Endophytes in Southeast Asia and Japan: their taxonomic diversity and potential applications

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Endophytes were isolated by placing plant parts on agar following extensive washing in running tap water and sterilization by dipping in 75% ethanol and 5.3% sodium hypochlorite. Four hundred and two plants, including endemic plants, collected in Hokkaido, Japan, Indonesia and Malaysia were subjected to this treatment. At least one taxon was isolated from each healthy surface-sterilized stem from each plant examined, indicating that more than one microorganism live together inside a single plant. In total, 1133 strains of endophytic fungi and 678 strains of bacteria were isolated and are stored at -80ºC. These isolates were screened for useful products. Many endophytic fungi and bacteria strains produced useful extracellular enzymes that degrade xylan and mannan, common constituents of plant cells, into xylo-oligosaccharides and manno-oligosaccharides. About 10-30% of endophytes showed antifungal or antibacterial activities in their supernatants. Some fungal strains produced bioactive substances which showed testosterone-5-α-reductase inhibition or promotion in proliferation of mouse hair follicle cells *in vitro*. Some fungal and bacterial strains isolated from Indonesian plants also produced amylolytic enzymes and/or mannanase. These results demonstrate that endophytes are potential sources for discovering useful metabolites, such as oligosaccharides, antibiotics, and enzymes. Phylogenetic analyses of amplified 18S rDNA and the ITS region including 5.8S rDNA of 25 selected endophytic fungi indicated that they belonged to diverse genera. Certain endophytic fungi isolated from individual trees in the Botanical Garden and around the campus of Hokkaido University were found to be specific symbionts of *Ulmus davidiana* var. *japonica*.

Keywords: bacteria, fungi, microbial resource, mutual association, molecular phylogeny.

Introduction

Microorganisms used for biotechnological applications have almost exclusively been isolated from soil (Hyde, 2001). Microorganisms, however, survive in environments, many of which still remain unexplored (Staley *et al*., 1997). My research team has a particular interest in fungi and bacteria inhabiting aerial parts of plants, which have various associations with their

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hosts. These microorganisms are thought to have novel capabilities for biotechnological application (Dreyfuss and Chapela, 1994).

There have been extensive studies on endophytes of various plants in various countries over the past 15 years (Petrini, 1986; Romero *et al*., 2001; Suryanarayanan and Vijaykrishna, 2001; Kumaresan and Suryanarayanan, 2002). In Japan, investigations of endophytic fungi have been carried out on conifers, rye grasses and ericaceous plants (Koga *et al*.; 1993; Hata and Futai, 1993, 1995, 1996, Okane *et al*., 1996). It has been shown that some endophytic fungi from monocotyledonous plants produce substances that are toxic to cattle and insects that feed on the host grasses (Clay, 1988). The production of biologically active substances by endophytic fungi from conifers have also been reported (Stierle *et al*., 1993; Findlay *et al*., 1995; Schultz *et al*., 1995). There is, however, no unified definition of "endophytes" and no extensive systematic survey of endophytes has been conducted.

The endemic plants in subtropical or tropical regions are particularly important because of their richness in species diversity (e.g. Hyde *et al*., 2001; Photita *et al*., 2001; Yanna *et al*., 2001; 2002). However only limited studies have been performed due to the difficulty to access to most of the areas where these endemic species inhabit. The isolation of endophytes from these endemic plants is particularly important because habitat and species are threatened with imminent destruction and loss from human intrusions.

The association and infection cycles of seed-transmitted grass endophytes, e.g. *Neotyphodium* spp., with their host plants are well investigated. Grass endophytes have also been used in agriculture and industry (Clay, 1988; Koga *et al*., 1993). There are, however, few studies that have examined the temporal change of infection of endophytes, especially for endophytes in the twigs of deciduous trees. Many species of fungi asymptomatically inhabit living bark on twigs and small branches of coniferous and broad-leaved trees, but very little is known of their infection cycle and mutual association on the basis of physiology (Wilson, 2000).

In this study, endophytes were isolated from various plants in Southeast Asia and Japan. Isolates were screened for their capability to produce antibiotics, and also oligosaccharides from various natural polysaccharide sources. Seventeen filamentous fungi were selected and identified by rDNA analysis. Endophytes with different physiological states were also isolated from seeds and branches of *Ulmus davidiana* var. *japonica*. They were classified and enumerated and provide an example for discussion of mutual associations between endophytes and their host plants.

