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FULL PAPER

Recognition and characterization of four Thai xylariaceous fungi inhabiting various tropical foliages as endophytes by DNA sequences and host plant preference

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Abstract A total of 202 strains of xylariaceous fungi (183 endophytic strains isolated from 25 plant species of 24 genera in 21 families and 19 saprobic strains), segregated into four clades, were examined by nuclear rDNA internal transcribed spacer (ITS) sequence and beta-tubulin coding region analyses to clarify their taxonomic status and species boundaries. Three of the four species clades were assigned to *Xylaria cubensis* (100 strains), *Xylaria grammica* (33 strains), and *Nemania diffusa* (48 strains). Another fungus was tentatively assigned to *Nemania* cf. *bipapillata* (21 strains). Comparison of the host plants revealed that *X. cubensis* inhabited healthy leaves of at least 24 plant species (23 genera of 21 families) as endophytes; *N. diffusa* was found on 19 plant species (18 genera of 15 families), *Nemania* cf. *bipapillata* on 11 species (10 genera of 9 families), and *X. grammica* on 8 species (8 genera of 7 families). The present results suggest that the major xylariaceous endophytes in tropical plants are likely to be non-host specific, or have a wide range of host plant preferences.

Keywords Endophytic fungi · *Nemania* · Tropical plants · *Xylaria*

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Introduction

The family Xylariaceae includes a group of fungi that play important roles in the forest ecosystem as decomposers of plant materials and plant pathogens; some are associated with insects and many are endophytes (Petrini and Petrini 1985; Rogers et al. 2005). Xylariaceous endophytes have been found in all investigated major groups of terrestrial plants (Brunner and Petrini 1992; Davis et al. 2003). Some Xylariaceae species are considered 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 the lifestyle and life cycles of endophytic or saprobic Xylariaceae have been published (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, because of difficulties in species-level identification, especially for endophytic isolates, the biological significance of these fungi is still unclear despite their ubiquitous existence inside living plant tissues.

In a study of the diversity and ecology of xylariaceous endophytes in Khao Yai National Park in Thailand, 21 xylariaceous fungi, which were recognized based on ribosomal DNA sequence analysis, were found to be endophytes of tropical foliage (Okane et al. 2008). Most of these 21 xylariaceous fungi possessed the ability to endophytically infect several different plants. Four species clades among the 21 xylariaceous fungi were likely to be major tropical plant endophytes, and each clade consisted of both endophytic strains and several saprophytic strains. The four clades were considered to be important xylariaceous endophytes at the site and were thought to possess the

ability to inhabit the foliage of a wide range of tropical plants. However, xylariaceous fungi rarely exhibit their morphological characteristics in culture. To identify xylariaceous isolates, in particular the endophytic ones, rigorous cultural and isozyme diagnoses were attempted (Whalley and Greenhalgh 1975; Petrini and Petrini 1985; Brunner and Petrini 1992; Rodrigues et al. 1993). Profiling of secondary metabolites was also conducted to identify the fungi (Whalley and Edwards 1995). However, the precise identification of certain xylariaceous endophytes is difficult, even when applying such methods. DNA sequence analyses are powerful tools for genus- or species-level identification, especially for endophytic strains. Moreover, precise identification and rigorous species delimitation is required to estimate a host range and preference for each species. In the present study, xylariaceous strains that were segregated into four clades by phylogenetic analysis based on nuclear 28S rDNA D1/D2 region sequences (Okane et al. 2008) were examined by nuclear rDNA internal transcribed spacer (ITS) sequence analysis and beta-tubulin gene coding regions to clarify their taxonomic status and species boundaries. The host plants for the endophytic phase of the four xylariaceous fungi were also investigated to examine their host specificity or host plant preferences.

Materials and methods

Strains examined

A total of 202 strains that were segregated into four clades by 28S rDNA D1/D2 region sequence analysis in a previous study (Okane et al. 2008) were included in this study. Among the 202 strains, 181 endophytic strains except for 2 strains were isolated in a study by Okane et al. (2008); the others (19 strains) are saprophytic strains that were previously isolated from fruit bodies by Thai researchers and maintained in the BIOTEC Culture Collection (BCC). The endophytic strains were isolated from tropical foliage in a permanent plot of Khao Yai National Park in Thailand in 2005 and 2006. The plants tested belong to 24 species of 23 genera in 20 families including pteridophytes. Table 1 lists representative strains deposited in the NITE Biological Resource Center (NBRC) collection and selected from each clade, together with their host plants.

