Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species

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Diverse fungal species live inside plant tissues, some of which presumably occur in a mutualistic association. Some fungal endophytes are widespread and can be found in many different plant species, whereas others are highly specific to single hosts. In this study, we investigated the taxonomic identities and phylogenetic relationships of fungal endophytes isolated from three plant species, *Artemisia capillaris*, *A. indica*, and *A. lactiflora*, using a combination of morphological and molecular approaches. Morphological differences among the fungal isolates indicate that diverse distinct morphotypes might be present within the hosts. Thirty-four fungal isolates were selected for further molecular phylogenetic analysis using nuclear ribosomal DNA sequences, including both the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene region. The 34 endophytes were identified to various taxonomic levels, and some to the species level based on fungal sequences with known identities in GenBank. Our results suggest that *Alternaria*, *Colletotrichum*, *Phomopsis*, and *Xylaria* species are the dominant fungal endophytes in the *Artemisia* hosts, and some of these endophytes exhibit host and tissue specificity. This aspect can be further explored to understand the relationships between plant hosts and their fungal endophytes.

Key words: *Artemisia*, endophytic fungi, molecular identification, phylogenetic analysis.

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Introduction

Endophytes commonly refer to a group of fungi that reside asymptomatically inside the living plant tissues (Sánchez Márquez *et al*., 2007, 2008; Hyde and Soytong, 2008). Recent surveys of various host plants have demonstrated that fungal endophytes are ubiquitous in plant species (Kumar *et al*., 2004; Zhang *et al*., 2006; Sánchez Márquez *et al*., 2007; Huang *et al*., 2008; Oses *et al*., 2008) and even lichens (Li *et al*., 2007). Globally, there are at least one million species of endophytic fungi (Ganley *et al*., 2004), which represent an important genetic resource for biotechnology. Endophytes have been recognized as potential sources of novel natural products for pharmaceutical, agricultural and industrial uses, especially those secondary metabolites produced by fungal endophytes colonizing medicinal plants (Strobel and Daisy, 2003; Hyde and Soytong, 2008; Mitchell *et al*., 2008). Much attention is now being paid to endophytic biodiversity, the chemistry and bioactivity of endophytic metabolites, and the relationship between endophytes and their host plants (Tan and Zou, 2001; Schulz *et al*., 2002; Kumar *et al*., 2005; Rungjindamai *et al*., 2008; Tao *et al*., 2008).

The genus *Artemisia* (*Asteraceae*) is widely distributed in the world, with about 185 species in China and is highly valued for its medicinal properties (Zou *et al*., 2000). *Artemisia capillaris* is a traditional oriental medicinal herb, and has been used for treatment of various liver diseases such as hepatitis, jaundice and fatty liver (Hong *et al*., 2004). *Artemisia indica* allegedly possesses anti-malarial activity, and has been reported in the guideline of Thai medicinal plants used in primary health care (Chanphen *et al*., 1998). *Artemisia lactiflora*, an edible plant in

Southeast Asia, has inhibitory effects on a variety of tumor promoter-induced biological responses such as oxidative stress as well as tumor promotion in ICR mouse skin (Nakamura *et al*., 1999). As part of our ongoing research towards finding novel anti-oxidants from natural resources, we investigated the secondary metabolites of endophytes isolated from these three *Artemisia* medicinal plants and found that some of the endophytic fungal isolates exhibited strong anti-oxidant capacity (Huang *et al*., 2007). It is important to further identify these bioactive fungi for potential utilization using morphological and/or molecular techniques.

Fungal taxonomy is traditionally based on comparative morphological features (e.g., Lodge *et al*., 1996; Sette *et al*., 2006; Crous *et al*., 2007; Zhang *et al*., 2008). However, special caution should be taken when closely related or morphologically similar endophytes are identified, because the morphological characteristics of some fungi are medium-dependent and cultural conditions can substantially affect vegetative and sexual compatibility (Zhang *et al*., 2006; Hyde and Soytong, 2007). Furthermore, the conventional methods cannot be applied for identifying fungal isolates that fail to sporulate in culture, which are categorized as mycelia sterilia (Lacap *et al*., 2003). Various optimization of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures (Guo *et al*., 2000). Nevertheless, a large number of fungi still do not sporulate in culture, and these mycelia sterilia are considerably frequent in the endophyte studies (Lacap *et al*., 2003).

In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al*., 2006). Several recent studies have shown that genetic methods can be successfully used in the studies of endophytic fungi (Gao *et al*., 2005; Wang *et al*., 2005; Arnold *et al*., 2007; Ligrone *et al*., 2007; Sánchez Márquez *et al*., 2007; Morakotkarn *et al*., 2007). Most of the endophytic fungi were detected and identified by comparative analyses of the ribosomal DNA sequences, especially the ITS region. For example, Harney *et al*. (1997) identified arbuscular mycorrhizal fungi from *Artemisia californica* using the ITS region. Also based on ribosomal DNA sequences, Guo *et al*. (2000) and Lacap *et al*. (2003) evaluated the endophytic fungal 'morphotype' concept concerning mycelia sterilia. A high diversity of endophytic fungal communities was revealed from either *Heterosmilax japonica* or *Livistona chinensis* using a cultivation-independent approach by analyzing fungal DNA sequences extracted from plant tissues (Guo *et al*., 2001; Gao *et al*., 2005). Ganley *et al*. (2004) studied morphologically similar endophytes and parasites based on the ITS region, and found that the endophytic fungi in west white pine were actually most closely related to, but distinct from, the parasites. Peintner *et al*. (2003) first recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Queloz *et al*. (2005) monitored the spatial and temporal dynamics of the tree-root endophyte *Phialocephala fortinii* using the restriction fragment length polymorphism (RFLP) analysis.

In the present study, we isolated a total of 108 endophytic fungal isolates from different tissues of three medicinal *Artemisia* species (*A. capillaris*, *A. indica*, and *A. lactiflora*). These fungal endophytes were identified using a combination of morphological and molecular methods. Thirty-four representative isolates of various morphological endophytes were selected for DNA sequence analysis using the whole ITS region, including ITS1, 5.8S, and ITS2. Combining both morphological characters and DNA sequence data, we identified some of these endophytes to the species level and the others at least to the family level, and evaluated phylogenetic relationships among the endophytes. This study represents the first step for linking the biodiversity of endophytes, their taxonomic identities and phylogenetic positions with their bioactivities in the medicinal plant species.

Materials and methods

Plant material

Healthy plants of three medicinal *Artemisia* species growing in Hong Kong were sampled from June to October, 2005. *Artemisia capillaris* was collected from Ham Tin Wan, *A. indica* from Kadoorie Farm and Botanic Garden, and *A. lactiflora* from the Hong Kong Island. Fresh samples were taken to the laboratory and treated within 8 hours. Digital photographs were taken of each species, and all the species samples were deposited in School of Biological Sciences, the University of Hong Kong.

Isolation of endophytic fungi

A total of 20 samples of both leaves and stems from the three *Artemisia* species as well as inflorescences from *A. capillaris* plants were first washed in running water. The leaves were cut into segments (5×5 mm), and stems or inflorescences were cut into pieces (10 mm in length). Surface sterilization and isolation of endophytic fungi followed a modified procedure as described by Schulz *et al*. (1993), and the details of the procedure were also given in Huang *et al*. (2007).

DNA extraction, amplification and sequencing of fungal ITS

A total of 34 representative isolates of various morphological endophytic fungi isolated from each *Artemisia* species were selected for molecular identification. Fungal genomic DNA was prepared with the NucleoSpin® DNA extraction Kit (Macherey-Nagel, Düren, Germany). About 10 mg of fungal mycelia were scraped with a sterile nipper from fresh cultures growing on PDA plates at 28˚C for 5-15 days and suspended in 400 µl of buffer C1. All the rest of extraction procedure followed the instructions given in the Kit User's Manual. The extracted DNA pellet was then kept in 50 µl TE buffer at –20˚C.

