Endophytic *Pestalotiopsis* species associated with plants of *Podocarpaceae, Theaceae* and *Taxaceae* in southern China

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A survey of endophytic *Pestalotiopsis* associated with ten plant species belonging to three families in southern China was carried out from 2001 to 2003. Colonization frequencies of endophytic *Pestalotiopsis* species varied with host plants, ages, tissues and sites. A total of 24 endophytic *Pestalotiopsis* species were isolated, of which 18, 16 and four species were obtained from *Podocarpaceae*, *Theaceae* and *Taxaceae*, respectively. The Shannon-Wiener diversity indices of *Pestalotiopsis* species in the three families were 2.22, 2.29 and 1.07, respectively. The number of *Pestalotiopsis* species isolated from different hosts varied from two to 16, with an average of 5.3 species per host. The community similarity of endophytic *Pestalotiopsis* species was higher between *Podocarpaceae* and *Theaceae* than between *Podocarpaceae* and *Taxaceae* as well as *Theaceae* and *Taxaceae*. There were higher community similarities of endophytic *Pestalotiopsis* species between Hangzhou and Nanning and between Hangzhou and Kunming than between Nanning and Kunming. Molecular phylogeny based on the analysis of ITS region (ITS1, 5.8S and ITS2) sequences of 41 *Pestalotiopsis* strains indicated that some *Pestalotiopsis* species could have endophytic and pathogenic stages in their life cycle. Our results support the conclusion that *Pestalotiopsis* species are not generally specific to host plants.

Key words: diversity, endophyte, host recurrence, *Pestalotiopsis*, phylogenetic relationships

Introduction

Pestalotiopsis species are broadly distributed in the world, occurring on a wide range of substrata (Wang *et al*., 2005). Most species are plant pathogens

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(Zhu *et al*., 1991; Zhang *et al*., 2003) and some are saprobes in soil (Agarwal and Chauhan, 1988) and in plant debris (Osono and Takeda, 2000; Tang *et al*., 2003). Studies on *Pestalotiopsis* diversity in China has revealed about 120 pathogenic and saprobic species with some new species and new combinations (Zhu *et al*., 1991; Chen *et al*., 2002, 2003; Wang *et al*., 2002; Zhang *et al*., 2003).

Endophytic *Pestalotiopsis* have often been reported and considered as a main part of the *Pestalotiopsis* community in nature (Strobel *et al*., 1996, 1997; Okane *et al*., 1997, 1998; Suryanarayanan *et al*., 1998; Cannon and Simmons, 2002; Toofanee and Dulymamode, 2002; Wei and Xu, 2003a, b, 2004; Kumar and Hyde, 2004; Photita *et al*., 2004; Wang and Guo, 2004, Wei *et al*., 2005a; Liu *et al*., 2006). Endophytic *Pestalotiopsis* have been commonly isolated particularly in the subtropical and tropical regions. Ramos-Mariano *et al*. (1997, 1998) reported that *P. palmarum* was the dominant endophytic fungus in coconut (*Cocos nucifera*) in Brazil. Kumaresan and Suryanarayanan (2000) pointed out that *Pestalotiopsis* sp. was the dominant endophyte in the reproductive parts of *Rhizophora apiculata* (*Rhizophoraceae*) in India. *Pestalotiopsis* was isolated as the most dominant endophytic fungus from the leaves of *Cordemoya integrifolia* with an isolation frequency of 22.6% in Mauritius (Toofanee and Dulymamode, 2002). Therefore, investigations of the diversity of *Pestalotiopsis* species cannot be sufficient without endophyte studies. As host plant diversity is great in southern China, particularly in the part under Yangtze River, including temperate (only on some high mountain), subtropical and tropical regions, there should also be abundant endophytic *Pestalotiopsis* species.

The traditional identification of *Pestalotiopsis* species was previously more or less dependent on host association (Chen *et al*., 2002, 2003; Wang *et al*., 2002). Recently, phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association showed that isolates from the same host were not phylogenetically closely related and that there is a close phylogenetic relationship between isolates possessing similar morphological characters (Jeewon *et al*., 2004; Wei *et al*., 2005b). It was proposed that *Pestalotiopsis* species are not specific to host plants (Zhu, 1989; Jeewon *et al*., 2004; Wei *et al*., 2005b)

The aim of this paper is to demonstrate species diversity of endophytic *Pestalotiopsis* on plants of *Podocarpaceae*, *Theaceae* and *Taxaceae* in southern China and to answer the questions if there are relationships between endophytes and pathogens, and if endophytic *Pestalotiopsis* species are specific to host plants.

Materials and methods

Study sites and sample collection

The twigs and leaves of ten plant species belonging to three families, i.e. *Podocarpaceae*, *Taxaceae* and *Theaceae*, were collected in Hangzhou of Zhejiang, Nanning of Guangxi and Kunming of Yunnan in southern China.

Hangzhou site: the Hangzhou Botanical Garden and the Longjing Tea Garden located at 120º12′E, 30º10′N and an altitude of 100 m above sea level, where the annual average temperature, relative humidity and precipitation are 16.3ºC, 80% and 1513.6 mm, respectively. Fifteen samples were collected from *Camellia japonica* and *C. sasanqua*, respectively, ten samples from *C. sinensis* (*Theaceae*), 20 samples from *Podocarpus macrophyllus* and *P. nagi* (*Podocarpaceae*), respectively and 20 samples from *Taxus chinensis* var. *mairei* (*Taxaceae*).