DNA sequence 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 placed into 2-ml plastic tubes using a spatula. DNA was extracted using a Nucleon PhytoPure DNA extraction kit (GE Healthcare UK, Buckinghamshire, England) or DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Sequence analysis of nuclear rDNA ITS and beta-tubulin gene coding regions

The ITS regions of rDNA were amplified by polymerase chain reaction (PCR) using TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan) or Blend Taq Plus (Toyobo, Fukui, Japan) as a single fragment with the standard primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). 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: 3 min at 95°C, 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 using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a thermal cycler employing the following ramp: 3 min at 96°C, 25 cycles of 10 s at 96°C, 5 s at 50°C and 2 min at 60°C, followed by a 4°C soak. Nucleotide sequences were determined in both directions using the primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGGAGC-3′), ITS4, and ITS5 (White et al. 1990). Sequences were obtained with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

The beta-tubulin gene coding regions 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 T1 (5′-A ACATGCGTGAGATTGTAAGT-3′) and T22 (5′-TCT GGATGTTGTTGGGAATCC-3′) (O'Donnell and Cigelnik 1997). Amplification of the desired fragment was performed using the method described for the rDNA ITS analysis. Amplified DNA was sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as described for the rDNA ITS analysis. Nucleotide sequences were determined in both directions using the primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′), Bt2b-R (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (modified Bt2b), T12 (5′-AACAACTGGGCCAAGGGT CAC-3′), Bt1a (5′-TTCCCCCGTCTCCACTTCTTCATG-3′), T21 (5′-GGTTTGCCAGAAAGCAGCACC-3′), Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′), T224 (5′-G AGGGAACGACGGAGAAGGTGG-3′), T1 and T22 (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997), and 1853R (5′-ATCTGGTCCTCAACCTCCTTC-3′) (designed in this study). The beta-tubulin sequencing of one

Table 1 List of representative strains selected from each clade of the four xylariaceous endophytes

NBRC no.	BCC no.	Species	Source (host plant)	Plant family	Accession no.		Identities with voucher	
					Internal transcribed spacer (ITS)	Beta-tubulin	ITS sequences[a]	Beta-tubulin sequences[b]
104653	1027	*Xylaria cubensis*	Wood	–	AB625412	AB625355	547/563 (97%)	1514/1546 (97%)
104655	1144	*Xylaria cubensis*	Wood	–	AB625413	AB625356	547/563 (97%)	1508/1546 (97%)
104661	1219	*Xylaria cubensis*	*Tectona grandis*	Verbenaceae	AB625415	AB625358	547/563 (97%)	1512/1545 (97%)
104669	1303	*Xylaria cubensis*	*Tectona grandis*	Verbenaceae	AB625418	AB625361	546/563 (96%)	1490/1521 (97%)
104681	18768	*Xylaria cubensis*	*Saprosma longifolium*	Rubiaceae	AB440085	AB625372	556/582 (95%)	1442/1525 (94%)
104722	20988	*Xylaria cubensis*	*Ardisia nervosa*	Myrsinaceae	AB440125	AB625402	547/563 (97%)	1509/1545 (97%)
104724	20991	*Xylaria cubensis*	*Ardisia nervosa*	Myrsinaceae	AB440127	AB625403	545/565 (96%)	1516/1545 (98%)
107985	1227	*Xylaria cubensis*	*Tectona grandis*	Verbenaceae	AB625416	AB625359	547/563 (97%)	1510/1545 (97%)
107986	1255	*Xylaria cubensis*	*Tectona grandis*	Verbenaceae	AB625417	AB625360	546/563 (96%)	1518/1548 (98%)
107987	18723	*Xylaria cubensis*	*Gonocaryum lobbianum*	Icacinaceae	AB625419	AB625362	545/565 (96%)	1505/1545 (97%)
107988	18730	*Xylaria cubensis*	*Salacia chinensis*	Celastraceae	AB625420	AB625363	546/563 (96%)	1512/1545 (97%)
107991	18758	*Xylaria cubensis*	*Pteris decrescens*	Pteridaceae	AB625423	AB625368	547/563 (97%)	1514/1545 (97%)
107992	18762	*Xylaria cubensis*	*Selaginella inaequalifolia*	Selaginellaceae	AB625424	AB625369	557/579 (96%)	1489/1545 (96%)
107994	18765	*Xylaria cubensis*	*Thelypteris triphylla*	Thelypteridaceae	AB625426	AB625371	547/563 (97%)	1509/1545 (97%)
107995	18792	*Xylaria cubensis*	*Polyalthia simiarum*	Annonaceae	AB625427	AB625373	547/563 (97%)	1520/1546 (98%)
107996	18803	*Xylaria cubensis*	*Miliusa lineata*	Annonaceae	AB625428	AB625376	547/563 (97%)	1511/1547 (97%)
107997	18872	*Xylaria cubensis*	*Saprosma longifolium*	Rubiaceae	AB625429	AB625379	558/579 (96%)	1515/1546 (97%)
107998	18879	*Xylaria cubensis*	*Ancistrocladus tectorius*	Ancistrocladaceae	AB625430	AB625381	557/579 (96%)	1516/1545 (98%)
107999	18880	*Xylaria cubensis*	*Melicope pteleifolia*	Rutaceae	AB625431	AB625382	545/565 (96%)	1502/1545 (97%)
108000	18881	*Xylaria cubensis*	*Dipterocarpus gracilis*	Dipterocarpaceae	AB625432	AB625383	547/563 (97%)	1488/1521 (97%)
108007	20957	*Xylaria cubensis*	*Tectaria maingayi*	Dryopteridaceae	AB625440	AB625396	558/579 (96%)	1472/1522 (96%)
108008	20964	*Xylaria cubensis*	*Microlepia herbacea*	Dennstaedtiaceae	AB625441	AB625398	556/581 (95%)	1445/1525 (94%)
104680	18756	*Xylaria grammica*	*Pteris decrescens*	Pteridaceae	AB440084	AB625367	558/563 (99%)	1541/1555 (99%)
104688	18797	*Xylaria grammica*	*Ardisia nervosa*	Myrsinaceae	AB440093	AB625375	558/563 (99%)	1541/1555 (99%)
104697	18886	*Xylaria grammica*	*Saprosma longifolium*	Rubiaceae	AB625433	AB625384	556/563 (98%)	1542/1555 (99%)
104709	20932	*Xylaria grammica*	*Salacia chinensis*	Celastraceae	AB440112	AB625390	560/563 (99%)	1547/1555 (99%)
104711	20936	*Xylaria grammica*	*Salacia chinensis*	Celastraceae	AB440114	AB625391	558/563 (99%)	1541/1555 (99%)
104721	20987	*Xylaria grammica*	*Ardisia nervosa*	Myrsinaceae	AB440124	AB625401	557/563 (98%)	1545/1555 (99%)
104727	20995	*Xylaria grammica*	*Polyalthia simiarum*	Annonaceae	AB440130	AB625405	560/563 (99%)	1546/1555 (99%)
104730	20998	*Xylaria grammica*	*Ardisia nervosa*	Myrsinaceae	AB440133	AB625407	556/563 (98%)	1541/1555 (99%)
104731	20999	*Xylaria grammica*	*Ardisia nervosa*	Myrsinaceae	AB440134	AB625408	560/563 (99%)	1553/1555 (99%)
104732	21000	*Xylaria grammica*	*Ardisia nervosa*	Myrsinaceae	AB440135	AB625409	559/563 (99%)	1546/1555 (99%)
107983	1002	*Xylaria grammica*	Wood	–	AB625411	AB625354	560/563 (99%)	1544/1555 (99%)
107984	1170	*Xylaria grammica*	Wood	–	AB625414	AB625357	553/563 (98%)	1491/1556 (95%)
107989	18746	*Xylaria grammica*	*Licuala spinosa*	Palmae	AB625421	AB625365	559/563 (99%)	1513/1521 (99%)
104675	18739	*Nemania* cf. *bipapillata*	*Schizostachyum longispiculatum*	Gramineae	AB440079	AB625364	393/456 (86%)	1343/1539 (87%)
104696	18873	*Nemania* cf. *bipapillata*	*Melicope pteleifolia*	Rutaceae	AB440102	AB625380	393/456 (86%)	1348/1547 (87%)
104714	20950	*Nemania* cf. *bipapillata*	*Selaginella inaequalifolia*	Selaginellaceae	AB440117	AB625395	393/456 (86%)	1348/1547 (87%)
104726	20993	*Nemania* cf. *bipapillata*	*Polyalthia simiarum*	Annonaceae	AB440129	AB625404	393/456 (86%)	1349/1548 (87%)
104728	20996	*Nemania* cf. *bipapillata*	*Ardisia nervosa*	Myrsinaceae	AB440131	AB625406	393/456 (86%)	1348/1547 (87%)
104733	21001	*Nemania* cf. *bipapillata*	*Miliusa lineata*	Annonaceae	AB440136	AB625410	393/456 (86%)	1350/1549 (87%)
107993	18763	*Nemania* cf. *bipapillata*	*Selaginella inaequalifolia*	Selaginellaceae	AB625425	AB625370	393/456 (86%)	1350/1548 (87%)
108004	20926	*Nemania* cf. *bipapillata*	*Gonocaryum lobbianum*	Icacinaceae	AB625437	AB625389	392/457 (85%)	1348/1547 (87%)