PCR reactions for sequencing were carried out in a PTC-100™ thermal cycler (MJ Research, Inc., Watertown, MA, USA) with universal primers ITS5 (5′-GGAAGTAAAAG TCGTAACAAGG-3′) and ITS4 (5′-TCCTC CGCTTATTGATATGC-3′) (White *et al*., 1990). The amplified fragment includes ITS1, 5.8S and the ITS2 of rDNA. Amplification was performed in a 50 µl reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primers, 0.5 unit of Taq polymerase, and 2µl of fungal genomic DNA. The thermal cycling program was as follows: 2 min initial denaturation at 94˚C, followed by 35 cycles of 30 s denaturation at 94˚C, 30 s primer annealing at 55˚C, and 45˚s extension at 72˚C, and 5 min at 72˚C for a final extension. The PCR product was purified with High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). Direct DNA sequencing was performed using primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS5 (White *et al*., 1990) on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.).

DNA sequence assembly and alignment

Sequence similarity searches were performed for each of the 34 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). The ITS1-5.8S-ITS2 sequences of endophytic fungal isolates were aligned with the sequences of selected reference taxa using the Multi-Alignment Editor of GeneTool 1.0 software (www.biotools.com), and the alignment was inspected and adjusted manually where necessary. The nucleotide sequences of reference taxa along with their GenBank accession numbers are listed in Table 2. GenBank accession numbers of the 34 representative endophytic fungal sequences from this study and their top BLAST match sequences are given in Table 3.

Phylogenetic analysis

Phylogenetic analysis was conducted based on both the ITS and 5.8S gene data using both maximum parsimony (MP) and neighbor joining (NJ) approaches. MP and NJ searches were carried out using PAUP*4.0 b10 (Swofford, 2003) on a Power Macintosh G3 computer. All characters were equally weighted and unordered. Alignment gaps were treated as missing data. MP analysis was

conducted using a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 100 random addition sequence replicates. Statistical support for the internal branches was estimated by bootstrap analysis based on 5,000 replications. NJ trees were constructed based on the total character differences and bootstrap values were calculated from 1,000 replications. Gaps were coded according to the simple indel coding method of Simmons and Ochoterana (2000) using SeqState (Müller, 2005). The MP and NJ analyses using the gap-coded dataset were compared to those based on the noncoded dataset.

Bayesian posterior probability support for the clades was obtained using MrBayes v3.1.2 (Ronquist and Heulsenbeck, 2003). The selection of the best model for the datasets was based on Akaike Information Criterion (AIC; Akaike, 1981) using MrModeltest v2.2 (Nylander, 2004). For the Bayesian analysis, two independent runs with four Markov chains were run for 2 million generations and trees were sampled every 100th generation. The first 10% of the sampled trees were discarded as the burnin phase and the remaining trees were summarized. The tree topologies obtained with Bayesian analysis were similar to those based on parsimony analysis. The Bayesian posterior probability supports can thus be added in the clades of parsimony based trees in all the figures.

Results

Isolation and morphological grouping of endophytic fungi from three Artemisia species

Morphological identification of the 108 fungal isolates from three medicinal *Artemisia* species was first carried out according to colony or hyphal morphology of the fungal culture, characteristics of the spores, and reproductive structures if discernible (see Wei, 1979; Carmichael *et al*., 1980; Barnett and Hunter, 1998). Based on these features, the 108 endophytes could be classified into 27 different morphological taxa (Table 1). Most of the fungal isolates (42 isolates) were obtained from host *A*. *capillaris*, 5 isolates isolated from its leaves, 15 isolates from stems, and 22 isolates from inflorescences. Thirty-nine fungal isolates were isolated from host *A*. *indica*, 18 from its leaves and 21 from stems. Twenty-seven fungal isolates were isolated from host *A*. *lactiflora*, 16 from its leaves and 11 from stems. Using the traditional morphological techniques, only some of the fungal isolates could be identified to the genus level. Among them, *Alternaria*, *Colletotrichum* and *Phomopsis* were common, especially *Alternaria* spp. with 27 isolates (25% relative isolation frequency) and *Colletotrichum* spp. with 22 isolates (20.4%). Some species of *Aureobasidium*, *Ephelis* and *Pestalotiopsis* were also isolated from the *Artemisia* hosts, and one isolate from the stems of *A*. *capillaris* was similar to *Drechslera* sp. Other fungi could be identified only to the family or higher levels, such as *Xylariales*, coelomycetes, and ascomycetes. The remaining 26 fungal isolates (23.9%) lacking sporulating structures were grouped into mycelia sterilia. These mycelia sterilia were further divided into 9 different 'morphospecies' according to their cultural characteristics.

Molecular identification and phylogenetic analysis of representative endophytic fungi

In addition to the morphological characterization, molecular analyses were carried out to confirm the identification of 34 representative fungal isolates from the three *Artemisia* species. The ITS1-5.8S-ITS2 sequences of these isolates were compared to 27 corresponding sequences of reference fungal taxa in the database. In the noncoded dataset, there are 350 variable characters (51.2%), 283 (41.4%) being parsimony-informative. While in the gap-coded dataset, 710 (55.9%) out of 872 (68.6%) variable characters are parsimony-informative. Heuristic search of the gap-coded database found 4,992 equally most parsimonious trees with a length of 1,888 steps (consistency index (CI) = 0.584, retention index (RI) = 0.842, rescaled consistency index (RC) = 0.492, and homoplasy index (HI) = 0.416). One of the most parsimonious trees which is most consistent in topology with the NJ and Bayesian analysis (Fig. 1) was given to show the diversity of the endophytic fungi associated with these *Artemisia* species and to illustrate the phylogenetic placement of these endophytes. The tree was rooted with a species of *Trypethelium*, based on the phylogenetic

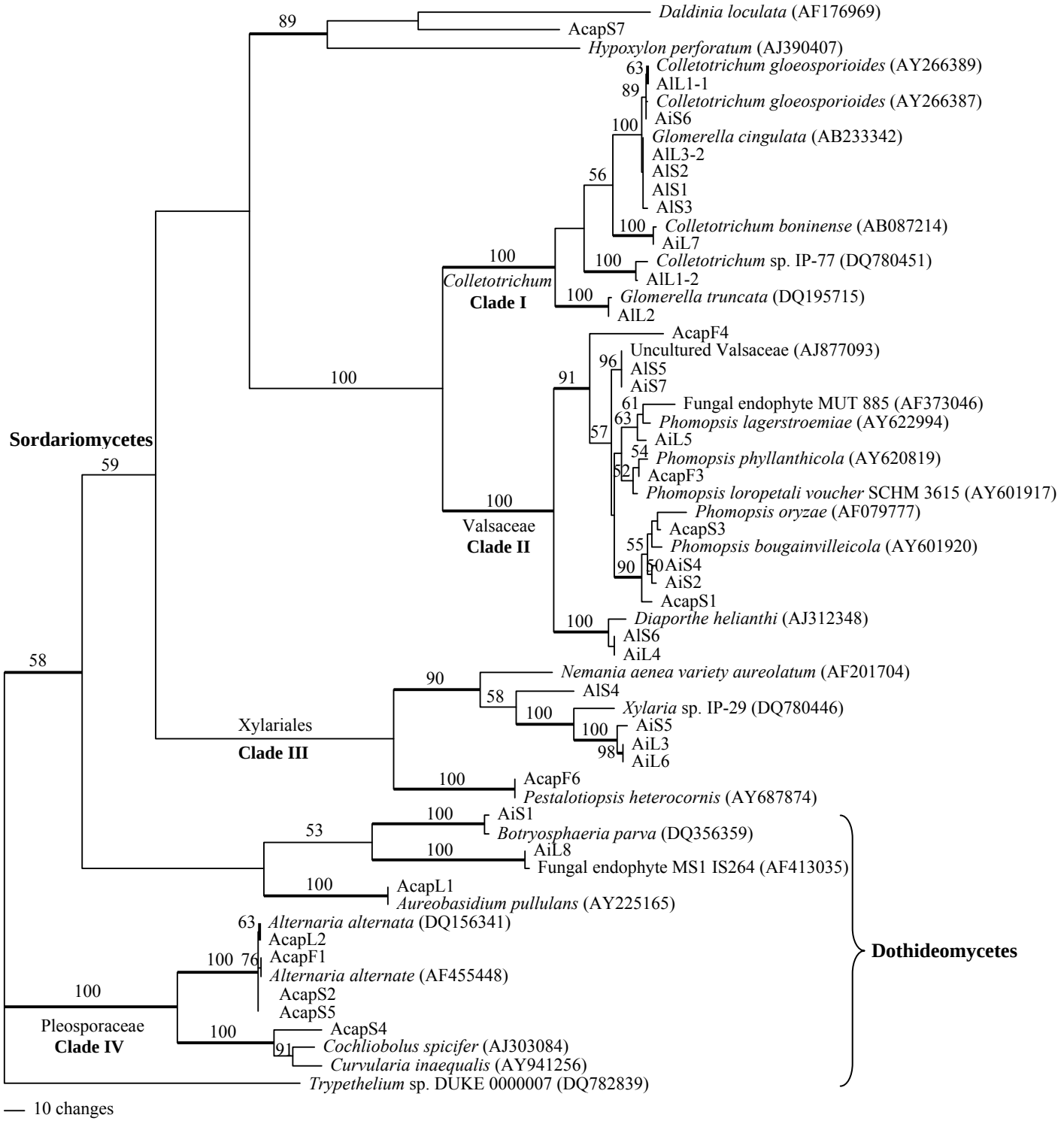

Fig. 1. One of 4,992 MP trees (Length = 1,888, CI = 0.584, RI = 0.842, RC = 0.492, HI = 0.416) most consistent in topology with NJ and Bayesian analyses. All 34 endophytic fungal strains and 27 reference taxa were included. Thickened branches represent clades with over 0.95 Bayesian posterior probability support. Bootstrap support values (≥ 50%) are shown above the branches.