Nanning site: the Botanical Garden of Guangxi Forestry Academy of Sciences and the Golden Flower Tea Park located at 108º21′E, 22º49′N and an altitude of 72.2 m above sea level, where the annual average temperature, relative humidity and precipitation are 21.6ºC, 80% and 1300.6 mm, respectively. Twenty samples were collected from *C. japonica* and *C. sasanqua*, respectively, ten samples from *C. nitidissima* and *C. oleifera* (*Theaceae*), respectively, and 20 samples from *P. macrophyllus* and *P. nagi* (*Podocarpaceae*), respectively.

Kunming site: the Kunming Botanical Garden of Chinese Academy of Sciences and the Kunming International Horticultural Exposition located at 102º41′E, 25º01′N and an altitude of 1190 m above sea level, where the annual average temperature, relative humidity and precipitation are 17.4ºC, 73% and 1012 mm, respectively. Fifteen samples were collected from *C. japonica* and *C. sasanqua*, respectively, ten samples from *C. reticulata* and *C. sinensis* (*Theaceae*), respectively, 15 samples from *P. macrophyllus* and *P. nagi* (*Podocarpaceae*), respectively, and 20 samples from *T. yunnanensis* (*Taxaceae*).

Isolation and identification

Thirty segments (5 × 5 mm) of leaves and twigs were cut from each sample and in total 18000 segments (6000 segments from each site) were used for isolation from three sites in this study. The surface sterilization of plant tissues was according to Wei and Xu (2004) and Kumar and Hyde (2004). The sterilized segments were transferred to PDA medium in Petri dishes. Plates

were incubated at 25°C for 3-20 days and checked regularly. Further isolation was carried out using single spore following the methods by Choi (1999) and Promputtha *et al*. (2005). Endophytic *Pestalotiopsis* strains were grown on autoclaved segments of carnation leaves (*Dianthus caryophyllus*), which were placed on cultures for sporulation (Strobel *et al*., 1996). *Pestalotiopsis* species were identified based on their morphological characters (Steyaert, 1949; Guba, 1961; Nag Raj, 1993). The living cultures were deposited in China General Microbiological Culture Collection Center (CGMCC) in Beijing, China.

Data analysis

Colonization frequency (CF%) was calculated as the total number of plant tissue segments infected by *Pestalotiopsis* species divided by the total number of segments incubated. The Shannon-Wiener diversity index (*H′*) was used to estimate the species diversity of the fungal assemblages recovered from a particular type of sample (leaf and twig) and from different sampling sites. The *H′* was calculated according to the following formula: $H' = -\sum_{i=1}^{k} pi \times \ln pi$, where *k* is the total number of fungal species, and *pi* is the proportion of individuals that species *i* contributes to the total (Pielou, 1975).

To evaluate the degree of community similarity of endophytic *Pestalotiopsis* species between two sampling sites, Sorenson's coefficient (C_S) was employed and calculated according to the following formula: $C_S = 2j/(a+b)$, where *j* is the number of endophytic *Pestalotiopsis* species coexisted in the both sampling sites, *a* is the total number of endophytic *Pestalotiopsis* species in one sampling site, and *b* is the total number of endophytic *Pestalotiopsis* species in the other sampling site.

Statistical analysis was made by SPSS for Windows. The same letters on the bars of the same graph are not significantly different according to the Least Significant Difference (LSD) test ($p < 0.05$).

DNA extraction, PCR amplification and DNA sequencing

About 50 mg fresh fungal mycelia were scraped from the surface of the PDA plate and genomic DNA was extracted following the protocol of Wang *et al*. (2005) and Cai *et al*. (2005). The DNA pellet was then re-suspended in 100 μL TE buffer (10 mM Tris-HCL, 1 mM EDTA) and the final concentration of total DNA was *ca*. 100 ng μL^{-1}.

Primers ITS5 and ITS4 were used to amplify the internal transcribed spacers and 5.8S gene of rDNA (White *et al*., 1990). The DNA fragment was

amplified in an automated thermal cycler (PTC-100^{TM}, MJ Research, Inc.). Amplification was performed in a 50-μL reaction volume which contained PCR buffer (10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCL, pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCL_2$, 200 μM of each deoxyribonucleotide triphosphate, 15 *p*mol of each primer, *ca*. 100 ng template DNA, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). The thermal cycling program was as follows: 3 minutes initial denaturation at 95˚C, followed by 35 cycles of 40 seconds denaturation at 94˚C, 50 seconds primer annealing at 52˚C, 1 minutes extension at 72˚C, and a final 10 minutes extension at 72˚C. A negative control using water instead of template DNA was included in the amplification process. Four microlitres of PCR products from each PCR reaction were examined by electrophoresis at 75 V for 2 h in a 0.8% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0) and visualized with UV light after staining with ethidium bromide (0.5 μg mL^{-1}).

PCR products were purified and directly sequenced in an automated sequencer (377, ABI Inc., USA) followed the manufacturer's instructions.

Sequence data analysis

The ITS region (ITS1, 5.8S gene and ITS2) sequences of 44 strains (Table 1) were aligned using Clustal X program (Thompson *et al*., 1997) and the matrix was adjusted manually where necessary to maximize alignment. The alignment data were subsequently used for maximum-parsimony analysis in which searches for most parsimonious trees were conducted with the heuristic search algorithms with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b1a (Swofford, 1988). For each search, 1000 replicates of random stepwise sequence addition were performed. Maxtrees set to 10000, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. A preliminary analysis showed no major topographical differences between trees obtained with gaps treated either as fifth character state or as missing data (results not shown). Character states were all treated as unordered and of equal weight. Statistical support for the internal branches was estimated by bootstrap analysis with 1000 replications.