Table 1 continued

NBRC no.	BCC no.	Species	Source (host plant)	Plant family	Accession no.		Identities with voucher	
					Internal transcribed spacer (ITS)	Beta-tubulin	ITS sequences[a]	Beta-tubulin sequences[b]
108005	20944	*Nemania* cf. *bipapillata*	*Polyalthia* aff. *evecta* var. *evecta*	Annonaceae	AB625438	AB625393	393/456 (86%)	1349/1548 (87%)
108006	20948	*Nemania* cf. *bipapillata*	*Licuala spinosa*	Palmae	AB625439	AB625394	393/456 (86%)	1350/1548 (87%)
108010	20981	*Nemania* cf. *bipapillata*	*Dipterocarpus gracilis*	Dipterocarpaceae	AB625443	AB625400	393/456 (86%)	1348/1547 (87%)
104686	18793	*Nemania diffusa*	*Polyalthia* sp.	Annonaceae	AB440091	AB625374	531/561 (95%)	1433/1527 (93%)
104689	18853	*Nemania diffusa*	*Calamus palustris*	Palmae	AB440095	AB625377	542/559 (96%)	1447/1526 (94%)
104692	18863	*Nemania diffusa*	*Abrus precatorius*	Legminosae	AB440098	AB625378	542/559 (96%)	1449/1527 (94%)
104703	20842	*Nemania diffusa*	*Licuala spinosa*	Palmae	AB440106	AB625387	541/559 (96%)	1452/1526 (95%)
104713	20943	*Nemania diffusa*	*Schizostachyum longispiculatum*	Gramineae	AB440116	AB625392	542/559 (96%)	1447/1526 (94%)
104717	20960	*Nemania diffusa*	*Tectaria maingayi*	Dryopteridaceae	AB440120	AB625397	542/559 (96%)	1447/1526 (94%)
107990	18754	*Nemania diffusa*	*Pteris decrescens*	Pteridaceae	AB625422	AB625366	543/559 (97%)	1444/1528 (94%)
108001	18890	*Nemania diffusa*	*Pteris decrescens*	Pteridaceae	AB625434	AB625385	543/559 (97%)	1447/1526 (94%)
108002	18900	*Nemania diffusa*	*Cinnamomum subarenicum*	Lauraceae	AB625435	AB625386	543/559 (97%)	1451/1526 (95%)
108003	20846	*Nemania diffusa*	*Aglaia elaeagnoidea*	Meliaceae	AB625436	AB625388	542/559 (96%)	1449/1526 (94%)
108009	20970	*Nemania diffusa*	*Knema elegans*	Myristicaceae	AB625442	AB625399	542/559 (96%)	1449/1526 (94%)