Materials and methods

Isolation of endophytes

Our aim was to obtain diverse strains of endophytes from different plant specimens. The following methods for isolation were adopted after examining the effect of sterilization time and isolation media on endophytes obtained (Karim *et al*., 1998). The plant specimens used were young healthy branches (one or two years old) and after washing in running water for 10 minutes, they were dried using a sterile paper towel. Branches were then cut into several pieces of about 1 cm and surface-sterilized by the immersion sequence in 75% ethanol for 1 minute, 5.3% sodium hypochlorite solution for 5 minutes and 75% ethanol for 0.5 minutes. Grass stems were surface-sterilized by immersion in 75% ethanol for 2 minutes only. After drying, the pieces were cut on a sterilized glass to expose their inner tissues and placed in Petri dishes containing CMM medium (17g cornmeal agar (Difco), 20g malt extract, 2g yeast extract, 50mg chloramphenicol per liter water) or NA medium (5g meat extract, 10g peptone, 5g NaCl, 15g agar, 100 mg nystatin per liter water). After 3 days to 4 weeks of incubation at room temperature, fungi were isolated on to potato dextrose agar (PDA) slants, and bacteria on to nutrient agar (NA).

Production of oligosaccharides

One loop of endophytic bacteria cultivated on a nutrient agar slant at 27ºC for 2 days with shaking were inoculated in 5ml of xylan, inulin and mannan media (enzyme production medium in Table 1) and cultivated at 27ºC for 2 days. One plug of agar containing each endophytic fungus cultivated on a PDA slant was inoculated to 5ml of each enzyme production media and cultured in the same way as endophytic bacteria for 5 days. The culture broth was centrifuged at 2000 × g, 4ºC for 20 minutes and the supernatant was used as a crude enzyme. Oligosaccharides were detected by thin layer chromatography (TLC) using a silica gel plate (Silica gel 60, Merck) with a solvent system of 1-butanol-2-propanol-water-acetic acid (7:5:4:2, v/v). Spots were detected with a reagent containing *p*-anisaldehyde-H_2SO_4-ethanol (1:1:18, v/v) using the hydrolysate of polysaccharides as standards.

Production of amylolytic enzymes

Amylolytic microorgansims were screened using PAS and Dhawale medium (Dhawale *et al*., 1982). The plate was incubated for 2-3 days at 27^{o}C

Table l. Media for screening of microorganisms

Enzyme production medium		Antibiotic production media			
		F-4 medium		PD-Y medium	
NH_4NO_3(Mannan, Xylan) or $NaNO_3$(Inulin)	2 g/l	Glycerol	40 g/l	Potato dextrose broth	24 g/l
$MgSO4 \cdot 7H_2O$	0.5 g/l	Soy bean meal	25 g/l	Yeast extract	2 g/l
KCl	0.5 g/l	Yeast extract	5 g /l		
KH_2PO_4	0.5 g/l	Cornsteep liquor	1 g/l		
$FeSO4 \cdot 7H_2O$	0.01 g/l	NaCl	0.5 g/l		
Mannan, Xylan or Inulin	10 g/l				

and then Lugol's medium was added and amylolytic activity detected by the presence of a clear zone.

Production of antibiotics and bioactive substances

Endophytic fungi were cultivated for 5 days, and bacteria for 2 days at 27ºC with shaking in antibiotic production medium (Table 1). The culture broth was centrifuged at 2000 × g, 4ºC for 20 minutes and the supernatant was used for a paper disk diffusion assay using *Alternaria sp*., *Bacillus subtilis*, *Salmonella sp*., and *Saccharomyces cerevisiae* as test organisms (Nyunt, 1997)). This supernatant was also screened for testosterone-5-α-reductase inhibition (Okano *et al*., 1994) and the promotion in proliferation of mouse hair follicle cells *in vitro* (Tanigaki *et al*., 1989).

Analysis of rDNA gene

Endophytic fungi were grown in 500 ml Sakaguchi flasks containing 100ml modified Czapek Dox liquid medium (33.4g modified Czapek Dox liquid medium (Oxoid), 2g yeast extract per liter water) or potato dextrose broth (Difco) with shaking at 27 ºC for 3 days. The mycelia were harvested by centrifugation (6000 × g, 4ºC, 10 minutes), and washed with TEN buffer (10 mM Tris-HCl, 1mM EDTA, and 0.1M NaCl, pH 8.0). The washed mycelia with TEN buffers were filtered with a filter paper (Toyo No.1) in a Buchner funnel connected to water pump. The pre-dried 'mycelial cake' was peeled off from the filter paper, frozen at -80ºC and lyophilized. Genomic DNA was isolated from 50mg of lyophilized mycelia according to the methods of Raeder and Broda (1985). The 18S rDNA and internal transcribed spacer (ITS) region including 5.8S rDNA were selectively amplified by PCR using the specific