Results

Colonization frequency of endophytic Pestalotiopsis

Results of colonization frequencies of endophytic *Pestalotiopsis* species in ten plants belonging to three families are shown in Fig. 1. Colonization

Table 1. Taxa used for ITS (ITS1, 5.8S, ITS2) region sequence analysis in this study

Taxa	Collection site	Host plant	Habit	GenBank accession No.
Pestalotiopsis adusta 20	Nanning, Guangxi	*Podocarpus macrophyllus* var. *maki* (leaf)	Pathogen	AY687298
P. heterocornis 358	Hangzhou, Zhejiang	*P. macrophyllus* (twig)	Endophyte	AY681489
P. disseminata 43	Nanning, Guangxi	*P. macrophyllus* (leaf)	Pathogen	AY687869
P. heterocornis 391	Hangzhou, Zhejiang	*P. macrophyllus* (fruit)	Endophyte	AY681491
P. heterocornis 380	Hangzhou, Zhejiang	*P. macrophyllus* (bark)）	Endophyte	AY681490
P. microspora 319	Nanning, Guangxi	*P. macrophyllus* (twig)	Endophyte	AY687882
P. olivacea 263	Kunming, Yunnan	*P. nagi* (leave)	Pathogen	DQ417182
P. neglecta 401	Nanning, Guangxi	*P. nagi* (twig)	Endophytic	AY682932
P. lawsoniae 57	Nanning, Guangxi	*Pinus massoniana* (leaf)	Pathogen	AY687871
P. lawsoniae 1018	Shaoguan, Guangdong	*P. massoniana* (twig)	Endophyte	AY681472
P. yunnanensis 8171	Kunming, Yunnan	*P. macrophyllus* (twig)	Endophyte	AY526872
P. yunnanensis 789	Kunming, Yunnan	*P. macrophyllus* (twig)	Endophyte	AY373375
P. funereoides	GenBank	/	/	AF377289
P. thujae	GenBank	/	/	AF377295
P. funerea	GenBank	/	/	AF405299
P. maculans	GenBank	/	/	AF405296
P. karstenii 201	Hangzhou, Zhejiang	*Camellia japonica* (leaf)	Pathogen	AY681472
P. karstenii 307	Nanning, Guangxi	*C. japonica* (twig)	Endophyte	AY681473
P. hangzhouensis 742	Kunming, Yunnan	*C. sinensis* (twig)	Endophyte	AY526871
P. hangzhouensis 390	Hangzhou, Zhejiang	*C. sinensis* (twig)	Endophyte	AY526870
P. kunmingensis 766	Kunming, Yunnan	*P. macrophyllus* (old leaf)	Endophyte	AY373376
P. jesteri	GenBank	/	/	AF377282
P. oxyanthi 209	Hangzhou, Zhejiang	*P. macrophyllus* (leaf)	Pathogen	AY687875
P. crassiuscula 356	Hangzhou, Zhejiang	*P. macrophyllus* (leaf)	Endophyte	AY687868
P. photiniae 302	Nanning, Guangxi	*C. japonica* (twig)	Endophyte	AY682940
P. zonata 392	Hangzhou, Zhejiang	*P. macrophyllus* (fruit)	Endophyte	AY687305
P. photiniae 204	Hangzhou, Zhejiang	*C. japonica* (leaf)	Pathogen	AY682936
P. foedans 1017	Shaoguan, Guangdong	*P. massoniana* (twig)	Endophyte	AY687309
P. briosiana 689	Kunming, Yunnan	*C. sasanqua* (twig)	Endophyte	AY687308
P. virgatula 211	Hangzhou, Zhejiang	*P. macrophyllus* (leaf)	Pathogen	AY687879
P. photiniae 697	Kunming, Yunnan	*C. sasanqua* (twig)	Endophyte	AY682945
P. menezesiana 320	Nanning, Guangxi	*P. macrophyllus* (leaf)	Endophyte	AY687302
P. diospyri 121	Menglun, Yunnan	*P. macrophyllus* (leaf)	Pathogen	DQ417181
P. virgatula 1016	Shaoguan, Guangdong	*P. massoniana* (twig)	Endophyte	AY687880
P. photiniae 286	Tiane, Guangxi	*P. massoniana* (leave)	Pathogen	AY682939
P. theae 99	Menglun, Yunnan	*C. reticulata* (leaf)	Pathogen	AY681478
P. theae 310	Nanning, Guangxi	*C. nitidissima* (leaf)	Endophyte	AY681480
P. theae 205	Hangzhou, Zhejiang	*C. sinensis* (leaf)	Pathogen	AY681479
P. theae 312	Nanning, Guangxi	*C. nitidissima* (twig)	Endophyte	AY681481
P. theae 318	Nanning, Guangxi	*P. macrophyllus* (twig)	Endophyte	AY681482
P. theae 83	Hangzhou, Zhejiang	*C. sinensis* (leaf)	Pathogen	AY681477
Truncatella angustata (7)	GenBank	/	/	AF377300
T. angustata (5)	GenBank	/	/	AF405306
Amphisphaeria sp.	GenBank	/	/	AF375998

Note: /, no data.