[a] *Xylaria cubensis* GU373810, *Xylaria grammica* GU300097, *Nemania bipapillata* GU292818, *Nemania diffusa* GU292817

[b] *Xylaria cubensis* GQ502702, *Xylaria grammica* GQ487704, *Nemania bipapillata* GQ470221, *Nemania diffusa* GQ470220

strain, BCC 18800, isolated from *Ardisia nervosa* E. Walker (Myrsinaceae), was unsuccessful.

For the phylogenetic analysis, sequence data for the rDNA ITS and beta-tubulin gene coding regions of voucher specimens studied by Hsieh et al. (2010) were retrieved from the DDBJ/EMBL/GenBank nucleotide sequence databases, namely, ITS (accession numbers GU373810 and GU991523) and beta-tubulin (accession number GQ502702) of *Xylaria cubensis* (Mont.) Fr., ITS (GU300097) and beta-tubulin (GQ487704) of *X. grammica* (Mont.) Mont., ITS (GU292818) and beta-tubulin (GQ470221) of *Nemania bipapillata* (Berk. & M.A. Curtis) Pouzar, and ITS (GU292817) and beta-tubulin (GQ470220) of *N. diffusa* (Sowerby) Gray.

Phylogenetic analyses were conducted using Clustal X 1.83 (Thompson et al. 1997) to generate the evolutionary distances [the K_{nuc} value (Kimura 1980)] and the similarity values, and to perform the neighbor-joining (NJ) analysis (Saitou and Nei 1987) from the K_{nuc} values by bootstrap resampling (Felsenstein 1985) with 1,000 replicates for the evaluation of the topology of the phylogenetic tree. The NJ plot (Perrière and Gouy 1996) was used to generate the phylogenetic tree.

A comprehensive preliminary phylogenetic analysis using ITS and beta-tubulin sequence data of vouchers of other xylariaceous fungi analyzed by Hsieh et al. (2010) was performed to clarify the species-level definition of the strains examined (data not shown). Four clades were thus confirmed to be the closest to the aforementioned four xylariaceous fungi: *X. cubensis*, *X. grammica*, *N. bipapillata*, and *N. diffusa*.

In addition to the phylogenetic analysis, the sequence data corresponding to the strains analyzed was subjected to a BLAST search for identification and comparison to the sequence data of the voucher specimens.

Sequence data of the selected strains obtained in the present study (shown in Table 1) were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database. The corresponding accession numbers are shown in Table 1. The alignments were deposited in TreeBASE (http://www.treebase.org/treebase-web/home.html) under the Study ID number S11436.

Results and discussion

The 202 strains were clearly segregated into four clades, with the segregation supported by high bootstrap values. These clades had been similarly identified by 28S rDNA sequence analysis in a previous study (Okane et al. 2008). These data are also in agreement with prior comprehensive analyses performed with voucher specimens of other xylariaceous fungi by Hsieh et al. (2010) (data not shown). Both phylogenetic trees inferred from the ITS and beta-

tubulin sequences distinctly show the four clades, and the tree based on beta-tubulin sequences is shown in Fig. 1.

In ecological studies of endophytic fungi (Arnold and Lutzoni 2007; Higgins et al. 2007; Arnold et al. 2009), ITS data were used to designate operational taxonomic units (OTU) for analyses, and 90–95% ITS sequence similarity was often used as a proxy for species boundaries in fungi. In this study, a similarity comparison of ITS sequences between the strain examined and the vouchers was carried out using a BLAST search, and 95% ITS sequence similarity was the threshold for delimitation of species. We used the sequence similarity (>90%) of beta-tubulin to determine the species boundaries.

The clade that had been presumed to be *X. cubensis* (Okane et al. 2008) matched the species based on the ITS and beta-tubulin region sequence analyses. ITS sequence similarities between the strains of the clade and a voucher specimen of *X. cubensis* (data accession number GU373810) ranged from 95% (identities approximately 556/582) to 97% (identities approximately 547/563). Beta-tubulin sequence similarities between the strains analyzed and a voucher specimen of *X. cubensis* (data accession number GQ502702) ranged from 94% (identities approximately 1442/1525) to 98% (identities approximately 1520/1546). As a result, 100 strains (89 endophytic and 11 saprobic) included in this clade were assigned to *X. cubensis*. The similarity data of selected strains are shown in Table 1, in addition to the similarity data of three clades.

