Diversity and ubiquity of xylariaceous endophytes in live and dead leaves of temperate forest trees

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Abstract To test the hypothesis that xylariaceous endophytes were ubiquitous on live and dead leaves of various tree species in the field, xylariaceous fungi were isolated from live leaves and bleached and nonbleached portions of dead leaves of a total of 94 tree species in a cool temperate forest in Japan. The biodiversity of xylariaceous endophytes was evaluated as the richness of operational taxonomic units (OTUs) determined by phylogenetic analysis of the nucleotide sequence of the D1/D2 region of the LSU rDNA of fungal isolates. A total of 326 isolates of xylariaceous fungi were isolated from live and dead leaves and classified into 15 OTUs. The three major OTUs, *Xylaria* sp.1, *Nemania* sp., and *Biscogniauxia* sp., accounted for 94% (308 isolates) of the total number of isolates, and were isolated from various live and dead leaves. *Xylaria* sp.1 was frequently encountered on bleached portions (which were produced due to the selective decomposition of lignin) of dead leaves of broad-leaved deciduous tree species. The results suggest that xylariaceous endophytes did not show host specificity and had a saprobic phase on dead leaves in their life cycles and that *Xylaria* sp.1 was capable of decomposing lignin in the field conditions.

Keywords diversity • fungi • lignin • Xylariaceae

Introduction

Endophytic fungi are defined as those that can colonize internal plant tissues at some time in their life without causing apparent harm to their host (Sieber 2007). Endophytic fungi on leaves of forest trees play ecological roles as presumed mutualists (Saikkonen 2007), latent pathogens (Sieber 2007), and saprobic decomposers after leaf death (Osono 2006; Promputtha et al. 2007). Previous studies have shown that a few groups of endophytic fungi in the Rhytismataceae and Xylariaceae in Ascomycota take part in the decomposition of lignin (Osono 2002; Koide et al. 2005; Osono and Hirose 2010). As lignin is a major structural component often limiting decomposition (Hirobe et al. 2004; Osono and Takeda 2005), these ligninolytic endophytes are of particular importance in terms of their roles in carbon turnover and nutrient cycling in forest ecosystems and deserve further studies on their ecology and functioning.

Osono and Hirose (2009) reviewed the ecology of endophytic fungi associated with leaf litter decomposition and recognized two groups of ligninolytic endophytes. The first is Rhytismataceous endophytes, which are relatively host-specific, usually colonize dead leaves for less than one year, and cause lignin decomposition in the initial stage of decomposition. The second is xylariaceous endophytes, which appear to have low host specificity and are found on leaves of various tree species (Whalley 1985, 1996; Petrini and Petrini 1985; Petrini et al. 1995). Xylariaceous endophytes are primarily saprobic and persist until the late stages of decomposition (Osono 2006). Osono and Takeda (2001) demonstrated that an endophyte, *Xylaria* sp., was frequently isolated from bleached portions on dead leaves of Japanese beech (*Fagus crenata*), which were produced due to the selective decomposition of lignin by the fungal colonizer. It is unclear, however, whether the ligninolytic activity of xylariaceous endophytes occur on leaf litter of other tree species with different leaf traits. Further studies are needed regarding the biodiversity, host range, and functioning of xylariaceous endophytes associated with leaf litter decomposition and, particularly, with lignin decomposition.

The purpose of the present study was to evaluate the diversity and ubiquity of xylariaceous endophytes in live and dead leaves of trees in a cool temperate forest. Thus, we isolated xylariaceous endophytes from live and dead leaves for a total of 94 tree species with one of four types of leaf traits (79 broad-leaved deciduous, 8 broad-leaved evergreen, 2 coniferous deciduous, and 5 coniferous evergreen species). The biodiversity of xylariaceous endophytes was evaluated as the richness of operational taxonomic units (OTUs) examined with phylogenetic analysis of the nucleotide sequence of the D1/D2 region of the LSU rDNA of fungal isolates. Fungi in Xylariaceae have been extensively subjected to molecular phylogenetic analysis (i.e., Lee et al. 2000; Davis et al. 2003; Okane et al. 2008; Peláez et al. 2008; Guedegbe et al. 2009) and are suitable for molecular identification of fungal isolates obtained from live and dead leaves of different host trees.