primers for filamentous fungi proposed by White *et al*. (1990). In most strains, NS l-NS6, NS5-NS8 and ITS5-ITS4 segments were amplified and purified with MicroSpin columns S300HR system (Pharmacia Biotech). Nucleotide sequences of the PCR products were determined in both directions by the dideoxynucleotide chain termination method using a Thermo Sequenase Core Sequence kit, fluorescent primer (NSl, NS3, NS5, NS6 and NS8) and ALF express DNA Sequencer (Pharmacia Biotech) following the conditions provided by the manufacturer. The entire sequences of 18S rRNA genes and ITS region were compiled by using GENETYX-MAC and Gene Assembler. They were compared with those in the GenBank, EMBL and DDBJ databases through the DNA Information and Stock Center (National Institute of Agrobiological Resources, Tsukuba, Japan) using FASTA (Pearson and Lipman, 1988) and BLAST SEARCH (Altschul *et al*., 1990). The sequences of the 18S rDNA and ITS region were aligned respectively with the sequences of similar fungi retrieved from databases using CLUSTAL W (Tompson *et al*., 1994). A phylogenetic tree was constructed from the evolutionary distance data by a neighbor-joining clustering method with PHYLIP, version 3.57 c 8 (distributed by Felsenstein, J. Department of Genetics, University of Washington, Seattle). Confidence values for individual branches of the resulting tree were determined by a bootstrap analysis in which 1000 bootstrap trees were generated from resampled data.

Isolation of endophytes from Ulmus davidiana var. japonica, classification and enumeration of isolates

Twigs were collected from elm trees at the Botanical Garden and campus of Hokkaido University, Sapporo in Japan. In order to examine the variation of endophyte infections among the different biological states of twigs: healthy and less than 1 year old twigs, withered twigs and twigs without leaves in June, were cut from the trees. For the enumeration of endophytic fungi throughout the year, four twigs, about 20 cm in length, 1 to 3 years old, were cut from each of two mature trees at the intervals of 2 or 3 months; in April (pre-bud burst leaves and before blooming), June (post-bud burst leaves and blooming), September, November (leaf abscission), January and March (under snowfall). After washing under running water for 10 minutes and removing water with a paper towel, the terminal 1 cm of twig length was cut off, then 12 segments of 1 cm were cut from the remaining twigs. Surface sterilization was carried out in the same way as previously described in “Isolation Method”. Approximately 30 seeds were collected from twigs and the ground underneath the tree from which segments were cut. Insect damaged and rotten seeds were removed.

Surfaces of seeds were sterilized with 75% ethanol for 1 minute, 5.3% sodium hypochlorite solution for 2 minutes, and 75% ethanol for 30 seconds, before cutting a seed in half and placing halves on an isolation agar medium.

Classification and identification of endophytic fungi were carried out based on morphological characteristics and the sequence analysis of 18S rDNA and internal transcribed spacer (ITS) region including 5.8S rDNA. The colonization frequency was calculated from the numbers of endophytes colonizing segments and all segments examined as follows

$$\text{Colonization frequency (\%)} = \frac{\text{The number of samples colonized by endophyte}}{\text{The total number of samples examined}} \times 100$$

Confrontation test

The growth patterns of dominant endophytic fungi and other groups of endophytic or saprobic fungi were characterized when they were inoculated simultaneously at opposite sides of CMM plates and cultured for 10 days by measuring the width of the zone between the edge and the radius of both colonies.

Results and discussion

We obtained 282 endophytic fungi and 173 endophytic bacteria from 57 plants in Hokkaido, 251 fungi and 129 bacteria from 146 plants in Indonesia and 600 fungi and 376 bacteria from 199 plants in Malaysia. We isolated at least one endophytic microorganism from each plant sample examined. This number is rather low when compared to other endophytic studies (e.g. Fröhlich *et al*., 2000; Toofanee and Dulymamode, 2002), and indicates the strength of our surface sterilisation regime which was designed to remove epiphtyes.

Many of the endophytic fungi and bacteria were found to produce useful extracellular enzymes that degrade xylan and mannan, common constituents of plant cell walls, into xylo-oligosaccharides and manno-oligosaccharides (Table 2). About 10% to 30% of endophytes showed antifungal or antibacterial activities in their supernatants. Seventeen filamentous fungi were selected as candidates for further study (Table 3). One isolate effectively produced xylobiose from xylan and we have successfully isolated and characterized the enzyme (Suto *et al*., 2002) Some fungal strains produced bioactive substances which exhibited the inhibition of testosterone-5-α-reductase activity,

Table 2. Screening of endophytes

Endophytes tested	Production of oligosaccharides from			Biological activity	
	Mannan	Xylan	Inulin	Antifungal	Antibacterial
Plants in Hokkaido					
Fungi	110*/282#	251/282	79/282	24/211	76/211
Bacteria	96/173	67/173	10/113	11/161	47/161
Plants in Indonesia					
Fungi	21/189	125/189	52/189	45/189	13/189
Bacteria	59/129	40/129	17/129	3/30	2/30
Plants in Malaysia					
Fungi	227/427	328/427	98/427	48/252	11/252
Bacteria	70/378	68/378	30/378	53/292	58/292

*numbers of active strains.
#numbers of endophytes tested.

promotion in proliferation of mouse hair follicle cells *in vitro* or some allelopathic effects (Tanaka *et al*., 2002). Some fungal or bacterial strains isolated from Indonesian plants also produced amylolytic enzymes (Anindyawati *et al*., 2001) and/or mannanase. These results demonstrate that endophytes are potential microbial resources that can produce biotechnologically useful substances such as oligosaccharides, antibiotics and enzymes as suggested by (Dreyfuss and Chapela, 1994; Tanaka *et al*., 1999).