Although our previous study was not successful at the species-level identification of one of the four clades (Okane et al. 2008), the sequence analyses of the present study revealed that this clade was assignable to *X. grammica*. ITS sequence similarities between the strains included in the clade and a voucher specimen of *X. grammica* (data accession number GU300097) were approximately 98–99% (identities 556/563–560/563). Beta-tubulin sequence similarities between the strains and a voucher specimen of *X. grammica* (data accession number GQ487704) ranged from approximately 95% to 99% (identities 1491/1556–1553/1555). Thus, the clade containing 33 strains (25 endophytic and 8 saprobic) was assigned to *X. grammica*. Several BCC strains isolated from fruit bodies that were morphologically identified as *X. grammica* were included in this clade.

In the clade that was determined to be close to *N. diffusa* in Okane et al. (2008), ITS sequence similarities between the strains included in the clade and a voucher specimen of *N. diffusa* (data accession number GU292817) were approximately 95–97% (identities 531/561–543/559). The range of similarity of the beta-tubulin sequences between the strains and a voucher specimen of *N. diffusa* (data accession number GQ470220) was approximately 93–95% (identities 1433/1527–1452/1526). This clade of 48 endophytic strains was therefore assigned to *N. diffusa*.

The remaining fourth clade was previously assumed to be *N. bipapillata* in Okane et al. (2008) based on the result of a BLAST search showing approximately 99% similarity of the ITS sequences between the isolates obtained in that study and the 2 strains deposited as *N. bipapillata* in the databanks: AY541610 and AJ390429. However, in the present study, the ITS sequence similarity between these strains and a voucher specimen of *N. bipapillata* (data accession number GU292818) was quite low, i.e., approximately 86% (identities 393/456). Moreover, the beta-tubulin sequence similarities between the strains and a voucher specimen of *N. bipapillata* (data accession number GQ470221) were also low at approximately 87% (identities 1343/1539). We therefore concluded that this clade was not assignable to *N. bipapillata*, although all the strains were recognized as belonging to the same species. Because there were no data of voucher specimens of xylariaceous fungi other than *N. bipapillata* that were nested within the clade, this clade including 21 endophytic strains was considered as *Nemania* cf. *bipapillata* in this study.

Subsequent to the species delimitation of the four clades by sequence analysis, the plant species from which the four xylariaceous fungi had been isolated were compared from an ecological point of view, in particular considering their host plant preferences (Table 2).

Endophytic strains of *X. cubensis* were isolated from plants of 23 species in 22 genera, 20 families (Table 2). This fungus was not isolated from one of the 24 different plants examined, namely, *Aphanamixis polystachya* (Wall.) R. Parker (Meliaceae). In addition to the plants identified in the present study, *Tectona grandis* L.f. (Verbenaceae) is also likely to harbor this fungus according to previous studies carried out in Thailand (Mekkamol et al. 1997; Mekkamol 1998). Two endophytic strains, BCC 1219 (=NBRC 104661) and BCC 1303 (=NBRC 104669), which were isolated from *T. grandis* by Mekkamol et al. (1997) and identified as *X. cubensis*, were also examined in this study. *Xylaria cubensis* is known to be widely distributed in tropical, subtropical, and temperate regions of the world, and is usually found in decaying angiospermous wood (Rogers 1984). In Thailand, this fungus is one of the most frequently encountered xylariaceous fungi, and it forms fruit bodies on decomposing wood in the forest (Thienhirun 1997; Thienhirun and Whalley 2004). The fungus is also known to be one of the most abundant endophytes isolated from palms (Rodrigues and Samuels 1990; Rodrigues and Petrini 1997). In the present study, *X. cubensis* was found to inhabit a wide range of plants belonging to all the 21 families examined, suggesting that this fungus may inhabit a greater variety of plants than those included in this study. In previous studies analyzing endophytes of teak in Thailand, several xylariaceous fungi were isolated (Mekkamol et al. 1997; Mekkamol 1998; Chareprasert et al. 2006).