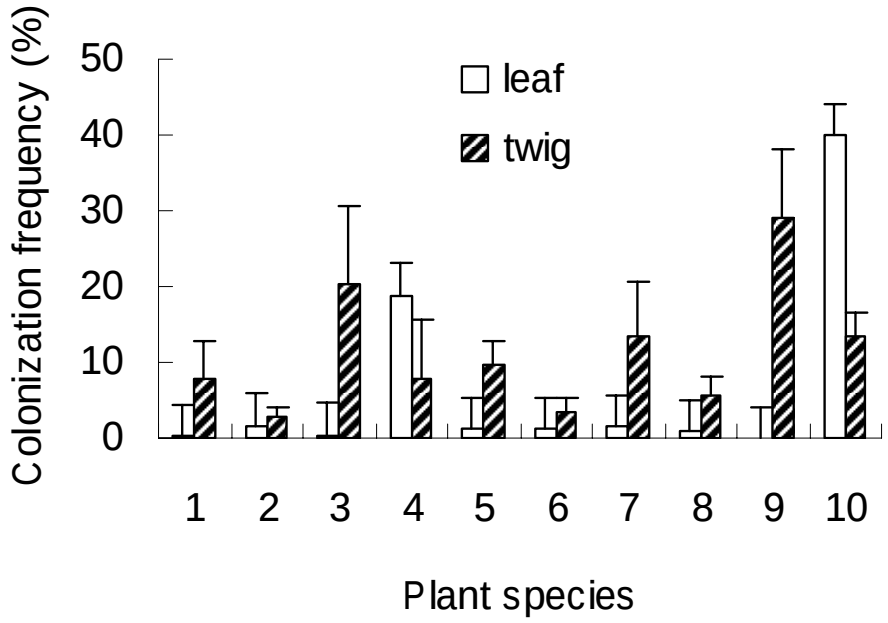

Fig. 1. Colonization frequencies of endophytic *Pestalotiopsis* species in leaves and twigs of ten plant species collected from Nanning, Hangzhou and Kunming. The sequence from 1 to 10 is *Camellia japonica*, *C. nitidissima*, *C. sasanqua*, *C. oleifera*, *C. sinensis*, *C. reticulata*, *Podocarpus macrophyllus*, *P. nagi*, *Taxus chinensis* var. *mairei* and *T. yunnanensis*, respectively.

frequency of endophytic *Pestalotiopsis* species in leaves of *Taxus yunnanensis* was significantly higher than the other nine plants, but there was no significant difference among the nine plants. There was no significant difference of the colonization frequencies of endophytic *Pestalotiopsis* species in twigs among the ten plants.

Generally there were higher colonization frequencies of endophytic *Pestalotiopsis* species in twigs than in leaves, except for *Camellia oleifera* and *T. yunnanensis*. Furthermore, the colonization frequency was significantly higher in twigs than in leaves of *C. sasanqua* and *T. chinensis* var. *mairei*.

The effect of tissue age on colonization frequency

Colonization frequencies of endophytic *Pestalotiopsis* species in different age twigs of three plants *C. japonica*, *C. nitidissima* and *C. sasanqua* in *Theaceae* collected from the Golden Flower Tea Park in Nanning are shown in Fig. 2. The colonization frequencies of endophytic *Pestalotiopsis* species

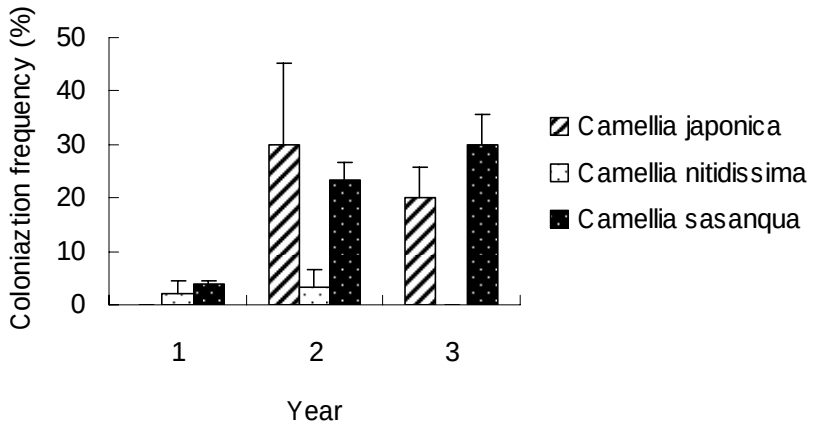

Fig. 2. Colonization frequencies of endophytic *Pestalotiopsis* species in 1-, 2- and 3-yr-old twigs of *Camellia japonica*, *C. nitidissima* and *C. sasanqua* of the *Theaceae* collected at the Golden-flower Tea Park in Nanning.

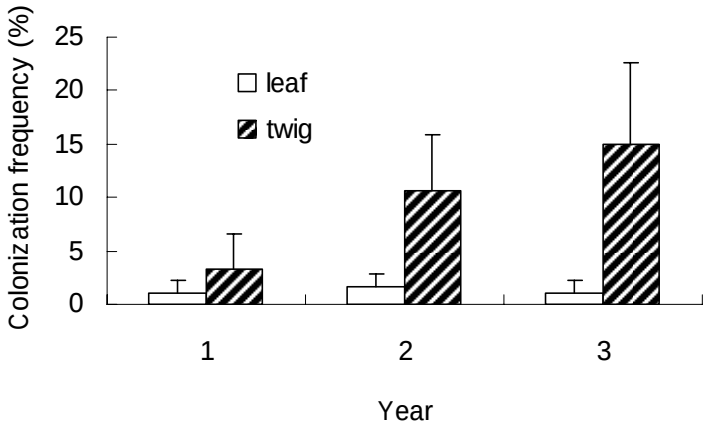

Fig. 3. Colonization frequencies of endophytic *Pestalotiopsis* species in 1-, 2- and 3-yr-old leaves and twigs of *Podocarpus macrophyllus* collected in Nanning.

were significantly higher in both 2- and 3-year-old twigs than in 1- year-old twigs of *C. japonica* and *C. sasanqua*, but there was no significant difference between 2- and 3- year-old twigs. There was no significant difference of the colonization frequencies of endophytic *Pestalotiopsis* species among 1-, 2- and 3- year-old twigs of *C. nitidissima*.