The sequences of the 25 selected endophytes were approximately 1,700 nucleotides long. They were aligned with 18S rRNA gene sequences retrieved from the GenBank, EMBL and DDBJ data libraries. Figure 1 shows the phylogenetic relationships of endophytes and known related fungi. The endophytic fungi belonged to *Pyrenomycetes*, *Loculoascomycetes, Discomycetes* and *Hymenomycetes.* The *pyrenomycetous* endophytic strains clustered with members of the *Diaporthales, Xylariales, Phyllachorales* and *Hypocreales*. The *loculoascomycetous* endophytic strains clustered with members of *Pleosporaceae, Phaeosphaericeae, Leptospheriaceae, Botryosphaericeae,* anamorphic *Mycosphaerellaceae,* and *Coccodiniaceae.* The phylogenetic tree based on ITS region also illustrated the high species diversity of endophytes encountered (Fig. 2). These results demonstrate that plants are good sources for a high diversity of fungi that have interesting metabolite producing capabilities. The sequence analysis of rDNA is a useful means to classify and identify endophytes, because their reproductive stages are often difficult to be observed on culture media (Guo *et al*., 2000; Lacap *et al*., 2003).

Table 3. Selected strains of interest

Endophyte	Characteristic	Host plant	Location
7A	Xylan → X2	*Rosa rugosa*	Hokkaido Japan
2A	Inulin → F5. Antifungal	*Zanthoxylam piperitum*	□
3B	Xylan → X2. Antibacterial	*Ulmus davidiana*	□
5D	Xylan → X1,X2,X3. Antifungal	*Syringa vulgaris*	Hokkaido Japan
11A	Xylan → X1,X2,X3 and Inulin → F6 Antifungal and antibacterial	*Actinidia kalomikta*	□
12A	Xylan → X2 and Inulin → F6 Antifungal	*Humulus lupulus var. cordifolius*	□
15B	Xylan → X2 and Inulin → GF4 Antifungal	*Rubus idaeus var. aculeatissimus*	□
14A	Antifungal and antibacterial	*Pueraria lobata*	□
17B	Inulin → F5 and Xylan → X2, X3	*Abelmoschus sp*	□
44	Antifungal	*Unknown*	Indonesia
71	Mannan → M4	*Unknown*	□
I12-3	Lepidimoide production	*Coleus galeatus*	Indonesia
IT81	Amylolytic enzyme production	*Dillenia execellaa*	□
IT72	Amylolytic enzyme production	*Dillenia suffruticosa*	□
M40	Testosterone 5 αreductase inhibition	*(Kemuncong)*	Malaysia
M42	Testosterone 5 αreductase inhibition	*Lasianthus sp.*	Malaysia
I1-3	Glucomannan → M2,M3	*Hotounia cordata*	Indonesia

X1: xylose, X2: xylobiose, X3: xylotriose, M2: mannobiose, M4: mannotetraose, M5: mannopentaose, F5: inulopentaose, F6: inulohexaose, Gf4:glycosyl inulotetraose

Few endophytic fungi were isolated from the stems of young twigs and seeds of *Ulmus davidiana* var. *japonica* (Table 4). The C.F. value in branches more than 1-year-old was almost 100% and did not decrease even in the winter season. *Coniothyrium fuckelii* and *Phomopsis* sp. were dominant taxa isolated with high frequency (Fig. 3). The anamorph of *Paraphaeosphaeria* sp., *Phoma* sp., *Alternaria* sp., and anamorphic *Botryosphaeriaceae* were also isolated with a frequency of 4%-22% throughout one whole year. *Phaeopshaeria* sp., *Fusarium* sp., *Epicoccum* sp., *VerticillIum* sp., anamorphic Basidiomycetes and xylariaceous taxa were less frequently isolated from healthy branches. Saprobic fungi such as *Botrytis* sp., *Mucor* sp., *Pestalotiopsis* sp., *Scopulariopsis* sp., and *Trichoderma* sp., were isolated from the twigs without