Materials and Methods

Study site

Leaf materials used for fungal isolation were collected in Ashiu Experimental

Forest of Kyoto University (35°18'N, 135°43'E, 355-660 m a.s.l.), Kyoto Prefecture, central Japan. During the past 29 years, the mean annual temperature was 11.7°C and mean monthly temperature ranged from 0.4°C in January to 25.5°C in August at the office of the Ashiu Experimental Forest at 355 m a.s.l. The mean annual precipitation during the past 29 years was 2353 mm. The study area is covered with snow from December to April. The Ashiu Experimental Forest is in a mountainous area, with natural stands of warm temperate forests dominated by evergreen oaks *Quercus salicina* Bl. and *Q. acuta* Thunb. ex Murray. below approximately 600 m a.s.l., and natural stands of cool temperate forests dominated by a deciduous beech, *Fagus crenata* Bl., and a deciduous oak, *Q. crispula* Bl., above the warm temperate region. The area is thus an ecotone of two climatic regions and hence has high richness of plant species, including 243 tree species recorded in the Ashiu Experimental Forest.

Sample collection

Live and dead leaves of a total of 94 tree species in 38 plant families were collected in the study site during the growing season from May to November in 2008 (Table 1). Broad-leaved deciduous tree species accounted for 84% (79 species) of the 94 species examined. Live, healthy-looking leaves of 74 tree species were collected in May, August, and November, mostly in August. On each sampling occasion, a total of 10 live, healthy-looking leaves were harvested for each tree species from two randomly chosen trees, two branches per individual tree, at an approximate height of 3-4 m. Two types of dead leaves were collected: those bearing bleached portions on the surfaces and those that were not bleached. The presence of bleached portions is associated with fungal colonization within leaf tissues and decomposition of lignin (Osono 2007). In the present study, the bleached portions were observed on dead leaves of 15 tree species, including 12 deciduous broad-leaved, 2 evergreen broad-leaved, and one evergreen coniferous tree species (Table 1). The bleached dead leaves were collected in May and July. Dead leaves without obvious bleached portions (denoted as nonbleached dead leaves) were collected for 63 tree species in May, June, and November. Bleached and nonbleached dead leaves were collected from the forest floor for each tree species on each sampling occasion. Sampling of a total of 1840 leaves was carried out during the study period. The leaves were placed in paper bags and taken to the laboratory. The leaves were processed within 24 hours after the collection. One leaf disk was punched out from the central part of each sample leaf, avoiding the primary vein, with a sterile cork borer (5.5 mm in diameter). A total of 10 leaf disks were used for each tree species, each leaf type, and on each sampling occasion, making a total of 1840 disks for the isolation of xylariaceous fungi.

Fungal isolation

A surface disinfection method (Kinkel and Andrews 1988) was used to isolate xylariaceous fungi. The leaf disks were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface-disinfected for 30 seconds in a solution of 15% hydrogen peroxide, and then submerged again for 1 min in 70% ethanol. The disks were rinsed with sterile, distilled water, transferred to sterile filter paper in Petri dishes (9 cm in diameter), and dried for 24 h to suppress vigorous bacterial growth after plating (Widden and Parkinson 1973). The disks were placed in 9-cm Petri dishes containing lignocellulose agar (LCA) modified by Miura and Kudo (1970), two disks per plate. LCA contains glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v). Note that the modified LCA of Miura and Kudo (1970) does not contain lignin or other recalcitrant compounds. The modified LCA was used because its low glucose content suppresses the overgrowth of fast-growing fungal species (Osono and Takeda 1999). Plates were incubated at 20°C in the dark and observed at 1, 4, and 8 weeks after surface disinfection. Putative xylariaceous fungi that produced on the plates conidia and conidiophores of anamorphic Xylariaceae, such as *Xylocoremium*, *Geniculosporium*, and *Nodulisporium*, and (or) dark pseudosclerotinial plates in submerged hyphae were subcultured on fresh LCA to establish pure cultures.