The results of the colonization frequencies of endophytic *Pestalotiopsis* species in different age leaves and twigs of *Podocarpus macrophyllus* in

Nanning are shown in Fig. 3. There was no significant difference of the colonization frequencies of endophytic *Pestalotiopsis* species between 1-, 2- and 3- year-old leaves and twigs, except that colonization frequency was significantly higher in 3- year-old twigs than in 3- year-old leaves. In addition, colonization frequencies of endophytic *Pestalotiopsis* species in twigs increased with age.

The effect of sampling site on colonization frequency

The colonization frequencies of endophytic *Pestalotiopsis* species of *C. japonica* and *Podocarpus macrophyllus* in different sites are shown in Figs. 4 and 5. Colonization frequencies of endophytic *Pestalotiopsis* species in leaves of *C. japonica* and *P. macrophyllus* were not significantly different between Nanning, Hangzhou and Kunming, respectively. There was no significant difference of colonization frequencies in twigs of *C. japonica* and *P. macrophyllus* between Nanning, Hangzhou and Kunming, respectively, except that colonization frequency in twigs of *P. macrophyllus* was significant higher in Kunming than in both Nanning and Hangzhou.

Diversity of the endophytic Pestalotiopsis in different hosts

Pestalotiopsis species were isolated from all ten plants studied in this survey, but the species numbers varied from two to 16 in different hosts, with an average of 5.3 species per host (Table 2).

A total of 302, 365 and 198 *Pestalotiopsis* isolates were obtained from plants of *Podocarpaceae*, *Theaceae* and *Taxaceae*, respectively (Table 3). Eighteen *Pestalotiopsis* species were isolated from *Podocarpaceae*, 16 from *Theaceae* and four from *Taxaceae*. The most common *Pestalotiopsis* species were similar between *Podocarpaceae* (*P. heterocornis*, *P. neglecta* and *P. photiniae*) and *Theaceae* (*P. heterocornis*, *P. karstenii*, *P. neglecta* and *P. photiniae*), and *P. neglecta* and *P. subcuticularis* were the most common species in *Taxaceae* (Table 3).

Shannon-Wiener diversity indices of endophytic *Pestalotiopsis* species in plants of both *Podocarpaceae* (2.2242) and *Theaceae* (2.287) were much higher than that in *Taxaceae* (1.069) (Table 3).

Community similarities of *Pestalotiopsis* species were analysed based on the Sorenson's coefficient (C_S), and the results indicated that there was a higher community similarity between *Podocarpaceae* and *Theaceae* (C_S = 0.5294) than between *Podocarpaceae* and *Taxaceae* (C_S = 0.3) as well as *Theaceae* and *Taxaceae* (C_S = 0.1818).

Table 2. Endophytic *Pestalotiopsis* species isolated from ten plant species of three families at three sites

Plant	Endophytic *Pestalotiopsis* species		
	Kunming	**Hangzhou**	**Nanning**
Theaceae			
Camellia japonica	*P. heterocornis*, *P. karstenii*, *P. mangifolia*, *P. photiniae*	*P. heterocornis*, *P. karstenii*, *P. photiniae*	*P. heterocornis*, *P. karstenii*, *P. photiniae*
C. reticulata	*P. mangifolia*, *P. osyridis*		
C. sasanqua	*P. briosiana*, *P. karstenii*, *P. mangifolia*, *P. olivacea*, *P. paeoniae*, *P. photiniae*, *P. subcuticularis*	*P. karstenii*, *P. photiniae*	*P. karstenii*, *P. photiniae*
C. sinensis	*P. hangzhouensis*, *P. microspora*, *P. neglecta*	*P. clavispora*, *P. hangzhouensis*, *P. neglecta*, *P. theae*	*P. clavispora*, *P. neglecta*
C. oleifera	/	/	*P. clavispora*, *P. heterocornis*, *P. suffocata*
C. nitidissima	/	/	*P. conigena*, *P. neglecta*, *P. oxyanthi*, *P. rhododendri*, *P. theae*
Podocarpaceae			
Podocarpus macrophyllus	*P. heterocornis*, *P. kunmingensis*, *P. menezesiana*, *P. microspora*, *P. neglecta*, *P. olivacea*, *P. oxyanthi*, *P. paeoniae*, *P. photiniae*, *P. rhododendri*, *P. yunnanensis*	*P. clavispora*, *P. crassiuscula*, *P. heterocornis*, *P. neglecta*, *P. photiniae*, *P. rhododendri*, *P. zonata*	*P. aquatica*, *P. clavispora*, *P. heterocornis*, *P. menezesiana*, *P. microspora*, *P. neglecta*, *P. photiniae*, *P. rhododendri*, *P. theae*
P. nagi	*P. photiniae*, *P. mangifolia*, *P. neglecta*, *P. olivacea*	*P. neglecta*, *P. paeoniicola*, *P. photiniae*	*P. neglecta*, *P. photiniae*
Taxaceae			
Taxus yunnanensis	*P. neglecta*, *P. paeoniae*, *P. subcuticularis*	/	/
T. chinensis var. *mairei*	/	*P. neglecta*, *P. photiniae*, *P. subcuticularis*	/
Total number of isolates	297	295	273
Total number of species	15	15	14
Shannon-Wiener index (H')	2.22	2.12	2.06

Note: /, no plant sample collected.