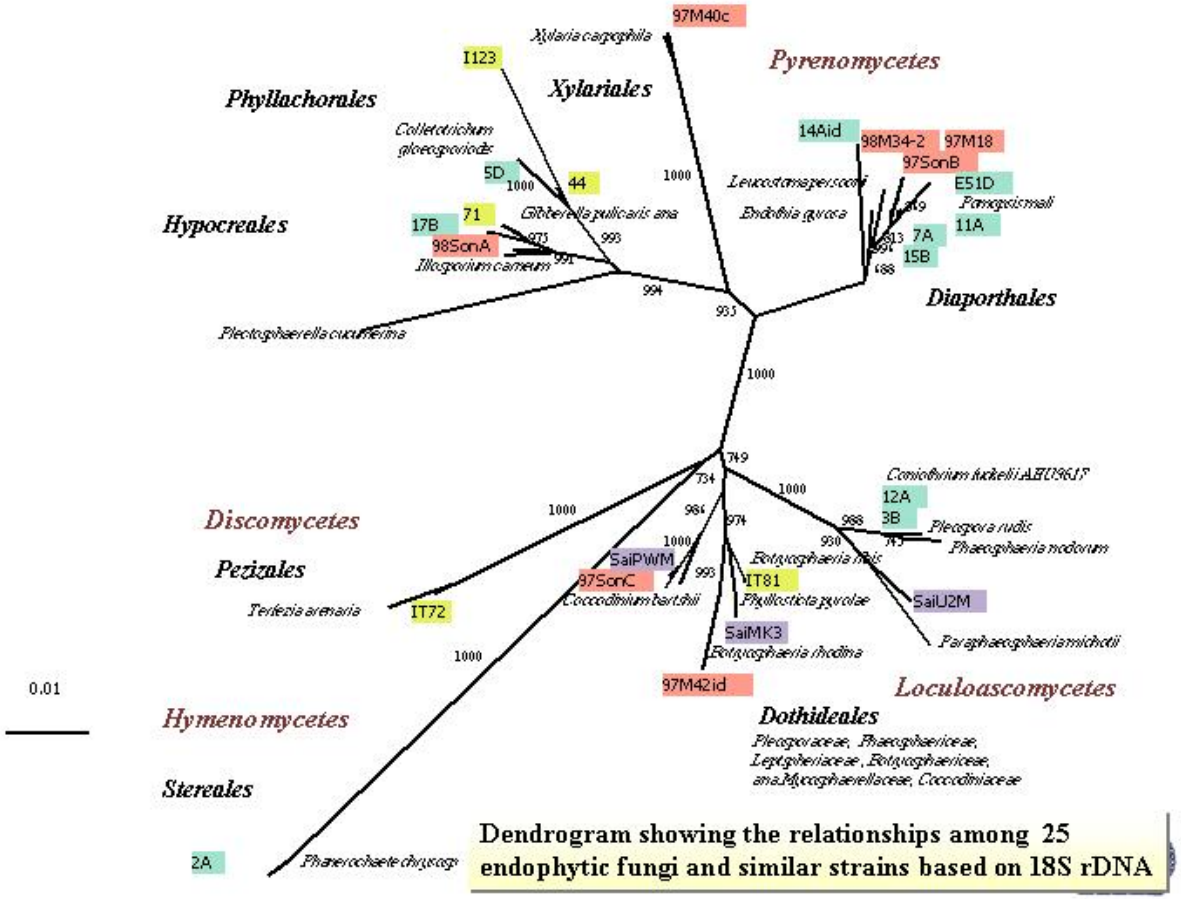


Fig. 1 Dendrogram showing the relationships among 25 endophytic fungi and similar strains based on 18S rDNA

Table 4. Infection frequency of endophytes in twigs and seeds

Plant	Fungi (%)	Bacteria (%)
Twigs less than 1-yr-old	0 - 73.3	0 - 12.5
Twigs more than 1-yr-old	93.7 - 100	14.6 - 39.5
Twig without leaf in June	97.9	ND*
Seeds	0	46.7

ND*: not done

leaves, and may have certain relationships with leaf abscission as mentioned by Kowalski and Kehrbranche (1996). The seasonal variation of C.F. in dominant taxa appeared to be significant. Fig. 4 shows that the C.F. value of *Coniothyrium fuckeli* decreased from April to the next March and that of *Phomopsis* sp. significantly increased from November to March. Wilson and Carrol (1994) proposed the infection cycle for *Discula quercina* in leaves of Olegon white oak as horizontal transmission via spores associated with rainfall and assumed that the most likely sources of conidia in rainfall under mature oak trees were from conidiomata of the endophyte that had been observed on dead fallen leaves and bark. For deciduous trees, the endophytes would have to find alternative substrate to colonize until new leaves emerge. However, our results suggested that the branches including bark and xylem could be a place where the endophytes occupy or overwinter in dormant state year round. *Phomopsis oblonga* colonized in elm bark has reported as a deterrent against elm bark beetle boring and feeding (Webber *et al*., 1984; Claydon *et al*., 1985). *Phomopsis* sp. isolated from *U. davidiana* var. *japonica* were shown to be different from *P. oblonga* by rDNA sequence analysis, but the increase of C.F. of *Phomopsis* sp. from November to March might be involved in the antagonism towards insects, that will lead to increased plant fitness.