Determination of OTUs

The pure cultures obtained were identified by molecular analysis. When fungal structures such as spores and sporocarps were produced on the medium, their morphological characteristics were observed with a Nikon Optiphot microscope (Nikon Inc., Tokyo, Japan). For molecular analysis, a small amount of mycelial tips from each culture were picked, crushed in 24 μl of distilled water in a tube, microwaved for 12 seconds, and used as templates for PCR. A reaction mixture (50 μl) containing 25 μl Qiagen GoTaq premix (Qiagen, Ontario, Canada) and 10 pmol of each primer and distilled water was added to the templates. The oligonucleotide primer-pair NL1 and NL4 (O'Donnell 1993) were used for PCR of ribosomal DNA large subunit D1/D2 region. The reactions were initiated with 4 min of denaturation at 95°C, followed by 40 cycles of two-step PCR, consisting of 20 seconds at 94°C and 60 seconds at 56°C with a final extension for 10 min at 72°C on a GeneAmp 9700 thermal cycler (Perkin-Elmer Applied Biosystems, California, USA). Amplification products were purified using a QIAquick PCR Purification Kit (Qiagen, Ontario, Canada), and sequenced with a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit ver. 3.1 and an ABI PRISM 3100 genetic analyzer (Perkin-Elmer Applied Biosystems, California, USA). Both strands of a fragment were sequenced. Sequence data sets were manually truncated both ends and edited using the program BioEdit sequence editor version 5.09 (Hall 1999). Homology searches were performed using each obtained sequences data on a BLAST program at the National Center for Biotechnology Information (NCBI). Neighbor joining trees were also constructed using MEGA version 5 (Tamura et al. 2011) with related sequences from NCBI database. Isolates with more than 99% homology of sequence and within the same cluster were treated as OTUs with tentative codes for data analysis. In the case that obtained sequences contained polymorphic sites, they are treated as the same OTUs with close relatives.

Statistical analysis

To assess the affinity of major fungal OTUs to leaf traits of host trees, Fisher's exact probability test was performed to examine the differences in the number of tree species from which the major OTUs were isolated between broad-leaved and coniferous trees and between deciduous and evergreen trees.

Results

Operational taxonomic units of xylariaceous fungi

A total of 326 isolates of xylariaceous fungi were isolated from live and dead leaves of 82 (87%) of the 94 tree species examined (Table 1). Xylariaceous fungi were isolated from live leaves of 74 (84%) of 88 tree species, from bleached dead leaves of 12 (80%) of 15 tree species, and from nonbleached dead leaves of 36 (57%) of 63 tree species (Table 1).

The fungal isolates were classified into 15 OTUs (Table 2). *Xylaria* sp.1 was the most dominant OTU (135 isolates), followed by *Nemania* sp. (123 isolates) and *Biscogniauxia* sp. (50 isolates). These three OTUs (308 isolates) accounted for 94% of the total number of isolates (326 isolates). The other 12 OTUs were isolated only infrequently, with the number of isolates ranging from 1 to 4.

The number of OTUs isolated from live leaves was 13, and those from bleached and nonbleached dead leaves were 3 and 5, respectively (Table 2). *Nemania* sp. was the most dominant OTU on live leaves, followed by *Xylaria* sp.1 and *Biscogniauxia* sp. *Xylaria* sp.1 accounted for 93% of the total number of isolates from bleached dead leaves. *Xylaria* sp.1 was the most dominant OTU on nonbleached dead leaves, followed by *Nemania* sp. and *Biscogniauxia* sp.