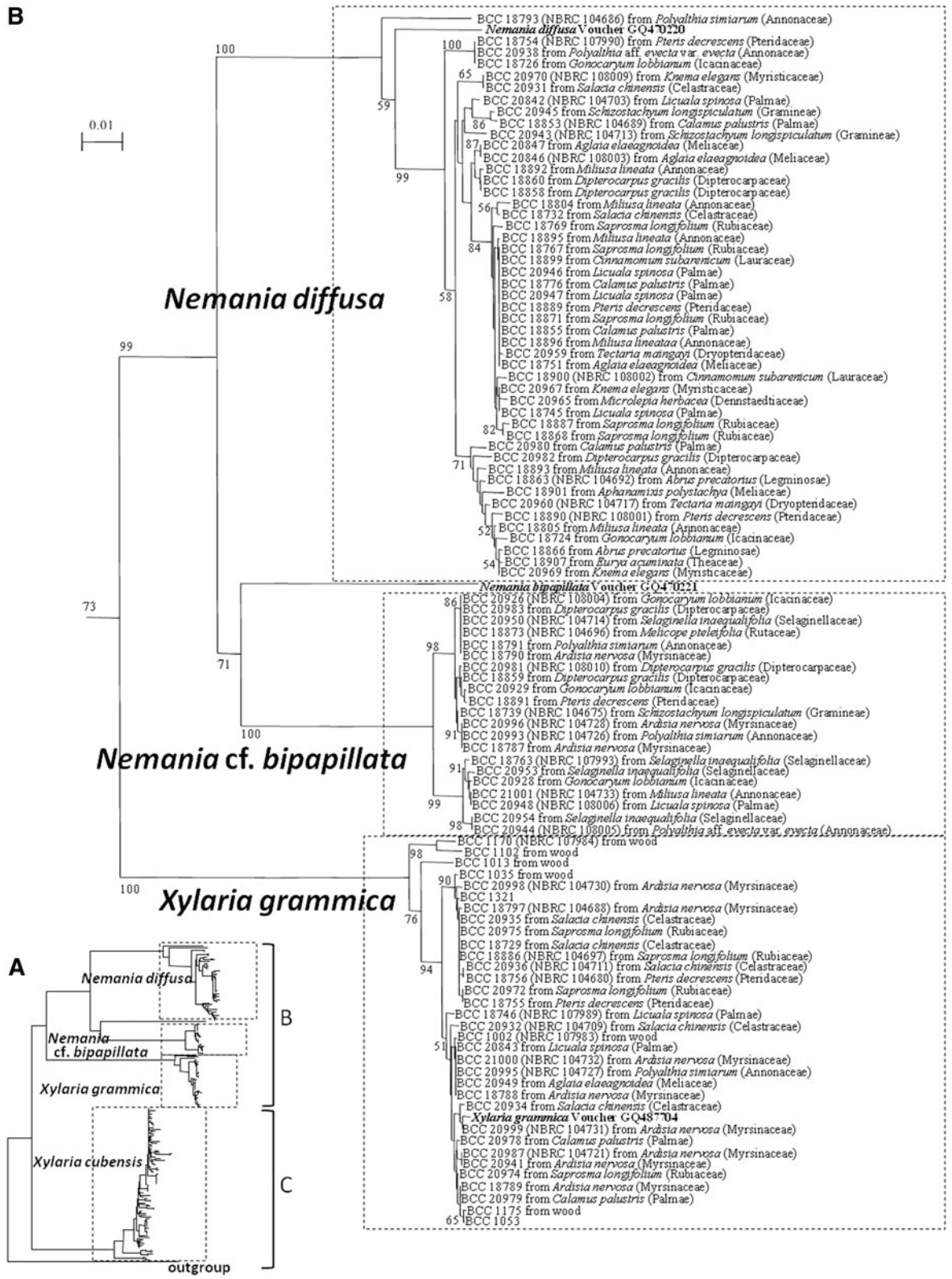

Fig. 1 Neighbor-joining tree generated from the alignment of the beta-tubulin gene coding regions of 201 strains (the sequencing of 1 strain was unsuccessful) and the data derived from vouchers. **a** An overview of the tree. **b** An enlargement of three clades other than *Xylaria cubensis*. **c** An enlargement of the *X. cubensis* clade. One strain that was identified as *Daldinia* sp., BCC 21002, was specified as the outgroup. Bootstrap values >50% are shown *above branches*. *Bar* 0.01 K_{nuc} in nucleotide sequences