ITS sequences of dominant taxa and anamorphic state of *Paraphaeosphaeria* sp. isolated each year were analyzed and compared within the same genera. Several strains in each genera had identical ITS sequences and it is presumed that each of these dominant strains are genetically conserved by repetitive infection (Table 5). Table 6 shows the growth patterns when dominant endophytic fungi and other groups of endophytic or saprobic fungi were inoculated simultaneously at opposite sides on a CMM plate to test the hypothesis that dominant taxa can contribute to a possible mutualistic relationship. Schults *et al*., (1995) isolated biologically active substances from these confrontation test *in vitro.* There were specific growth patterns between dominant and other taxa ; dominant strains had some inhibition effect to other endophytic fungal growth and some endophytic fungi produced substances which diffuse in the agar medium and inhibit the growth of dominant taxa.

Table 5. Identification of endophytic fungi from *Ulmus davidiana* using rDNA sequence.

Strains isolated*[3]	On the basis of 18S rDNA	Homology percentage	On the basis of ITS region	Homology Percentage	Probable taxa
E5-3B	*Coniothyrium fuckelii*	100%	*Coniothrium fuckelii**[1]	99.8%	*Coniothyrium fuckelii*
E11-4CC	AHU9617*[1]	(1746bps)	AHU9617	(551bps)	
E15-3CB					*Conithrium* sp.
U4A1-B	*Leptosphaeria maculans*	99.1%			
U4B2-C	(U04238)	(1746bps)			
U6B3-OC					
01U4B4-9F					
E5-3C	*Parahaeosphaeria michotii*	100%	*Paraphaeospaeria pilleata*	88.8 %	*Paraphaeospaeria* sp.
E15-3CC	(AF250817)*[2]	(1724bps)	(AF250821)	(590bps)	
U4A3-B					
U4B1-B	*Paraphaeospaeria pilleata*	100%	*Parahaeosphaeria michotii*	88.4%	
U11A1-4J	(AF250821)	(1718bps)	(AF250817)	(584bps)	
U11A3-1K		99%			
01U4B1-H		(1733bps)			
01U4A3-5G					
U4A2-A	*Phomopsis mali**[1]	99.9%	*Diaporthe phaseolorum*	97.5%	*Phomopsis* sp.
01U4A1-10J	IFO 31031	(1759bps)	(AF001016)	(593bps)	
00U9A2-YA					
U6A1-OB	*Endothia gyrosa*	98.7%	*Phomopsis mali*	93.0%	
E15-3CA	(L42443)	(1737bps)	IFO 31031	(598bps)	

*1: The sequence was analysed in this study.
*2: The number in parenthesis is an accession number in GenBank.
*3: The sequences of ITS region in each isolated strains were almost identical within a group.

Table 6. Results of confrontation test.

Group and taxon	Comparison of the radius□		Distance between the edges of both colonies □□□□		Growth pattern of colonies	
	***Conio.* sp.**	***Phomo.* sp.**	***Conio.* sp.**	***Phomo.* sp.**	***Conio.* sp.**	***Phomo.* sp.**
Coniothyrium sp.	1	1.1	3.9	0.8	A	A
Phomopsis sp.	1	1	0.8	0	A	A
Paraphaeosphaeria sp.	1.1	1.2	5.2	1.8	A	A,B
□ *Phoma* sp.	0.9	0.8	3.2	0.5	B	B
Alternaria sp.	0.9	0.9	0.4	0	B	B
Anamorphic Botryosphaeraceae	0.8	0.8	0	1.3	B	B
Pheaosphaeria sp.	1.2	1	8.6	11	D	D
□ *Fusarium* sp.	0.9	0.9	3.6	5.6	D	D
Xylariales	1	1	0	2.1	A,C	A,B
Basidiomycetes	0.5	0.7	0	0	C	C
Verticillium sp.	1.1	1.3	1.8	0	A	A
Botrytis sp.	0.8	0.6	0	0	C	C
□ *Scopulariopsis* sp.	0.6	0.7	0	0	C	C
Trichoderma sp.	0.4	0.4	6.9	0	C	C
Pestalotia sp.	0.8	0.8	1	0.6	B	B
Mucor sp.	0.7	0.8	0	0	C	C

□Comparison of the radius = Radius (mm) of dominant strain in dual culture

□ Average radius (mm) of dominant strain *n*=3

A: No effect on each other.