Table 1. Number and morphological taxonomic identification of the fungal endophytes isolated from three *Artemisia* species.

Fungal taxon	*Artemisia capillaris*			*Artemisia indica*		*Artemisia lactiflora*	
	inflorescence	leaf	stem	leaf	stem	leaf	stem
Alternaria spp.							
Alternaria sp. 1	15						
Alternaria sp. 2	1	4	5				
Alternaria sp. 3			2				
Ascomycete spp.							
Ascomycete sp. 1					5	1	
Ascomycete sp. 2			1		4		1
Ascomycete sp. 3				3			
Ascomycete sp. 4				1			
Aureobasidium sp.		1					
Coelomycetes sp.					4		
Colletotrichum spp.							
Colletotrichum sp.1						1	1
Colletotrichum sp.3				1		1	5
Colletotrichum sp.5					1	11	1
Drechslera-like sp.			1				
Ephelis sp.			2				
Mycelia sterilia spp.							
Mycelia sterilia sp. 1	2						
Mycelia sterilia sp. 2	3						
Mycelia sterilia sp. 3			1				
Mycelia sterilia sp. 4			2				
Mycelia sterilia sp. 5				9	2		
Mycelia sterilia sp. 6				1			
Mycelia sterilia sp. 7				1			1
Mycelia sterilia sp. 8						2	
Mycelia sterilia sp. 9				2			
Pestalotiopsis sp.	1						
Phomopsis spp.							
Phomopsis sp. 1							2
Phomopsis sp. 2					5		
Xylariales sp.			1				
Total	22	5	15	18	21	16	11

relationships reported in Spatafora *et al*., 2006. Other more distant outgroups were not used due to the potential difficulty in sequence alignment.

As shown in Fig. 1, all the isolates from *Artemisia* host plants could be classified as Sordariomycetes or Dothideomycetes. Sordariomycetes mainly included *Colletotrichum* (Clade I), *Valsaceae* (Clade II), and *Xylariales* (Clade III). *Pleosporaceae* (Clade IV) was included in Dothideomycetes. Within the Dothideomycetes, AiL8 and fungal endophyte MS1 IS264 formed a well supported clade, AiS1 and *Botryosphaeria parva* formed another well supported clade, and AcapL1 and *Aureobasidium pullulans* were basically identical. These clades indicated the identities or affiliations of the unknown isolates in reference to the previously identified taxa, in addition to their phylogenetic relationships. These clades formed a larger clade sister to the Sordariomycetes clade, indicating that the Dothideomycetes clade is not a monophyletic group. As MP, NJ and Bayesian analyses all showed the same relationships with strong bootstrap supports (100%) and Bayesian probability (1.00). It can be stated with confidence that isolate AiL8 was closely related to endophyte MS1 IS264, which was isolated from *Polygonum multiflorum* and most closely related to *Phyllostica* (Lacap *et al*., 2003). A BLAST search showed that endophyte MS1 IS264 (AF413035) was 99% identical to AiL8 with 97% query coverage. Isolate AiS1 was most likely *B*. *parva* (99% identical with both AY259098 (99% coverage) and DQ356359 (96% coverage) in BLAST searches), and isolate AcapL1 was *A*. *pullulans*

Table 2. Species and GenBank accession number used in the study.

Species	GeneBank accession number	Species	GeneBank accession number
Alternaria abutilonis	AF314578	Fungal endophyte MS1 IS264	AF413035
Alternaria aff. dianthicola RHR2	AY923859	Fungal endophyte MUT 885	AF373046
Alternaria aff. longipes RLC16.3	DQ156337	Fungal endophyte sp. J26	AY601897
Alternaria alternata	AF455406, AF455441, AF455444, AF455448, AF455516, AF455539, AB094666, DQ023279, DQ156341	Fungal endophyte WMS16	AY063312
Alternaria brassicae	DQ156344	Fungal endophyte WMS8	AY063304
Alternaria citri	AF314579	*Glomerella cingulata* (anamorph: *Colletotrichum gloeosporioides*)	AB042317, AB219012, AB233342, AF272779, AF489568, AY245021, AY266378, AY266387, AY266389, AY266393, AY714052, DQ062671
Alternaria gaisen	AF314574, AF314581	*Glomerella truncata* (anamorph: *Colletotrichum truncatum*)	DQ195715
Alternaria longipes	AY751457	*Hypoxylon perforatum*	AJ390407
Alternaria pomicola	AF314583	*Hypoxylon* sp.	DQ223762, DQ223763, DQ322128
Alternaria tenuissima	AF314572	*Nemania aenea*	AF201704, AJ390427, AJ390428
Annulohypoxylon annulatum	AJ390395	*Nodulisporium* sp. MF6245	AF201756, AF201760
Apiognomonia veneta	DQ994610	*Nodulisporium* sp. MF6378	
Arthroxylaria elegans	AF432179	*Pestalotiopsis heterocornis*	AY687874
Aureobasidium pullulans	AY225165, EF197817	*Pestalotiopsis microspora*	AF409958
Bipolaris papendorfii	AF163075	*Pestalotiopsis* sp. ICMP6088	AF409957
Bipolaris spicifera	AY253918	*Pestalotiopsis uvicola*	AY687297
Bipolaris tetramera	AF229477, AY004777	*Phomopsis averrhoae*	AY618930
Botryosphaeria parva	AY259098, DQ356359	*Phomopsis bougainvilleicola voucher* SCHM 3006	AY601920
Cochliobolus australiensis	AY923860	*Phomopsis conorum*	DQ116553
Cochliobolus cymbopogonis	AF071351	*Phomopsis eucommicola*	AY578071
Cochliobolus lunatus (anamorph: *Curvularia lunata*)	AF163082, DQ836799	*Phomopsis eucommii*	AY601921
Cochliobolus spicifer	AJ303084	*Phomopsis lagerstroemiae*	AY622994

Table 2 (continued). Species and GenBank accession number used in the study.

Species	GeneBank accession number	Species	GeneBank accession number
Colletotrichum boninense	AB042313, AB087214	*Phomopsis liquidambari*	AY601919
Colletotrichum crassipes	AY376530	*Phomopsis loropetali voucher* SCHM 3615	AY601917
Colletotrichum kahawae	AF534468	*Phomopsis magnoliae*	AY622995
Colletotrichum lindemuthianum	AB087222	*Phomopsis oryzae*	AF079777
Colletotrichum orbiculare	AY841133	*Phomopsis phyllanthicola*	AY620819
Colletotrichum sp. IP-42	DQ780413	*Phomopsis quercina*	AJ293876
Colletotrichum sp. IP-77	DQ780451	*Phomopsis* sp.	AB286211, DQ780461
Colletotrichum trifolii	AB087223	*Plectosphaerella cucumerina*	DQ227289
Colletotrichum truncatum	AY266384	*Pleosporaceae* sp. LM156	EF060514
Curvularia cymbopogonis	AF163079	*Podosordaria tulasnei*	AY572970
Curvularia gladioli	AF071337	*Rosellinia arcuata*	AB017660
Curvularia gudauskasii	AF071338	*Rosellinia mirabilis*	AY862572
Curvularia heteropogonicola	AF163080	*Rosellinia necatrix*	AB017658, AJ972672
Curvularia inaequalis	AF120261, AY941256	*Rosellinia quercina*	AB017661
Daldinia loculata	AF176969	*Tubercularia* sp. TF5	AY427785
Diaporthe conorum	DQ116551	Uncultured *Valsaceae*	AJ877093
Diaporthe helianthi	AJ312348, AJ312366	*Xylaria acuta*	AF163026
Diaporthe phaseolorum	AF001021, AF001028	*Xylaria apiculata*	AF163027
Diaporthe phaseolorum var. caulivora	AJ312360	*Xylaria arbuscula*	AF163028, AY183369
Diaporthe vaccinii	AY952141	*Xylaria cornu-damae*	AF163031
Drechslera andersenii	AM262415	*Xylaria longipes*	AF163038
Eutypa leptoplaca	AY684237	*Xylaria* sp.	AF153744, DQ780446

Table 3. Morphological identification, GenBank accession numbers and top BLAST match sequences of the fungal isolates from *Artemisia* species included in phylogenetic analysis.