Xylaria sp.1, *Nemania* sp., and *Biscogniauxia* sp. were isolated from live leaves of 43 (49%), 51 (58%), and 24 (27%), respectively, of 88 tree species examined (Table 2). *Xylaria* sp.1 was isolated from bleached dead leaves of 12 tree species (Table 2). *Xylaria* sp.1, *Nemania* sp., and *Biscogniauxia* sp. were isolated from nonbleached dead leaves of 22 (35%), 26 (41%), and 5 (8%) of 63 tree species examined, respectively (Table 2). The number of tree species from which *Xylaria* sp.1, *Nemania* sp., and *Biscogniauxia* sp. were isolated from both of live and dead leaves (bleached or nonbleached) was 20, 16, and 1 species, respectively.

Patterns of occurrence of major OTUs

The number of tree species from which the three major OTUs were isolated was summarized in Table 3 with respect to four types of leaf traits (i.e., broad-leaved deciduous, broad-leaved evergreen, coniferous deciduous, and coniferous evergreen). When live leaves were considered, the number of tree species from which *Xylaria* sp.1, *Nemania* sp., and *Biscogniauxia* sp. were isolated was not significantly different between broad-leaved and coniferous trees (P=0.25, P=0.31, and P=0.17, respectively) or between deciduous and evergreen trees (P=0.17, P=0.24, and P=0.15, respectively) (Table 3).

Xylaria sp.1 was isolated from bleached dead leaves of all of the 12 broad-leaved deciduous tree species examined, but not on those of broad-leaved or coniferous evergreen tree species (Table 3). *Nemania* sp. was not isolated from bleached dead leaves (Table 3). *Biscogniauxia* sp. was isolated from bleached dead leaves of one broad-leaved deciduous tree species (Table 3).

When nonbleached dead leaves were considered, the number of tree species from which *Xylaria* sp.1 and *Nemania* sp. were isolated was not significantly different between broad-leaved and coniferous trees (P=0.32 and P=0.24, respectively) or between deciduous and evergreen trees (P=0.34 and P=0.17, respectively) (Table 3). *Biscogniauxia* sp. was isolated from nonbleached dead leaves of 2 out of 58 broad-leaved tree species, which were significantly (P=0.002) lower than in coniferous trees (three out of five tree species) (Table 3). The number of tree species of which *Biscogniauxia* sp. was isolated from nonbleached dead leaves was not significantly different between deciduous and evergreen trees (P=0.06).

Discussion

In previous studies at the present study site, xylariaceous fungi were isolated from live and dead leaves of two major tree species, *Fagus crenata* and *Swida controversa* (Osono 2002, Osono et al. 2004). In the present study, 15 OTUs of xylariaceous fungi were found from live and dead leaves of 94 tree species, indicating that they are major components of endophytic and litter-inhabiting fungi in the cool temperate forest. The community structure of the fungal OTUs was highly skewed, with the top three OTUs accounting for 94% of the total number of isolates (Table 2). In a similar study of endophytic Xylariaceae from Thailand, Okane et al. (2008) isolated from live leaves of 25 tree species a total of 273 isolates that were assigned to 25 OTUs according to their 28S rDNA D1/D2 sequence. The top three OTUs with respect to the number of fungal isolates accounted for 31% of the total number of isolates in the study of Okane et al. (2008). Thus, the diversity of xylariaceous endophytes was lower in the cool temperate forest in the present study than in the tropical forest of Okane et al. (2008) in terms of the dominance of a few major OTUs and the lower prevalence of rare OTUs.