B: Dominant taxon has inhibitory effect on the other.

C: The other taxon grows over the dominant one.

D: The other taxon has an inhibitory effect on the dominant one.

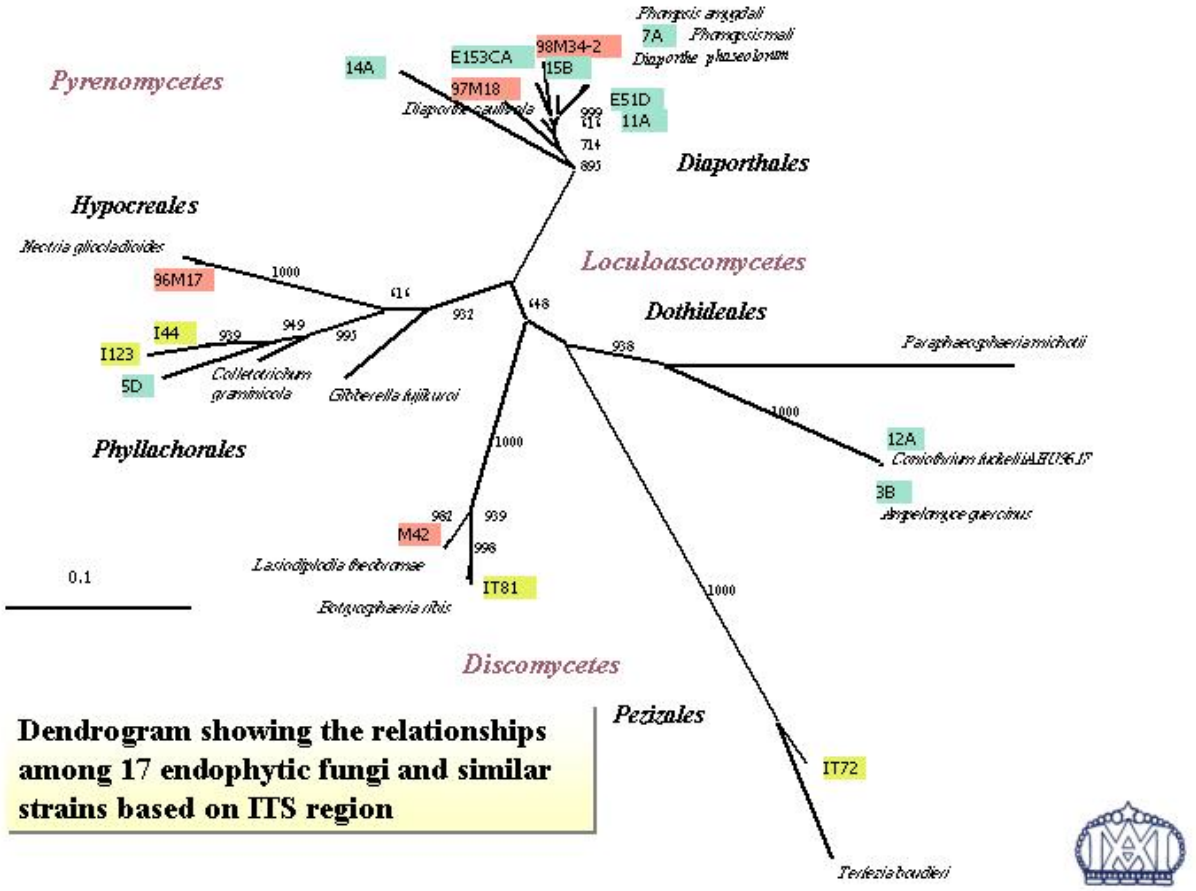
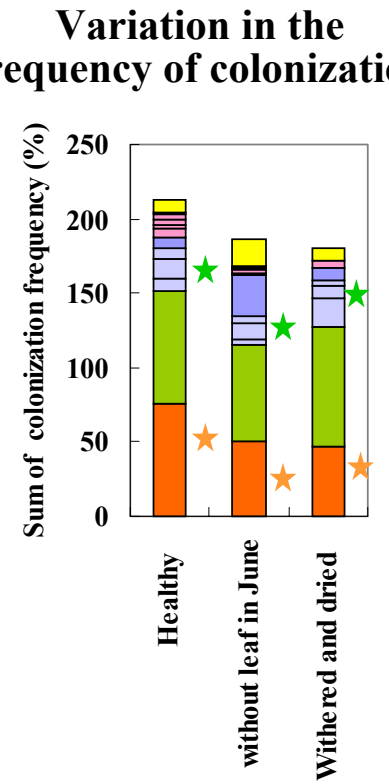


Fig. 2. Dendrogram showing the relationships among 17 endophytic fungi and similar strains based on ITS region