Fungal strain	Morphological identification	GeneBank accession No.	BLAST match sequence		
			Reference accession No.	coverage	Max ident
AcapF1	*Alternaria* sp. 1	EU054396	*Alternaria alternata* AF455406	100%	100%
AcapF3	Mycelia sterilia sp. 1	EU054397	*Phomopsis loropetali* AY601917	94%	99%
AcapF4	Mycelia sterilia sp. 2	EU054398	*Phomopsis* sp. IP-81 DQ780461	99%	94%
AcapF6	*Pestalotiopsis* sp.	EU054399	*Pestalotiopsis heterocornis* AY687874	96%	100%
AcapL1	*Aureobasidium* sp.	EU054400	*Aureobasidium pullulans* AY225165	97%	100%
AcapL2	*Alternaria* sp. 2	EU054401	*Alternaria aff. longipes* DQ156337	99%	100%
AcapS1	Mycelia sterilia sp. 4	EU054402	*Phomopsis bougainvilleicola* AY601920	96%	98%
AcapS2	*Alternaria* sp. 2	EU054403	*Alternaria gaisen* AF314581	100%	100%
AcapS3	Mycelia sterilia sp. 3	EU054404	*Phomopsis oryzae* AF079777	99%	98%
AcapS4	*Drechslera*-like sp.	EU054405	*Pleosporaceae* sp. LM156 EF060514	100%	97%
AcapS5	*Alternaria* sp. 3	EU054406	*Alternaria gaisen* AF314581	100%	100%
AcapS7	*Xylariales* sp.	EU054407	*Hypoxylon* sp. SUT237 DQ322128	82%	95%
AiL3	Mycelia sterilia sp. 5	EU054408	*Xylaria longipes* AF163038	98%	92%
AiL4	Ascomycete sp. 4	EU054409	*Diaporthe helianthi* AJ312366	96%	98%
AiL5	Mycelia sterilia sp. 6	EU054429	*Phomopsis eucommii* AY601921	92%	98%
AiL6	Mycelia sterilia sp. 7	EU054410	*Xylaria longipes* AF163038	98%	92%
AiL7	*Colletotrichum* sp. 2	EU054411	*Colletotrichum boninense* AB042313	99%	98%
AiL8	Mycelia sterilia sp. 5	EU054412	Fungal endophyte MS1 IS264 AF413035	97%	99%
AiS1	Ascomycete sp. 1	EU054413	*Botryosphaeria parva* AY259098	99%	99%
AiS2	Ascomycete sp. 2	EU054414	*Phomopsis oryzae* AF079777	99%	97%
AiS4	*Phomopsis* sp. 2	EU054415	*Phomopsis bougainvilleicola* AY601920	96%	98%
AiS5	Mycelia sterilia sp. 5	EU054416	*Xylaria longipes* AF163038	98%	93%
AiS6	*Colletotrichum* sp. 5	EU054417	*Glomerella cingulata* AB219012	99%	99%
AiS7	Ascomycete sp. 2	EU054418	*Phomopsis* sp. H1 AB286211	100%	99%
AlL1-1	*Colletotrichum* sp. 5	EU054419	*Colletotrichum gloeosporioides* AY714052	100%	99%
AlL1-2	*Colletotrichum* sp. 1	EU054420	*Colletotrichum* sp. IP-42 DQ780413	95%	99%
AlL2	Mycelia sterilia sp. 8	EU054421	*Colletotrichum truncatum* DQ195715	79%	99%
AlL3-2	*Colletotrichum* sp. 3	EU054422	*Colletotrichum gloeosporioides* AF272779	99%	100%
AlS1	*Colletotrichum* sp. 3	EU054423	*Colletotrichum gloeosporioides* AY266378	99%	100%
AlS2	*Colletotrichum* sp. 5	EU054424	*Colletotrichum gloeosporioides* AY266378	99%	100%
AlS3	*Colletotrichum* sp. 1	EU054425	*Colletotrichum gloeosporioides* AY266393	99%	99%
AlS4	Mycelia sterilia sp. 7	EU054426	*Xylaria* sp. MS1083 AF153744	83%	99%
AlS5	Ascomycete sp. 2	EU054427	*Phomopsis* sp. H1 AB286211	100%	99%
AlS6	*Phomopsis* sp. 1	EU054428	*Diaporthe helianthi* AJ312348	96%	98%

(100% identical to AY225165 (97% coverage) and 99% similar to EF197817 (100% coverage)).

Four of the isolates, AcapL2, AcapF1, AcapS2, and AcapS5, were grouped with *Alternaria alternata* accessions in Clade IV (Fig. 1) with 100% bootstrap support and Bayesian probability of 1.00, consistent with their morphological identification. In individual BLAST searches where the total length of each sequence was used, AcapS2 and AcapS5 were found to be 100% identical with *Alternaria gaisen* (AF314581, 100% coverage). AcapL2 was 100% identical with both *Alternaria aff. longipes* RLC16.3 (DQ156337) and *Alternaria alternata* (DQ156341) with 99% coverage. AcapF1 was 100% identical with *A. alternata* (AF455406, AF455441, AF455444, AF455448, AF455516, and AF455539); 99% coverage with *A. alternata*

(AB094666 and DQ023279); 100% coverage with *Alternaria longipes* (AY751457), and 99% coverage with *Alternaria abutilonis* (AF314578), *Alternaria aff. dianthicola* RHR2 (AY923859), *Alternaria brassicae* (DQ1563 44), *Alternaria citri* (AF314579), *Alternaria gaisen* (AF314574), *Alternaria pomicola* (AF314583), and *Alternaria tenuissima* (AF314572) (Table 3).

On the basis of its morphology, AcapS4 was classified as a *Drechslera*-like sp. (Table 3). It has relatively high sequence similarities, ranging from 92%-96%, with accessions of *Bipolaris*, *Cochliobolus*, *Curvularia* and *Drechslera*. In MP, NJ, and Bayesian analyses, AcapS4 was found to be most closely related to *Cochliobolus* and *Curvularia* of Clade IV. However, it was difficult to affirm its relationship in the family *Pleosporaceae* when more sequence data (4 accessions of *Bipolaris*, 5 of *Cochliobolus*, 6 of *Curvularia* and 1 of *Drechslera*) were included in further MP, NJ, and Bayesian analyses (trees not shown). In an individual BLAST search, its top match sequence was also a *Pleosporaceae* sp. LM156 (EF060514) with 100% coverage and 97% similarity. Thus, AcapS4 could only be verified to the family level, *Pleosporaceae*.

