists as a cryptic endophyte and another on which it is saprobic or pathogenic (Rogers 2000; Davis et al. 2003). On the other hand, Bayman et al. (1998) described that such endophytic infection of Xylariaceae may be a dead end for fungi, as was already mentioned. Many Xylariaceae are found only from living leaves as endophytes but are seldom found to produce their stromata on the leaves or isolated from fallen leaves (Bayman et al. 1998; Promputtha et al. 2007), while some were isolated even from fallen leaves and presumed to survive inside leaves and to play a role in decomposing leaves (Osono 2002; Osono et al. 2004). Further isolation tests and field surveys are still needed to find them and/or any forms of the fungi on fallen leaves and to reveal whether the lifestyles of such Xylariaceae are categorized as the foraging ascomycete lifestyle or a dead end. Concerning the dead end, in the case that some fungi die out afterward although they can penetrate in some way and quiescently inhabit host plant tissues, which may not be the primary host, it looks like a kind of abortive migration, that is, "expatriation" as employed in the ecology of fish and other animals (Iwata and Hosoya 2005; Onikura et al. 2008). It is expected that such endophytic fungi penetrating into plant tissues without the induction of host resistance will be able to survive adapting to environmental change or the extinction of their primary hosts. Further studies on anatomical features and on viability in plant tissues are also needed to identify their ecological functions as components of the forest ecosystem.

Clades consisting solely of endophytic strains

Nine clades consisted solely of strains derived from living plant tissue (clades 3, 4, 6, 8, and 10–14). Such endophytic clades could indicate that some of these behave mainly or exclusively as endophytes or parasitic symbionts during their life cycle, and some may be species with sequences not yet available from public databases. We need to examine other known species in detail and to conduct further field surveys to detect identical "free-living" fungi, some of which may produce only anamorphic stages on specific substrates. It is also possible that some of these fungi no longer produce teleomorphic stages, so future field work should also target anamorphic stages, not only teleomorphic stromata.

In this study using endophytic xylariaceous strains derived from various plants, host-specific fungi have not been found so far. Although most of the endophytic Xylariaceae have been known to be non-host specific (Læssøe and Lodge 1994; Bayman et al. 1998), further study with strains isolated from different plants is required to reveal any difference of host preference of the endophytic Xylariaceae.

As expected when introducing strains from other workers and from the sequence databases, differently named strains are in some cases nested in the same cluster. This result shows that there are contradictions between morphological and phylogenetic information, and some of them might be misidentified for lack of morphological information. Among them, endophytic strains that were isolated from teak and identified based on the morphology of stromata produced in culture on woody materials are also included (Mekkamol et al. 1997; Mekkamol 1998). Those morphological characters of the induced stromata might be insufficient to identify them precisely. Although the originally registered name of strains of BCC were generally used in this article, such questionable strains need to be rechecked by referring to the voucher specimens held in the BIOTEC herbarium and others.

To isolate endophytic Xylariaceae from foliage of tropical plants collected, selective direct-hyphal isolation was conducted. Although exhaustive isolation of all fungi growing from leaf tissues might be a more desirable way to isolate all xylariaceous endophytes, it is expected that we can efficiently isolate various xylariaceous endophytes by targeting typical mycelia of linear, nondendritic branching, silky, and with white hyphae at the initial growth (see Fig. 1).

More information about both endophytes and saprobes is necessary to elucidate the diversity and ecology of the Xylariaceae. Further studies of the species-level identification of the strains examined and of the intraspecies variation are required to reveal their ecological and physiological diversification. Studies on host preference of xylariaceous endophytes is also necessary to describe their lifestyle, life cycle, and ecological functions.

Acknowledgments We sincerely thank Dr. M. Tanticharoen Director of BIOTEC, Mr. K. Okuda Director-General of Dept. of Biotech. of NITE (NITE-DOB), and Dr. M. Miyazaki the former Director-General of NITE-DOB for their implementation of the Memorandum of Understanding between the two organizations and supporting our collaborative research work. We thank the BIOTEC staff of the Ecology Laboratory for their help in identifying the plants tested, the Mycology Laboratory for their kind cooperation in field and laboratory work, and the BCC for their help in preservation of isolates and making these available for our research. This study was carried out under a collaborative research project between the two culture collections, NITE Biological Resource Center (NBRC) in Japan and BIOTEC Culture Collection (BCC) in Thailand.