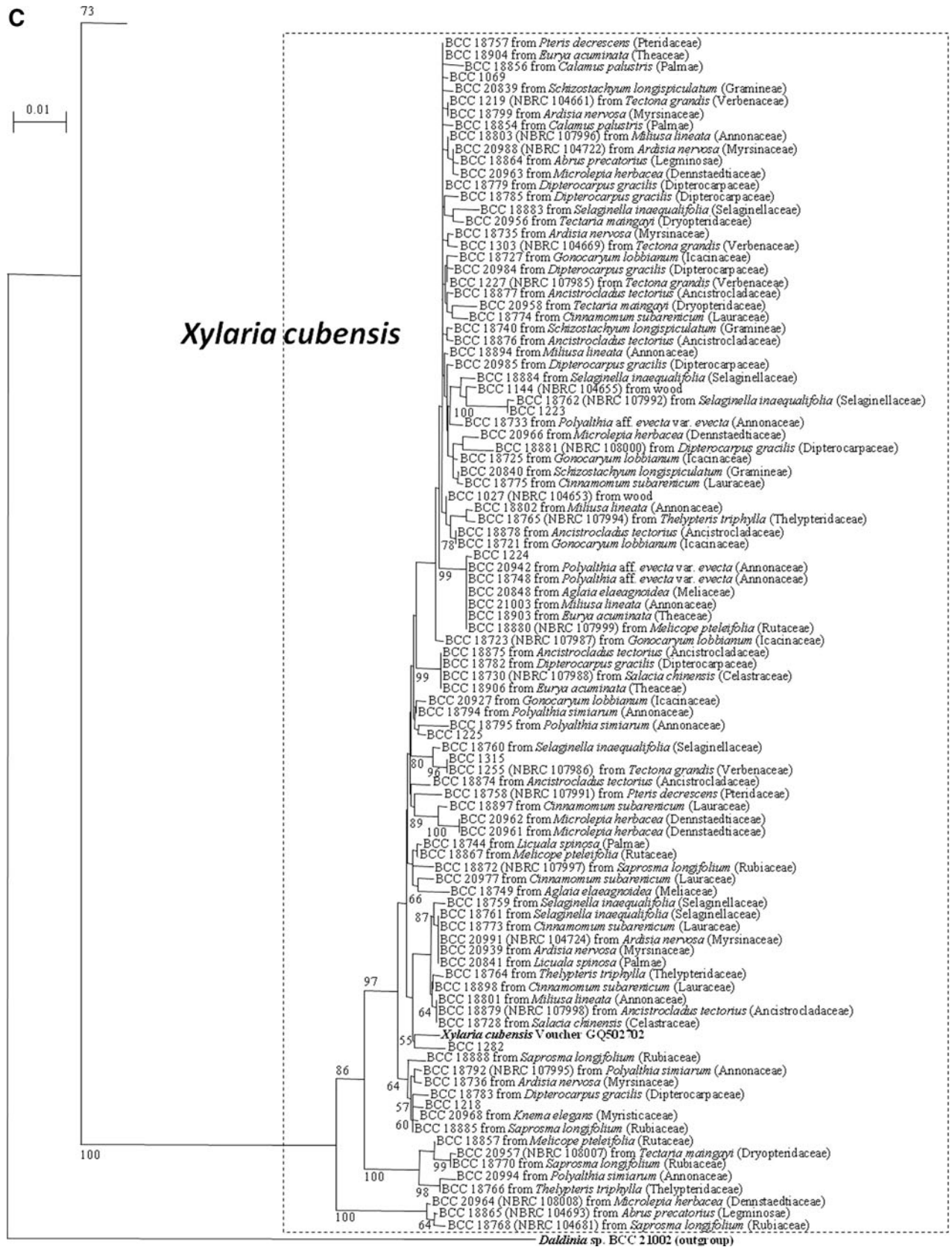

Fig. 1 continued

Chareprasert et al. (2006) reported the isolation of 11 unidentified endophytic species of *Xylaria*, among which *X. cubensis* might be included. According to Rodrigues et al. (1993), this fungus is a morphologically variable species, indicating that it could be a complex species composed of several taxa. Even among the endophytic strains genetically delimited as *X. cubensis* in our study, morphological variations in the production of anamorphic stage or blackish stroma-like structures were observed in the colony appearance on the PDA plate. A similar phenomenon was observed in the other 3 xylariaceous fungi analyzed.

Three xylariaceous fungi other than *X. cubensis* may also possess the ability to inhabit the living tissues of various plants. *Xylaria grammica*, which has been found on decomposing wood in tropical regions throughout the world (Rogers and Callan 1987), was isolated from 8 species of 8 genera within 7 families (Table 2). This fungus appears to be one of the prevalent xylariaceous endophytes in Thailand. Because this fungus has distinctive morphological characteristics in its ascomata, showing longitudinal stripes with raised lines on its stromata (Rogers and Callan 1987), it is probably easy to recognize the fungal occurrence on decomposing plant materials in the field. Therefore, *X. grammica* might be a useful species to study the distribution of fungal species, their population ecology, and the switching between saprobic and endophytic lifestyles in nature.

Nemania diffusa was isolated from various plants belonging to 19 species (18 genera, 15 families) (Table 2), following the widest host diversity of *X. cubensis*. *Nemania diffusa* is widespread and commonly found worldwide (Petrini and Petrini 1985; Pouzar 1985; Petrini and Müller 1986; Petrini and Rogers 1986; Ju and Rogers 1999, 2002). Although *N. diffusa* has been predominantly reported as inhabiting wood or bark, the present study revealed that the fungus has the ability to live within tissues of a wide range of plants as an endophyte.

Nemania cf. *bipapillata* was isolated from 11 species (10 genera, 9 families) of plants (Table 2). In Japan, many genetically identical strains of the same fungus have been isolated from fresh leaves of several subtropical plants collected in the southwest islands of Japan (I. Okane, unpublished data), and this fungus was the most frequently isolated xylariaceous endophyte at the site. Although its species-level identity has not yet been determined, this fungus probably inhabits plant tissues throughout tropical to subtropical regions.

One or two or more xylariaceous fungi were isolated from all plants examined in the course of the present study (data not shown). The four fungi subtypes analyzed in the present study were isolated from *Polyalthia simiarum* (Buch.-Ham. ex Hook. f. & Thomson) Benth. (Annonaceae), *Licuala spinosa* Wurmb (Palmae; the mangrove fan palm), and *Pteris decrescens* H. Christ (Pteridaceae) (Table 2). Several xylariaceous fungi other than the four species mentioned were also isolated from *L. spinosa* (data not shown). However, no xylariaceous fungi other than the four species analyzed were isolated from *Polyalthia simiarum* or *Pteris decrescens*. These results indicate the existence of host preference among xylariaceous endophytes. *Xylaria cubensis* was solely isolated from *Ancistrocladus tectorius* (Lour.) Merr. (Ancistrocladaceae) and *Thelypteris triphylla* (Sw.) K. Iwats. (Thelypteridaceae), suggesting that *X. cubensis* possesses a greater capacity to inhabit plant tissues or a wider host range than other xylariaceous fungi. Three or four xylariaceous fungi other than the present four species were also isolated from *Aglaia elaeagnoidea* (A. Juss.) Benth. (Meliaceae), *A. nervosa* (Myrsinaceae), *Saprosma longifolium* Pit. (Rubiaceae), and *L. spinosa* (Palmae) (data not shown).