Further phylogenetic analyses were conducted for the 15 sequences of sordariomycetes from Clade I (Fig. 1), together with other sequences of *Colletotrichum* (*Phyllachoraceae*), using *Plectosphaerella cucumerina* (*Phyllachoraceae*) as outgroup (Fig. 2). In the gap coded dataset, 52.9% of 210 variable characters are parsimony-informative, whereas in the noncoded dataset, 49.5% of 103 variable characters were parsimony-informative. Heuristic search found 12 equally most parsimonious trees of 130 steps (CI = 0.915, RI = 0.936, RC = 0.857, HI = 0.085) using the noncoded dataset, but only 3 equally most parsimonious trees of 271 steps (CI = 0.834, RI = 0.881, RC = 0.735, HI = 0.166) using the coded dataset. Six of the endophytic fungal isolates were placed into the same clade as all *Glomerella cingulata*/*Colletotrichum gloeosporioides* reference sequences, with one exception. The identity of *Colletotrichum kahawae* (AF534468) placed in this clade could be *C. gloeosporioides*, an anamorph of *G. cingulata*. Sequence diversity could exist among accessions of the same species, which was apparently the case among the six endophytes in the clades, with AlL1-1 and AiS6 being different from the other four endophytes (AlS1, AlS2, AlS3, and AlL3-2). AiL7 was placed in the clade of *Colletotrichum boninense* with strong bootstrap and Bayesian probability supports. AlL1-2 was sister to *Colletotrichum* sp. IP-42 and IP-77 in all phylogenetic trees. Isolate AlL2 was most likely to be *Glomerella truncata* (anamorph: *Colletotrichum truncatum*). These same grouping relationships were found in the corresponding NJ and Bayesian analyses with strong supports. On the basis of their morphology, all these nine endophytes could be identified as species of *Colletotrichum* except AlL2 (Table 3).

In Clade II of MP and NJ trees, AcapF4 was placed in a separate clade sister to all the other *Phomopsis* taxa, whereas in Bayesian analysis, AcapF4 was found to be most closely related to fungal endophyte MUT 885 with good support (0.87; figure not shown). MUT 885 was previously placed in the *Phomopsis*/*Diaporthe* clade and it was most similar to *Diaporthe caulivora* (AF000567) in ITS sequence (Girlanda, *et al*., 2002). Further MP, NJ, and Bayesian analyses were conducted for the 19 sequences in Clade II (*Valsaceae*) of Fig. 1 together with 14 other sequences belonging to the genus *Phomopsis* and/or *Diaporthe* in the family *Valsaceae*(*Diaporthales*), with *Apiognomonia veneta* (*Diaporthales*, *Gnomoniaceae*) as outgroup. In the noncoded dataset, there were 126 (23.2%) variable characters out of a total of 543 aligned bases, and 66 (12.2%) were parsimony-informative; in the gap coded dataset, there were 208 (33.2%) variable characters, and 107 (17.1%) were parsimony-informative. Heuristic search found 612 equally most parsimonious trees of 250 steps (CI = 0.616, RI = 0.761, RC = 0.469, HI = 0.384) with the noncoded dataset, and 2250 equally most parsimonious trees of 391 steps (CI = 0.604, RI = 0.744, RC = 0.449, HI =0.396) with the gap coded dataset. As shown in Fig. 3, eight of the endophytic fungal isolates from the *Artemisia* hosts were placed in the clade of *Phomopsis*. AcapF4 could be molecularly classified as either a taxon of *Phomopsis* or *Diaporthe*. In the MP, NJ, and

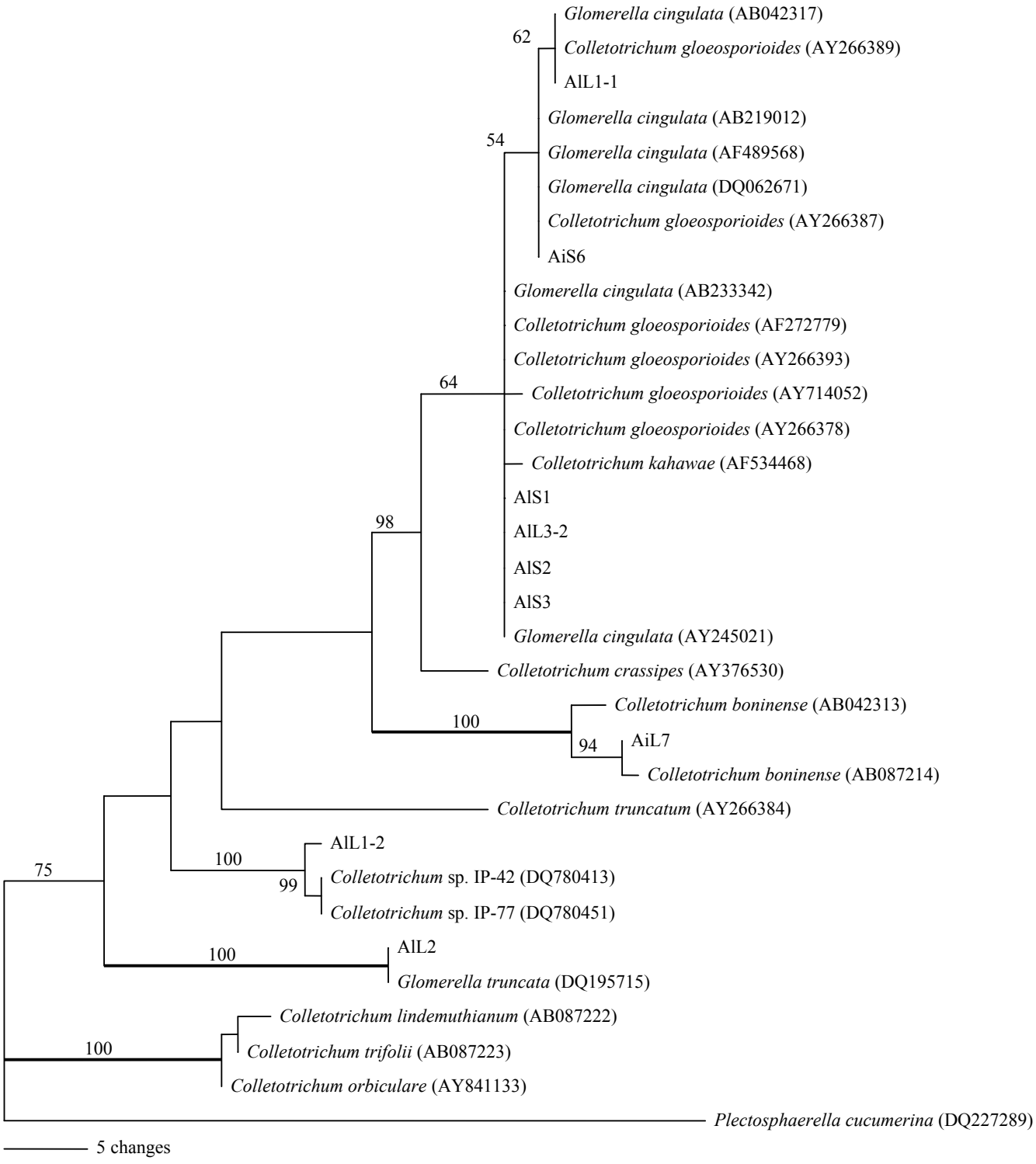

Fig. 2. One of 12 MP trees (Length = 130, CI = 0.915, RI = 0.936, RC = 0.857, HI = 0.085) including the 9 endophytic fungal strains and 23 reference sequences belonging to *Colletotrichum* (*Phyllachoraceae*). Thickened branches represent clades with over 0.95 Bayesian posterior probability support. Bootstrap support values (≥ 50%) are shown above the branches. Note: *Glomerella cingulata*'s anamorph is *Colletotrichum gloeosporioides* and *Glomerella truncata*'s anamorph is *Colletotrichum truncatum*.