Table 3. The number of isolates and relative abundance of endophytic *Pestalotiopsis* species in the three families

Species	No. of isolates (relative abundance %)		
	Podocarpaceae	***Theaceae***	***Taxaceae***
P. aquatica	2 (0.7)	/	/
P. briosiana	/	1 (0.3)	/
P. clavispora	10 (3.3)	19 (5.2)	/
P. conigena	/	10 (2.7)	/
P. crassiuscula	18 (6)	/	/
P. hangzhouensis	/	3 (0.8)	/
P. heterocornis	87 (28.8)	41 (11.2)	/
P. karstenii	/	55 (15.1)	/
P. kunmingensis	1 (0.3)	/	/
P. mangifolia	6 (2)	15 (4.1)	/
P. menezesiana	4 (1.3)	/	/
P. microspora	16 (5.3)	32 (8.8)	/
P. neglecta	49 (16.2)	59 (16.2)	72 (36.4)
P. olivacea	18 (6)	25 (6.8)	/
P. osyridis	/	2 (0.5)	/
P. oxyanthi	2 (0.7)	2 (0.5)	/
P. paeoniae	7 (2.3)	/	8 (4)
P. paeoniicola	3 (1)	/	/
P. photiniae	52 (17.2)	78 (21.4)	19 (9.6)
P. rhododendri	9 (3)	/	/
P. subcuticularis	/	12 (3.3)	99 (50)
P. suffocata	/	5 (1.4)	/
P. theae	2 (0.7)	6 (1.6)	/
P. yunnanensis	2 (0.7)	/	/
P. zonata	14 (4.6)	/	/
Total number of isolates	302	365	198
Total number of species	18	16	4
Shannon-Wiener index (H')	2.22	2.29	1.07

Note: /, no *Pestalotiopsis* species isolated.

Diversity of the endophytic Pestalotiopsis at three sites

There were similar numbers of endophytic *Pestalotiopsis* species and strains obtained among Kunming (15 species, 297 strains), Hangzhou (15 species, 295 strains) and Nanning (14 species, 273 strains). Shannon-Wiener diversity indices of endophytic *Pestalotiopsis* species were similar among the three sites (Table 2).

Community similarities of *Pestalotiopsis* species between sampling sites were analysed based on the Sorenson's coefficient (C_S), and the results indicated that there were higher community similarities between Hangzhou and

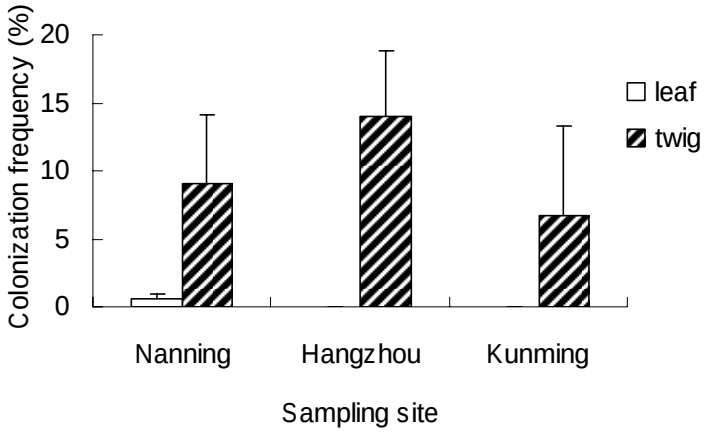

Fig. 4. Colonization frequencies of endophytic *Pestalotiopsis* species of leaves and twigs of *Camellia japonica* collected in Nanning, Hangzhou and Kunming.

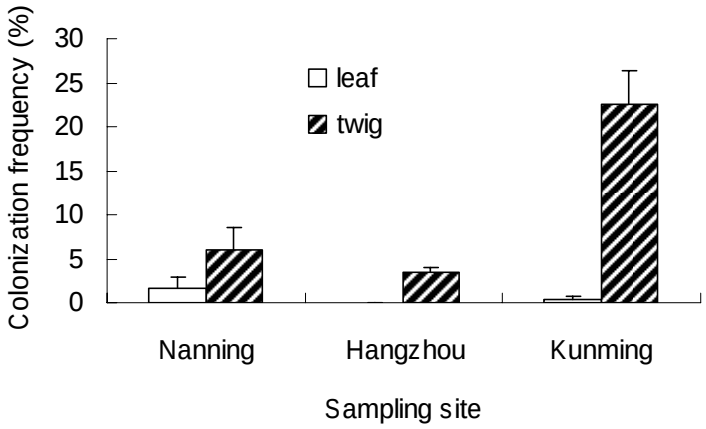

Fig. 5. Colonization frequencies of endophytic *Pestalotiopsis* species of leaves and twigs of *Podocarpus macrophyllus* collected in Nanning, Hangzhou and Kunming.

Nanning (C_S = 0.6207) and Hangzhou and Kunming (C_S = 0.6) than between Nanning and Kunming (C_S = 0.4138).

Host recurrence of endophytic Pestalotiopsis

Endophytes *P. neglecta* and *P. photiniae* were isolated from all the ten plants in the three families, and most species were distributed in more than one