Petrini and Petrini (1985) isolated several xylariaceous endophytes from various plants ranging from bryophytes or pteridophytes to monocots (Gramineae, Orchidaceae, etc.). Among these xylariaceous endophytes, *Hypoxylon fragiforme* (Pers.) J. Kickx f. and an unidentified *Nodulisporium* species were found to have a wide host range. In the current study, the four xylariaceous fungi were isolated from certain pteridophytes, whereas *X. cubensis* and *Nemania* cf. *bipapillata* were also found in *Selaginella inaequalifolia* (Hook. & Grev.) Spring (Lycopodiophyta) (see Table 2). In addition, the four fungi were isolated not only from dicots, but also monocots. These results suggest that certain endophytic xylariaceous fungi possess the ability to simultaneously inhabit the living tissue of plants that are phylogenetically far apart. Even *X. grammica,* which was found in a comparatively small number of plants, was isolated from a diversity of plants ranging from a type of pteridophyte (*P. decrescens*) to monocots (Palmae).

Considering that fallen leaves harboring the mycelium of xylariaceous endophytes can play a functional role as inocula for fungal colonization and production of their fruit bodies on suitable substrates, the fact that the aforementioned plants are hosts to many xylariaceous endophytes is significant for the distribution of the xylariaceous fungi. Moreover, the nonspecific infection of a wide range of plants might be an effective ecological strategy for these fungi. For example, the establishment of a large population of *X. cubensis* in tropical regions might result from the ability of this species to inhabit the living tissues of various plants without, or with less, host specificity. Further studies on the ecological role of the foliage harboring the fungi would help determine the validity of this hypothesis.

Table 2 List of plants from which the four xylariaceous endophytes were isolated

Plants	Families	*Xylaria cubensis*	*Xylaria grammica*	*Nemania diffusa*	*Nemania* cf. *bipapillata*	Common
Selaginella inaequalifolia	Selaginellaceae	○	–	–	○	2
Microlepia herbacea	Dennstaedtiaceae	○	–	○	–	2
Tectaria maingayi	Dryopteridaceae	○	–	○	–	2
Pteris decrescens	Pteridaceae	○	○	○	○	4
Thelypteris triphylla	Thelypteridaceae	○	–	–	–	1
Miliusa lineata	Annonaceae	○	–	○	○	3
Polyalthia simiarum	Annonaceae	○	○	○	○	4
Polyalthia aff. *evecta* var. *evecta*	Annonaceae	○	–	○	○	3
Knema elegans	Myristicaceae	○	–	○	–	2
Cinnamomum subarenicum	Lauraceae	○	–	○	–	2
Ancistrocladus tectorius	Ancistrocladaceae	○	–	–	–	1
Eurya acuminata	Theaceae	○	–	○	–	2
Dipterocarpus gracilis	Dipterocarpaceae	○	–	○	○	3
Ardisia nervosa	Myrsinaceae	○	○	–	○	3
Abrus precatorius	Legminosae	○	–	○	–	2
Salacia chinensis	Celastraceae	○	○	○	–	3
Gonocaryum lobbianum	Icacinaceae	○	–	○	○	3
Aglaia elaeagnoidea	Meliaceae	○	○	○	–	3
Aphanamixis polystachya	Meliaceae	–	–	○	–	1
Melicope pteleifolia	Rutaceae	○	–	–	○	2
Saprosma longifolium	Rubiaceae	○	○	○	–	3
Tectona grandis	Verbenaceae	○	–	–	–	1
Calamus palustris	Palmae	○	○	○	–	3
Licuala spinosa	Palmae	○	○	○	○	4
Schizostachyum longispiculatum	Gramineae	○	–	○	○	3
25 species of 24 genera	21 families	24 spp. (23 genera in 21 families)	8 spp. (8 genera in 7 families)	19 spp. (18 genera in 15 families)	11 spp. (10 genera in 9 families)	

○ isolated, – not isolated

According to Læssøe and Lodge (1994) and Bayman et al. (1998), most of the xylariaceous endophytes have been reported to be non-host specific. The present study confirmed that most of the major xylariaceous endophytes are likely to be non-host specific, or have a wide range of host plant preference, by using molecular techniques. Although the four xylariaceous fungi studied were revealed to inhabit phylogenetically diverse plants as endophytes, the phylogenetic analyses revealed that strains isolated from the same plant were not aggregated into subclusters within each clade. Although further genetic studies using different molecular markers or whole genomic data or rigorous inoculation experiments are required to clarify the host specificities at the intraspecies level, the present results indicate that these xylariaceous fungi might not possess strict host specificity in their endophytic phase: this means that "the specialization of parasitism" of some of plant pathogenic fungi was not recognized in these endophytic fungi. Thus, these particular fungi could be considered to be in a pleioxeny, the condition of plurivorous parasitism, in the endophytic phase.

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