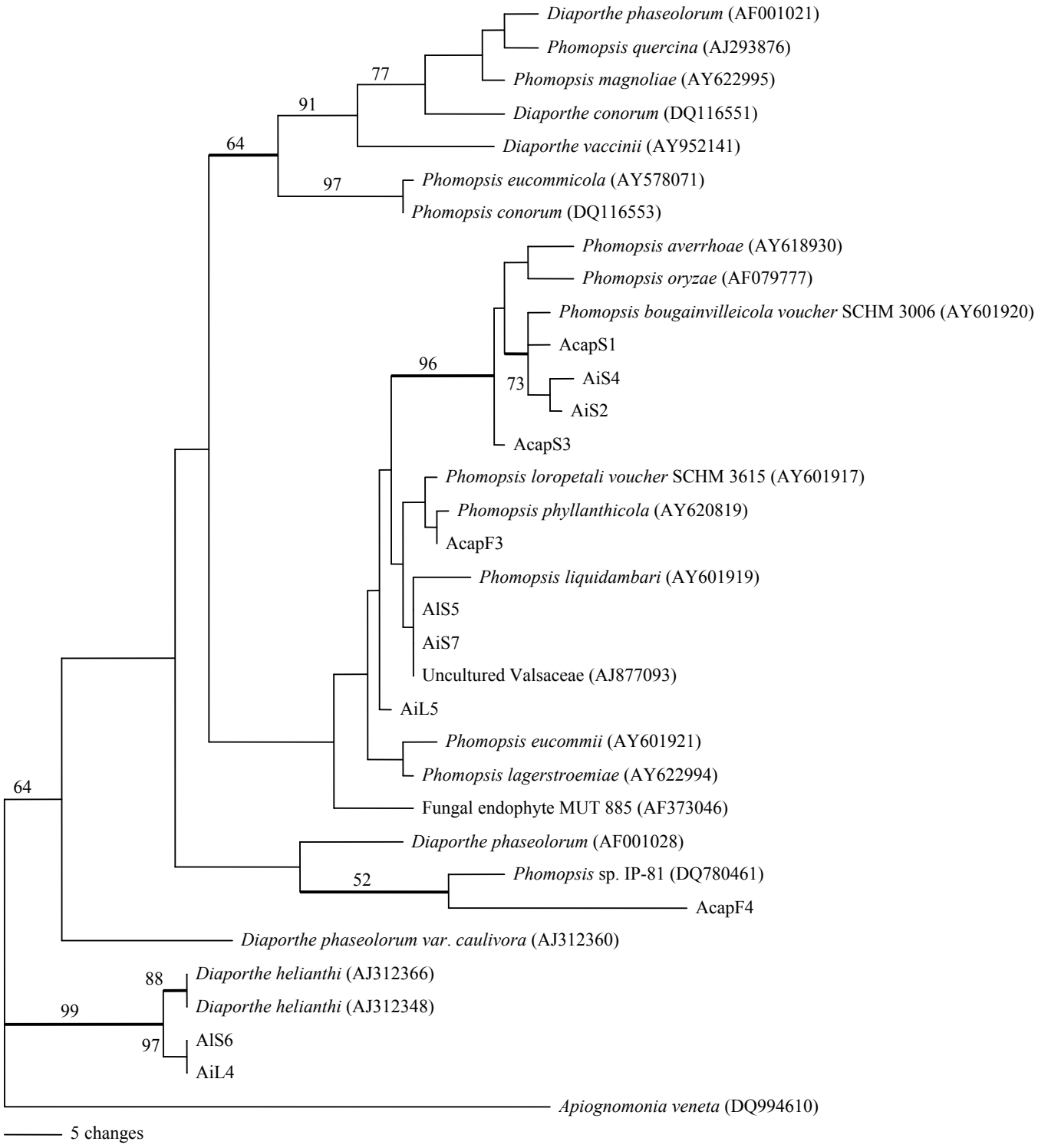

Fig. 3. One of 612 MP trees (Length = 250, CI = 0.616, RI = 0.761, RC = 0.469, HI = 0.384) including the 11 endophytic fungal strains and 22 reference sequences belonging to *Phomopsis* and/or *Diaporthe* of *Valsaceae* (*Diaporthales*). Thickened branches represent clades with over 0.95 Bayesian posterior probability support. Bootstrap support values (≥ 50%) are shown above the branches.

Bayesian analyses, AcapF4 was always placed sister to a *Phomopsis* sp. IP-81, with strong Bayesian probability support (1.00) (Fig. 3). Two of the fungal strains, AlS6 and AiL4, were grouped together into a small clade sister to two *Diaporthe helianthi* accessions. These taxa formed a well supported clade in all the analyses. In the clade of *Diaporthe*, AlS6 and AiL4 had identical ITS1-5.8S-ITS2 sequences, which was 98% similar to *Diaporthe helianthi* (AJ312348 and AJ312366) with 96% coverage in a BLAST search. Because some endophytic

fungi failed to sporulate in PDA culture or showed no discernible reproductive structures, only two (AlS6 and AiS4) of these 11 endophytic fungi could be morphologically identified as *Phomopsis* (Table 3). AcapS1, AiS2, AiS4 and AcapS3 were all placed in a clade containing *Phomopsis bougainvilleicola* voucher SCHM 3006 (AY601920) and *Phomopsis oryzae* (AF079777) (Fig. 1), as well as *Phomopsis averrhoae* (AY618930) (Fig. 3). Also in the clade of *Phomopsis*, AlS5 and AiS7 were found to be related to uncultured *Valsaceae*, which was an endophytic fungus molecularly identified as *Phomopsis phyllanthicola* (Gao *et al*., 2005). They were 99% similar to *Phomopsis* sp. H1 (AB286211, 100% coverage) and *P. phyllanthicola* (AY62 0819, 93% coverage), and 98% similar to *Phomopsis liquidambari* (AY601919, 92% coverage) in the BLAST searches. AcapF3 was placed closest to *P. phyllanthicola*, and together with *Phomopsis loropetali* voucher SCHM 3615 (AY601917), they formed a sister clade to AlS5 and AiS7 in both NJ and MP trees. In all the phylogenetic trees, AiL5 was placed within the clade of *Phomopsis*, and a BLAST search showed that it was most similar to *Phomopsis eucommii* (AY601921) and *Phomopsis lagerstroemiae* (AY622994) with 98% identity (both 92% coverage).

According to Clade III (*Xylariales*) of Fig. 1, AcapF6 could be identified as a species of *Pestalotiopsis*, as the clade was strongly supported in all phylogenetic analyses (100% bootstrap support and Bayesian probability of 1.00). Its sequences was found to be 100% identical to *Pestalotiopsis heterocornis* (AY68 7874), *Pestalotiopsis microspora* (AF409958), *Pestalotiopsis* sp. ICMP6088 (AF409957) (all with 96% coverage), and *Pestalotiopsis uvicola* (AY687297, 94% coverage), although only accession AY687874 was shown in Fig. 1. This molecular identification was consistent with its morphological classification (Table 3).

AcapS7 could not be placed within any of the three major clades of Sordariomycetes (Fig. 1). All the MP, NJ and Bayesian analyses found that AcapS7 and two *Xylariaceae* reference accessions (*Hypoxylon perforatum* and *Daldinia loculata*) formed a well supported clade within the big *Xylariales* clade. BLAST search also showed that AcapS7 was similar to some genera of *Xylariaceae*. To further verify its relationship with these fungi, a new dataset was constructed for phylogenetic analysis, including AcapS7, *Daldinia loculata* (AF176969), *Hypoxylon perforatum* (AJ390 407), the 6 sequences from Clade III, together with other sequences of *Xylariaceae* (*Annulo-hypoxylon*, *Arthroxylaria*, *Hypoxylon*, *Nemania*, *Nodulisporium*, *Podosordaria*, *Rosellinia*, and *Xylaria*), one sequence of *Eutypa leptoplaca* in the family *Diatrypaceae* of *Xylariales* (Trouillas and Gubler, 2004), three sequences of other fungal endophytes (AY063304, AY063312, AY601897), and *Tubercularia* sp. TF5 (AY427785), an endophytic fungus of *Taxus mairei* (Wang *et al*., 2000). The tree was rooted with *Tubercularia* sp., which belonged to *Hypocreales* in the same subclass Sordariomycetidae. Of the 652 total characters in the noncoded dataset, 254 (39.0%) were variable, and 180 (27.6%) were parsimony-informative; while in the gap coded dataset, 398 (39.9%) of 599 (60.1%) variable characters were pasimony-informative. Heuristic search found 71 equally most parsimonious trees of 598 steps (CI = 0.609, RI = 0.699, RC = 0.426, and HI = 0.391) with the noncoded dataset; and 38 equally most parsimonious trees of 1331 steps (CI = 0.533, RI = 0.634, RC = 0.338, and HI = 0.467) with the gap coded dataset. Fig. 4 showed that AlS4 was grouped into the small clade of *Nemania*, which was consistent with the NJ and Bayesian analyses. However, in a BLAST search, AlS4 was found to have 99% similarity with *Xylaria* sp. MS1083 (AF153744, 83% coverage). AiL3 and AiL6 were the same in sequence, and sister to AiS5 (all with 100% bootstrap and probability support). Their sequences were found to be 87%-94% similar to the sequences of some *Xylaria* species by BLAST searches. Again, AcapS7 could not be identified as the closest relative of any of the taxa included in Fig. 4. However, the NJ and Bayesian analyses placed AcapS7 sister to *H. perforatum* (figures not shown). AcapS7 was also morphologically identified to be a member of the *Xylariales* AcapS7 could not be identified as the closest relative of any of the taxa included in Fig. 4. However, the NJ and Bayesian analyses placed AcapS7 sister to *H. perforatum* (figures not shown). AcapS7 was also morphologically