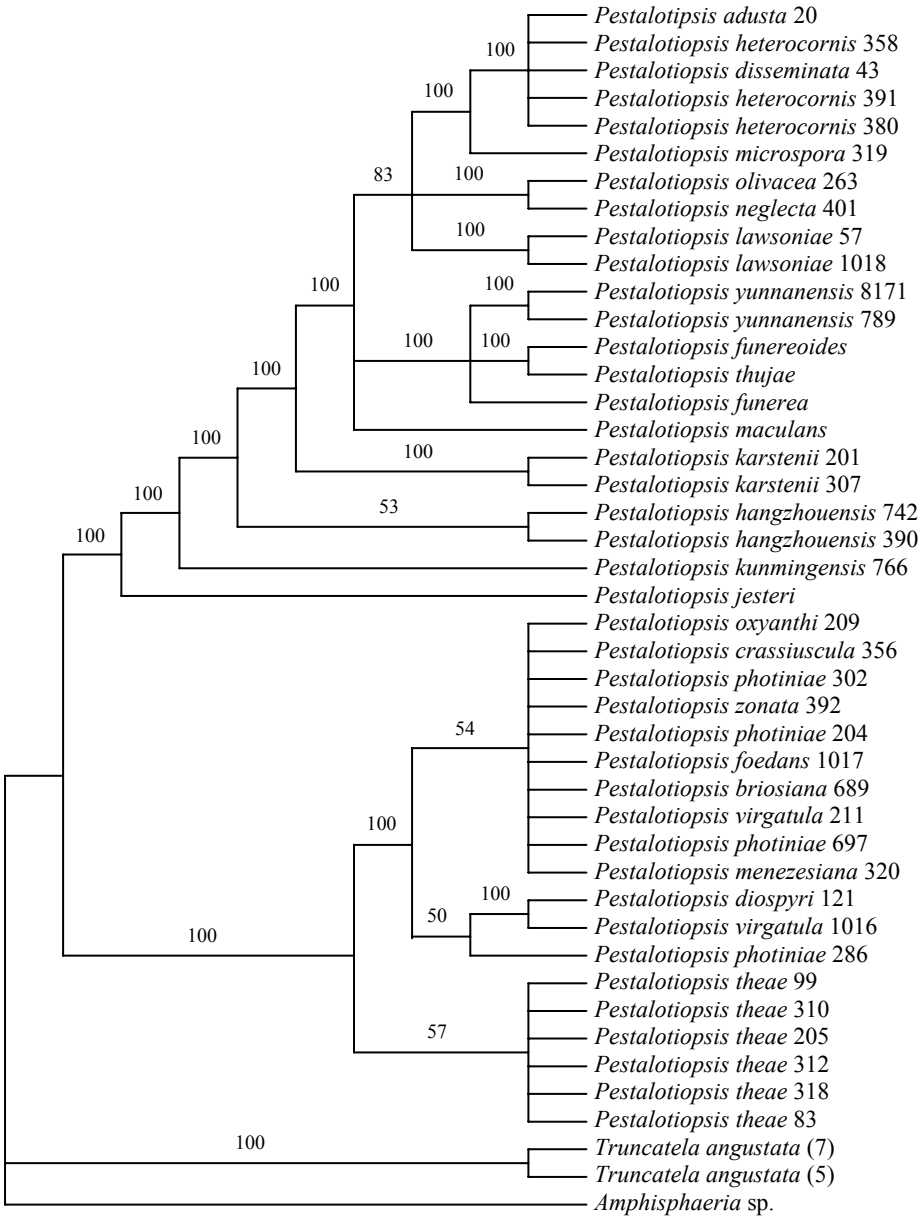

Fig. 6. A 50% majority consensus tree of maximum parsimony generated based on ITS region (ITS1, 5.8S, ITS2) sequences of 44 taxa. Rooted with *Amphisphaeria* sp., *Truncatella angustata* (5) and *T. angustata* (7) (TL = 229, CI = 0. 7293, HI = 0. 2707, RI = 0. 9353, RC = 0. 6821). Bootstrap values great than or equal to 50% (1000 replicates) are showed at branches. The strain numbers smaller than 300 are pathogenic *Pestalotiopsis* and larger than 300 are endophytic *Pestalotiopsis*.

host, except that *P. karstenii* was isolated from only *C. japonica* and *C. sasanqua* (*Theaceae*). The results indicated that endophytic *Pestalotiopsis* species are not specific to hosts generally and only a few species can colonize in a relatively narrow range of hosts (Table 2).

Relationship between endophytic and pathogenic Pestalotiopsis

The phylogenetic relationship between pathogenic and endophytic *Pestalotiopsis* species was analysed based on the ITS region sequences (Fig. 6). There were two clades in the tree. One clade included *Pestalotiopsis* species possessing conidia with brownish concolorous intermediate cells, the other clade included species possessing conidia with dark brown coloured intermediate cells.

Phylogeny indicates that two *P. karstenii* strains, an endophytic strain 307 and a pathogenic strain 201 isolated from the same host *Camellia japonica*, formed a terminal clade. In addition, DNA sequences from ITS1 and ITS2 were identical. Two *P. lawsoniae* strains, an endophytic strain 1018 and a pathogenic strain 57 isolated from *Pinus massoniana* in Guangdong and Guangxi, formed a terminal clade. In addition, DNA sequences from ITS1 and ITS2 had a similarity of 98.7%. Six *Pestalotiopsis theae* strains, including three endophytic strains and three pathogenic strains, formed a terminal clade, of which endophytic strains 310 and 312 were isolated from *C. nitidissima* and strain 318 from *Podocarpus macrophyllus*, and pathogenic strain 99 was obtained from *C. reticulata* and strains 83 and 205 from *C. sinensis*. There were relatively high ITS region sequence similarities (99.2%-100%) among the six strains. Four strains 99, 205, 310 and 312 have identical ITS region sequences, while there was one different base in strain 83 and two different bases in strain 318 comparing with others.

The different *Pestalotiopsis* species isolated from the same host were not phylogenetically closely related, for example, more than 15 species from *P. macrophyllus* were distributed in both clades and related to strains isolated from other hosts.

Discussion

Colonization frequency of endophytic Pestalotiopsis

Our results indicate that colonization frequencies of endophytic *Pestalotiopsis* species greatly varied with host species. Okane *et al*. (1998)

found endophytic *Pestalotiopsis* species colonizing seven species of *Ericaceae* with different colonization frequencies (0.7% to 17.1%) in Kyoto, Japan.