References

Bayman P, Angulo-Sandoval P, Baez-Ortiz Z, Lodge DJ (1998) Distribution and dispersal of *Xylaria* endophytes in two tree species in Puerto Rico. Mycol Res 102:944–948

Brunner F, Petrini O (1992) Taxonomy of some *Xylaria* species and xylariaceous endophytes by isozyme electrophoresis. Mycol Res 96: 723–733

Carroll GC (1999) The foraging ascomycete. 16th International Botanical Congress, St. Louis, MO, USA, abstracts, p 309

Chareprasert S, Piapukiew J, Thienhirun S, Whalley A, Sihanonth P (2006) Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World J Microbiol Biotechnol 22:481–486

Collado J, Platas G, Pelaez F (2001) Indentification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. Mycologia 93:875–886

Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. Am J Bot 90:1661–1667

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fröhlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. Mycol Res 104:1202–1212

Iwata A, Hosoya S (2005) Environmental assessment of the Shimanto estuary based on biodiversity of gobioid fishes. Aquabiology 27: 39–46

Ju YM, Rogers JD (1999) The Xylariaceae of Taiwan (excluding *Anthostomella*). Mycotaxon 73:343–440

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

Læssøe T, Lodge DJ (1994) Three host-specific *Xylaria* species. Mycologia 86:436–446

Lee JS, Ko KS, Jung HS (2000) Phylogenetic analysis of *Xylaria* based on nuclear ribosomal ITS1-5.8S-ITS2 sequences. FEMS Microbiol Lett 187:89–93

Lodge DJ, Fisher PJ, Sutton BC (1996) Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. Mycologia 88:733–738

Lumyong S, Thongantha S, Lumyong P, Tomita F (2000) Endophytic fungi from 13 bamboo species in Thailand. Biotechnol Sustainable Utilization Biol Resour Trop 14:96–101

Mekkamol S (1998) Endophytic fungi in *Tectona grandis* L. (Teak). PhD thesis. Liverpool John Moores University, Liverpool

Mekkamol S, Jonglaekha N, Hywel-Jones NL, Whalley AJS (1997) Endophytic fungi of teak in Thailand: a preliminary account. BIOTEC News 3:4–5

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB Interntional, Wallingford, pp 225–233

Onikura N, Nakajima J, Nishida T, Inui R, Eguchi K, Nakatani M, Oikawa S (2008) Spawning sites of *Eleotris acenthopoma* (Perciformes: Eleotridae) in the Kitagawa and Sumiegawa Rivers, Kyushu Island, Japan. Ichthyological Research doi: 10.1007/s10228-008-0058-x

Osono T (2002) Phyllosphere fungi on leaf litter of *Fagus crenata*: occurrence, colonization, and succession. Can J Bot 80:460–469

Osono T, Bhatta BK, Takeda H (2004) Phyllosphere fungi on living and decomposing leaves of giant dogwood. Mycoscience 45:35–45

Photita W, Lumyong S, Lumyong P, Hyde KD (2001) Endophytic fungi of wild banana (*Musa acuminata*) at Doi Suthep Pui National Park, Thailand. Mycol Res 105:1508–1513

Perrière G, Gouy M (1996) WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78:364–369

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrew JH, Hirano SS (eds) Microbial ecology of leaves. Springer-Verlag, New York, pp 179–197

Petrini O (1996) Ecological and physiological aspects of host-specificity in endophytic fungi. In: Redlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants. American Phytopathological Society (APS) Press, St. Paul, MN, pp 87–100

Petrini LE, Petrini O (1985) Xylariaceous fungi as endophytes. Sydowia 38:216–234

Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microb Ecol 53: 579–590

Rodrigues KF (1994) The foliar fungal endophytes of the Amazoniam palm *Euterpe oleracea*. Mycologia 86:376–385

Rodrigues KF, Petrini O (1997) Biodiversity of endophytic fungi in tropical regions. In: Hyde KD (ed) Biodiversity of tropical microfungi. Hong Kong University Press, Hong Kong, pp 57–69

Rodrigues KF, Samuels GJ (1990) Preliminary study of endophytic fungi in a tropical palm. Mycol Res 94:827–830

Rodrigues KF, Leuchtmann A, Petrini O (1993) Endophytic species of *Xylaria*: cultural and isozymic studies. Sydowia 45:116–138

Rogers JD (2000) Thoughts and musings on tropical Xylariaceae. Mycol Res 104:1412–1420

Rogers JD, Ju Y-M, Lehmann J (2005) Some *Xylaria* species on termite nests. Mycologia 97:914–923

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

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thienhirun S (1997) A preliminary account of the Xylariaceae of Thailand. PhD thesis. Liverpool John Moores University, Liverpool

Whalley AJS (1996) The xylariaceous way of life. Mycol Res 100: 897–922

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfaud DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322