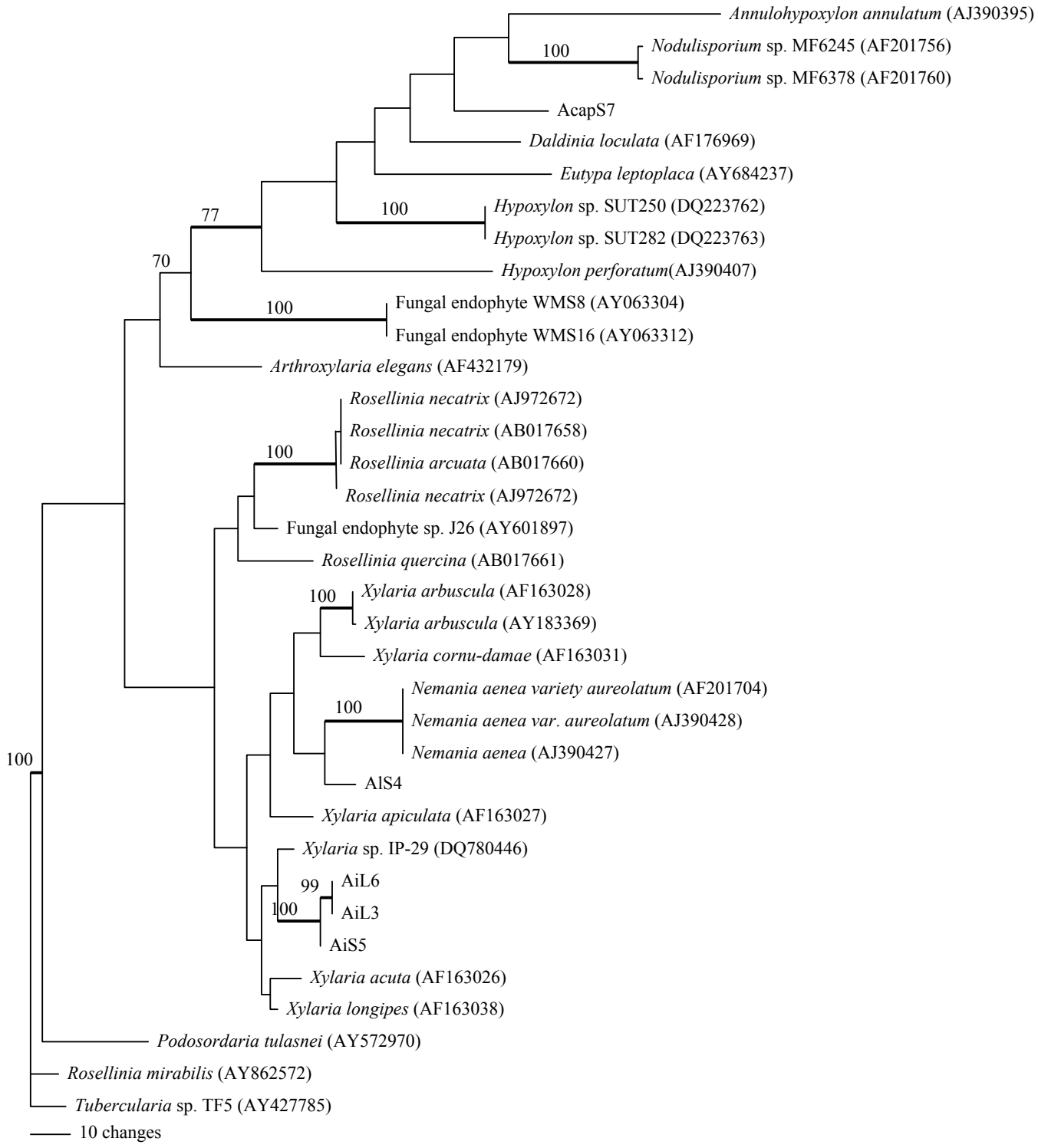

Fig. 4. One of 77 MP trees (Length = 598, CI = 0.609, RI = 0.699, RC = 0.426, HI = 0.391) including the 5 endophytic fungal strains and 30 reference sequences belonging to *Xylariales*. Thickened branches represent clades with over 0.95 Bayesian posterior probability support. Bootstrap support values (≥ 50%) are shown above the branches.

identified to be a member of the *Xylariales* (Table 3). Because AlS4, AiS5, AiL3 and AiL6 all failed to sporulate in PDA culture, they were classified as mycelia sterilia.

Discussion

The ubiquity of endophytes in the plant kingdom has been well established as they have been found in all investigated species

including algae, ferns, lichens, mosses, and vascular plants (Arnold *et al*., 2001; Li *et al*., 2007; Tejesvi *et al*., 2007). The colonization and propagation of endophytes may in some ways offer significant benefits to their host plants by producing a plethora of substances that provide protection or increase the fitness of the hosts, such as enhancement of stress-, insect-, and disease-resistance, productivity improvement, and herbicide activities (Redman *et al*., 2002; Arnold *et al*., 2003; Strobel *et al*., 2004; Tejesvi *et al*., 2007).

Endophytes could produce various bioactive secondary metabolites, especially those isolated from medicinal plants. Novel antibiotics, antimycotics, immuosuppressants, and anticancer compounds are only a few examples of their natural products (Strobel *et al*., 2004; Zhang *et al*., 2006). Lu *et al*. (2000), Liu *et al*. (2003), Ge *et al*. (2006) and Shen *et al*. (2006) reported on some novel and bioactive metabolites produced by endophytic fungi from the artemisinin-producing plant *Artemisia annua*, e.g., isoprenylindole-3-carboxylic acid, 3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene, 3β,5α-dihydroxy-6β-phenyl acetyloxy-ergosta-7,22-diene, leptosphaeric acid, paranolin, and new cytotoxic 10,13-cyclotrichothecane-derived macrolides. *Artemisia capillaris*, *A. indica*, and *A. lactiflora* of the same genus are also valuable medicinal plants. Our previous study showed that some antioxidant phenolic metabolites were produced by the fungal endophytes from these three *Artemisia* species (Huang *et al*., 2007).

Despite the ecological and biomedical importance of endophytic fungi, most of these organisms remain uncharacterized and not much is known about their phylogenetic relationships (Sette *et al*., 2006; Tao *et al*., 2008). Molecular analysis could help to identify the fungal endophytes isolated from plant hosts and to investigate their phylogenetic relationships.

Diversity of endophytes in this study

In this study, 108 endophytic fungal isolates were obtained from *A. capillaris*, *A. indica*, and *A. lactiflora*. For fungal identification, we relied on a combination of traditional and molecular methods. Thirty-four of the representative morphological isolates were further identified with molecular phylogenetic analysis of ITS1-5.8S-ITS2 sequences. Generally, there was a good agreement between morphological and ITS-sequence based approaches. The endophytic communities associated with the three *Artemisia* species showed high species diversity. In addition, fungal species compositions were distinct in different tissues. These endophytes could be identified to species, genus, or family level, mainly including *Aureobasidium pullulans*, *Botryosphaeria parva*, *Alternaria*, *Colletotrichum*, *Ephelis*, *Pestalotiopsis*, *Phomopsis*, *Phyllosticta*, *Nemania*, *Xylaria*, *Pleosporaceae*, and *Xylariaceae*. Among them, *Alternaria*, *Colletotrichum*, *Phomopsis*, and *Xylaria* were the most frequent endophytes either within or among the hosts, consistent with the findings reported in other studies of tropical endophytic fungi (Arnold *et al*., 2000, 2001; Wipornpan *et al*., 2001; Rodrigues *et al*., 2005). *Epichloe* species, the earlier morphs in the life cycle prior to asexual *Ephelis*, are seed transmitted endophytes symbiotic with many grasses (Kuldau *et al*., 1997). In this study, two endophytic fungal isolates of morphological *Ephelis* were isolated from stems of *Artemisia capillaris*. In addition, isolates of *Pestalotiopsis* sp. and *Aureobasidium pullulans* were obtained from inflorescences and leaves of *A. capillaris*, respectively. *Pestalotiopsis* sp. and *A. pullulans* are also common endophytic fungi in many species (Suryanarayanan *et al*., 2005; Tejesvi *et al*., 2006; Hu *et al*., 2007). Jeewon *et al*. (2003) reported the phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species.