The colonization frequencies of endophytic *Pestalotiopsis* species varied with different host tissues in the present study. Collado *et al*. (2001) found that colonization frequencies of endophytic *Biscogniauxia mediterranea* were the highest in bark of main stems, the lowest in leaves and intermediate in twigs of *Quercus ilex* (*Fagaceae*) and were obviously different with sites and seasons. Bayman *et al*. (1998) found that colonization frequencies of endophytic *Xylaria* of *Casuarina equisetifolia* (*Casuarinaceae*) and *Manilkara bidentata* (*Sapotaceae*) were obviously higher in leaves than in seeds in Puerto Rico. Concurrently, similar results have been reported in many previous endophyte studies (e.g. Fisher and Petrini, 1987; Fisher *et al*., 1994, 1995; Rodrigues, 1994; Collado *et al*., 2000). These differences might be a reflection of tissue recurrence in individual species and might reflect their capacity for utilizing or surviving within a specific substrate (Carroll and Petrini, 1983; Rodrigues, 1994). The distribution of endophytic fungi may be affected by tissue texture and changes in the tissue physiology and chemistry (Petrini and Carroll, 1981; Arnold *et al*., 2001).

In general, colonization frequencies of endophytic *Pestalotiopsis* species increased with age in the present study. Bernstein and Carroll (1977) investigated the endophytic fungi in Douglas fir foliage and reported that the colonization rates rose from almost 0 at 5 months to 20-30% at 10 and 12 months, and at 17 months they were present in 60%. Barengo *et al*. (2000) studied the endophytic fungi in *Betula pubescens* twigs and the results showed that the colonization rates were 94% of the 1-yr-old and all of the 4-yr-old twig segments. Higher colonization frequencies of endophytic fungi were consistently obtained from older, rather than younger, leaves of palm *Trachycarpus fortunei* collected from China, Australia and Switzerland (Taylor *et al*., 1999). The influence of the plant tissue ages on the endophyte infection has been reported in some previous studies (Bernstein and Carroll, 1977; Petrini and Carroll, 1981).

Species diversity of endophytic Pestalotiopsis

Various numbers of endophytic *Pestalotiopsis* species were isolated from different plant species in the present study. Cannon and Simmons（2002）studied species diversity of endophytic fungi from 12 plant species belonging to nine families in the Iwokrama Forest Reserve, Guyana, and the results showed that a number of different *Pestalotiopsis* species were associated with different plants. Strobel *et al*. (2000) obtained four endophytic

Pestalotiopsis species in bark of *Fragraea bodenii* in Papua New Guinea. Alva and Hyde (2000) found 14 endophytic *Pestalotiopsis* species in three plants, i.e. *Scaevola hainanensis* (*Goodeniaceae*), *Sesuvium portulacastrum* (*Aizoaceae*) and *Suaeda maritime* (*Chenopodiaceae*), and suggested that there was an average four to five endophytic *Pestalotiopsis* species that colonize one plant species. A similar result of an average of 5.3 *Pestalotiopsis* species from each plant was obtained in the present study.

The diversity of endophytic *Pestalotiopsis* species was similar among the three sites in this survey. The results indicate that the distribution of endophytic *Pestalotiopsis* species is ubiquitous and not largely infected by geographical factors. However, the number of *Pestalotiopsis* species isolated from Taxaceae was lower than from both *Podocarpaceae* and *Theaceae* in this study. This is mainly because low numbers of plant species in *Taxaceae* were investigated compared to plants from other families sampled.

Host recurrence and relationship between endophytic and pathogenic Pestalotiopsis

It had been claimed that endophyte communities (or at least community profiles) were usually specific at the host species level because most research on endophytes of single plant species was carried out (Petrini and Fisher, 1990; Petrini, 1996). Cannon and Simmons (2002) reported that in contrast to studies in temperate ecosystems, no distinct fungal communities were identified for individual plant species, suggesting that the degree of host recurrence was low. Bayman *et al*. (1998) found endophytic *Xylaria* species were not specific to plant species after they investigated endophytic fungi in *Casuarina equisetifolia* (*Casuarinaceae*) and *Manilkara bidentata* (*Sapotaceae*) from which four endophytic *Xylaria* species were obtained, and similar result was obtained from orchids. Zhu (1989) demonstrated that pathogenic *Pestalotiopsis* species was not specific to host through cross artificial inoculation. The present work based on the diversity and phylogenetic analysis supported the conclusion that endophytic *Pestalotiopsis* species were not specific to host plants. The results suggest that the naming of *Pestalotiopsis* species based on host association is generally not taxonomically valid.

Strains of *P*. *karstenii*, *P*. *lawsoniae* and *P*. *theae* are endophytes as well as pathogens in the present study. Brown *et al*. (1998) studied endophytic fungal communities of *Musa acuminata* species complex and found *Colletotrichum gloeosporioides*, *C*. *musae* and *Phyllosticta musicola* which had been certified as pathogens were endophytes. An endophyte *Deightoniella torulosa* isolated from wild banana (*Musa acuminata*) was able to cause leaf

spots on banana leaves *in vitro* (Photita *et al*., 2004), while the ITS sequences demonstrated that from the parsimony tree the pathogenic *Colletotrichum* isolates from banana were different from the endophytic isolates (Photita *et al*., 2005). Many pathogenic *Pestalotiopsis* species were certified as endophytes in our previous studies (Wei and Xu, 2003a, b; Wei *et al*., 2005a). The results indicated that more *Pestalotiopsis* species could have endophytic and pathogenic stages in their life cycle.

Conclusion

Colonization frequencies of endophytic *Pestalotiopsis* species varied with host plants, ages and organs. Endophytic *Pestalotiopsis* species are widely distributed in the all ten plants studied with an average of 5.3 species per host. Generally the endophytes were more frequently obtained from twigs than from leaves. Some *Pestalotiopsis* species could have endophytic and pathogenic stages in their life cycle. Our result supports the conclusion that *Pestalotiopsis* species are not generally specific to host plants.

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