Taxa	Healthy twigs	Twigs without leaf	Withered and dried twigs
Coniothyrium fuckelii	x	x	x
Phomopsis sp.	x	x	x
**Paraphaeospaeria* sp.	x	x	x
Phoma sp.	x	x	x
Alternaria sp.	x	x	x
Botryspaeraceae	x	x	x
Phaeosphaeria sp.	x	x	-
Fusarium sp.	x	x	-
Epicoccum sp.	x	x	-
Xylariales	x	-	x
Basiomycetes	x	-	-
Verticillium sp.	x	-	-
Botrytis sp.	-	x	-
Trichoderma sp.	-	x	-
Pestalotia sp.	-	x	-
Scopulariopsis sp.	-	x	-
Mucor sp.	-	x	-

*: anamorphic state, x: isolated, -: not isolated

Fig.3. Fungi isolated from various twigs

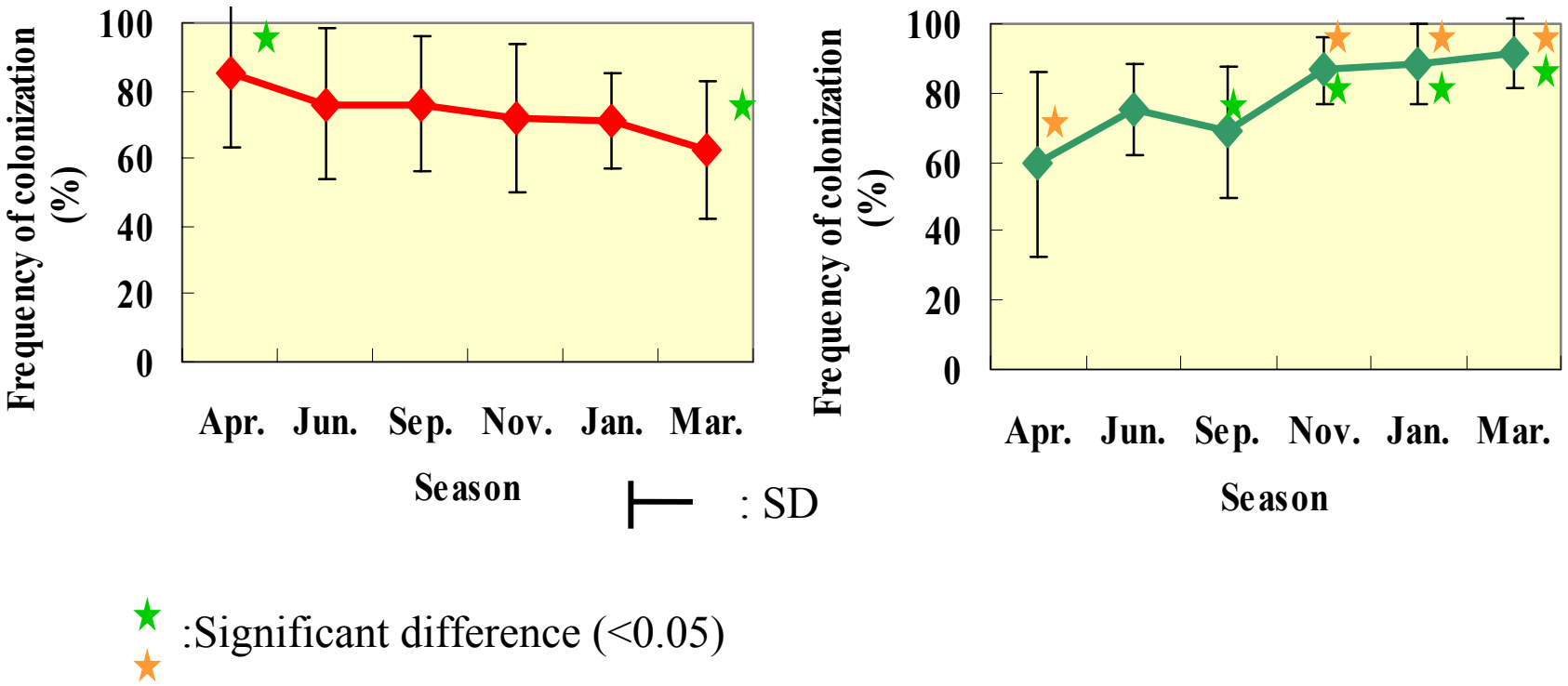


Fig. 4. Seasonal variation of colonization frequency in single taxon

These results presented here may indicate that (1) *Ulmus davidiana* var. *japonica* may have a particular interaction with *Coniothyrium fuckelii* and *Phomopsis* sp., which are the dominant endophytes; (2) the ability of endophytes to produce various enzymes means that the host supplies nutrients as well as a habitat for endophyte colonisation; and (3) antibiosis properties of endophytes may protect the host from pathogenic microbes.

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