Among the three plant hosts, the highest endophytic colonization rate occurred in *Artemisia capillaris*, which had highest fungal diversity. Five fungal isolates belonging to *Aureobasidium pullulans* (AcapL1), *Ephelis* (AcapS8, AcapS10), *Pestalotiopsis* (AcapF6), and *Pleosporaceae* (AcapS4), respectively, were only obtained from *A. capillaris*. Our previous study (Huang *et al*., 2007) found that *A. capillaris* possessed the strongest antioxidant capacity among these three *Artemisia* species. The endophytic fungal isolate AcapF3 (molecularly identified as *Phomopsis loropetali*) obtained from the inflorescence of *A. capillaries* also exhibited the strongest

antioxidant activity among 292 isolates from 29 medicinal plants. This interesting relationship between the bioactivities of the host and its fungal endophyte needs further investigation, which may shed light on host-endophyte co-evolution and interaction, and provide guidance for obtaining bioactive fungal isolates.

Taxonomy and phylogeny of endophyte isolates in this study

Most of the major polygenetic relationships were congruent based on MP, NJ, and Bayesian analyses of either the gap coded or noncoded dataset, except for minor differences in the placement of some small clades, which had not affected fungal strain identification.

All the 27 endophytic strains of *Alternaria* were obtained from host *A. capillaris*, which accounted for 64% of its total fungal isolates. Of the four isolates, AcapF1, AcapL2, AcapS2 and AcapS5, selected for molecular identification, only one or two nucleotides were different in their ITS1-5.8S-ITS2 sequences. AcapL2 and AcapS2 were similar in morphological characteristics, and the two isolates from the stem, AcapS2 and AcapS5, have the same ITS sequence. Sequences alignment showed that they were 100% identical to many closely related species of *Alternaria*, with *Alternaria alternata* as the most frequently matched species. *Alternaria alternata* was previously isolated from grapevine leaves, *and* it could inhibit sporulation of *Plasmopara viticola* and ultrastructurally alter grapevine downy mildew (Musetti *et al*., 2006). In the present study, the *Alternaria* isolates obtained from inflorescences, leaves, and stems differ in their ITS1-5.8S-ITS2 sequences, suggesting that they may belong to different fungal taxa. As indicated by their sequence homology to the corresponding GenBank accessions, the two isolates from stems (AcapS2 and AcapS5) could be *A. gaisen*, the one from leaves (AcapL2) might belong to *A. aff. longipes*, and the one from inflorescences (AcapF1) was most likely *A. alternate*. This study showed that *Colletotrichum* species are the most frequent endophytic fungi in *A. lactiflora*. Twenty-two of the 27 fungal isolates isolated from leaves or stems of *A. lactiflora* belong to *Colletotrichum*, including the 2 isolates of mycelia sterilia sp.8, with one of the isolates molecularly identified as a species of *Colletotrichum*. Another two *Colletotrichum* isolates were obtained from *A. indica*. Previous studies have also reported endophytic *Colletotrichum* isolates to be common \ly isolatd (Cannon and Simmons, 2002; Photita *et al*., 2003). Two other plants of the same genus, *Artemisia mongolica* and *A. annua* have also yielded *Colletotrichum* endophytes (Lu *et al*., 2000; Zou *et al*., 2000). In our phylogenetic analysis, most of the nine representative fungal isolates could be identified as *C. gloeosporioides*, which was asexual phases of *Glomerella cingulata*, and others likely belong to *C. boninense* or *C. truncatum*.

The coelomycetes *Phoma*, *Phomopsis*, *Phyllosticta*, and *Rhizosphaera* are common endophytic fungi (Muller and Hallaksela, 1998; Wipornpan *et al*., 2001; Lacap *et al*., 2003; Ganley *et al*., 2004; Aveskamp *et al*., 2008), which are difficult to distinguish from one another due to their similarity in morphological characteristics (Shenoy *et al*., 2007). In this study, 11 representative fungal isolates from the three *Artemisia* species could be molecularly identified as species of *Phomopsis* or *Diaporthe*, and one as a species of *Phyllosticta*, whereas only two of them could be morphologically identified to *Phomopsis* sp. and all others could only be classified as species of ascomycete or mycelia sterilia. *Phomopsis* species are considered to be the asexual phases of *Diaporthe* species, a teleomorphic genus in the family *Valsaceae* (Girlanda *et al*., 2002). Thus, the seemingly unresolved relationship between *Phomopsis* and *Diaporthe* in this study does not impact the results of molecular identification.

Xylaria species appeared to be the most common endophytic fungi in *A. indica*. Three representative isolates of mycelia sterilia from *A. indica* can be identified as species of *Xylaria* based on ITS data. A mycelia sterilia isolate (AlS4) from the stem of *A. lactiflora* was found to be most similar to *Nemania aenea*, which also belongs to the family *Xylariaceae*. *Xylaria* sp. MS1083 (AF153744) was a top BLAST match sequence of AlS4, and *N. aenea* together with AlS4 were placed within the clade of *Xylaria* species, indicating a close relationship

between *Nemania* and *Xylaria*. Some previous studies suggested that many endophytic mycelia sterilia fungi probably belong to the *Xylariales*. Guo *et al*. (2000, 2003) and Lacap *et al*. (2003) placed most of the mycelia sterilia endophytes in the genus *Xylaria* and some in *Nemania* of *Xylariaceae*. Other nine mycelia sterilia isolates of *A*. *indica* likely also belong to *Xylaria* since they were all similar to *Xylaria* in morphological characteristics. The isolate (AcapS7) isolated from the stem of *A*. *capillaris* could only be morphologically identified as *Xylariales* sp., whose relationship to other available taxa was difficult to establish (Fig.1 and Fig. 4). However, both NJ and Bayesian analyses revealed that AcapS7 was most closely related to *H*. *perforatum* of *Xylariaceae*, suggesting that AcapS7 is likely a species of *Hypoxylon*.

The use of molecular techniques in identifying endophytes

Molecular techniques have been successfully used for identifying endophytic fungi in recent studies (Promputtha *et al*., 2005; Sette *et al*., 2006; Tedersoo *et al*., 2006; Morakotkarn *et al*., 2007). Culture-independent DNA methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and ITS sequencing, have been developed for the investigation of complex microbial communities. For example, Bougoure *et al*. (2005, 2007) directly characterized the fungal community in hair roots using plant materials. Our study also shows that molecular identification based on ITS sequences can be used to complement or verify morphological identification of unknown endophytes. Some fungal isolates could be identified to the species level, and others to the level of genus or family. A high level of species diversity is present in the endophytic community of *Artemisia*, with 34 representative fungal isolates classified into 26 different molecular taxa, which was more sensitive than those identified with traditional methods (23 morphological species, see Table 3). Based on DNA analysis, problems associated with taxonomic identification of mycelia sterilia could be solved. Most of these morphotypes were filamentous ascomycetes belonging to the genera *Phomopsis* (*Diaporthe*) and *Xylaria*, consistent with previous reports (Guo *et al*., 2000; Lacap *et al*., 2003). However, molecular analysis alone also has limitations. It can not overcome the problem of over-isolating fast growing fungal species at the expense of slow growing taxa, nor isolating species that will not grow in culture (Duong *et al*.2006; Hyde and Soytong 2007). The use of ITS sequences also has limitations in phylogenetic analysis. Because the noncoding ITS sequence is fast evolving with many variable characters, it is usually difficult to achieve a perfect sequence alignment at high taxonomic levels. Moreover, it has been shown that 20-30% of sequences downloaded from GenBank for comparative analysis may not be accurate in their identification (see Nilsson *et al*., 2006; Hyde and Soytong, 2007). This inaccuracy in the database may have contributed to some of the unexpected observations in our study, such as *Alternaria alternata*, *Diaporthe phaseolorum*, and *Colletotrichum truncatum*, all having their accessions scattered across the phylogenetic tree, whereas some different species (e.g., several species of *Alternaria* and *Phomopsis*) or genera (e.g., *Xylaria* and *Nemania*) being clustered within the same clade. Further studies using different gene sequences can be conducted to resolve this type of difficulties in the phylogenetic analysis of fungi.

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