Xylaria sp.1 and *Nemania* sp. occurred on live and dead leaves of multiple tree species (Table 3), regardless of leaf traits (i.e. broad-leaved vs coniferous, deciduous vs evergreen), suggesting the low host specificity, which is consistent with previous studies of xylariaceous endophytes (Petrini et al. 1995; Cannon and Simmons 2002; Murali et al. 2007; Okane et al. 2008). Previous studies have shown that *Xylaria* sp.1 was also isolated from live and dead twigs (Fukasawa et al. 2009) and cupules (Fukasawa et al. 2012) of *F. crenata*, indicating its low tissue specificity. *Biscogniauxia* sp. was isolated from nonbleached dead leaves more frequently (in terms of the number of tree species isolated with respect to the total number of tree species examined) for coniferous than for broad-leaved tree species (Table 3), but the number of coniferous tree species examined was too low (i.e. five species) to be conclusive with the affinity of this OTU to the dead coniferous leaves.

Relating the endophytic fungal OTUs from live and dead leaves to their fruiting bodies is needed to evaluate their ecology and life cycles. Unfortunately, however, the teleomorphic states of the major OTUs in the present study have not yet been collected at the study site, making it difficult to evaluate the ecology and life cycle of the leaf-associated xylariaceous fungi in detail. Only a few rare OTUs have been phylogenetically related to teleomorphic states fruiting on woody tissues (i.e., *Xylaria hypoxylon* and *Hypoxylon fragiforme* in Table 2). Thus, further efforts are needed to search for fruiting bodies to identify the OTUs and to clarify their ecology and host- and tissue-specificity at the study site. It might also be important to take into consideration the possibility that the endophytic life stage of xylariaceous fungi is 'a dead-end' of the life cycle as it rarely ends with sexual reproduction on the leaf. Alternatively, some xylariaceous endophytes with *Geniculosporium* and *Nodulisporium* anamorphs can establish from conidia and grow and reproduce endophytically as anamorphic fungi (Rogers 1985).

The three major OTUs were isolated from not only live leaves but also bleached and nonbleached dead leaves of broad-leaved deciduous tree species (Table 2), indicating that these xylariaceous endophytes have a saprobic phase in their life cycles. The isolation of *Xylaria* sp.1 and *Nemania* sp. from both live and dead leaves of the same tree species suggested that these OTUs could persist in dead leaves from live leaves. *Xylaria* sp.1 was isolated from both bleached and nonbleached dead leaves, whereas *Nemania* sp. and *Biscogniauxia* sp. were mostly isolated from nonbleached dead leaves (Table 2). Because the lignin content is lower in bleached than in nonbleached portions (Osono 2007), *Xylaria* sp.1 is probably capable of decomposing lignin more actively than the other two OTUs. This is consistent with a pure culture test showing that *Xylaria* sp.1 contained isolates that decomposed lignin in *F. crenata* leaves more actively than *Nemania* sp. (as *Geniculosporium* sp., Osono and Takeda 2002). However, we cannot exclude a possibility that *Xylaria* sp.1 preferred bleached to nonbleached portions as substrata for colonization.

Xylaria sp.1 was isolated from bleached dead leaves of deciduous broad-leaved trees but not from those of evergreen broad-leaved or coniferous trees, despite its occurrence on live and nonbleached dead leaves of these evergreen trees (Table 3). However, the number of evergreen tree species examined for bleached dead leaves in the present study was too low to determine whether *Xylaria* sp.1 was truly absent from bleached dead leaves of evergreen trees. Further studies are needed to examine bleached dead leaves of evergreen trees for the occurrence of xylariaceous fungi and to explore possible mechanisms relating to the reduction of xylariaceous fungi in these leaves.

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Table 1 Tree species examined in the present study, month of collection in 2008, and the number of isolates of xylariaceous fungi on live leaves and bleached and nonbleached portions of dead leaves

			Live leaves		Bleached portions of dead leaves		Nonbleached portions of dead leaves	
Tree species	Family[a]	Abbr.	Month[b]	Number of isolates	Month	Number of isolates	Month	Number of isolates
Broad-leaved, deciduous								
Actinidia arguta	At	Aa	A	0	-	-	-	-
Acer carpinifolium	Ac	Ac	A	2	-	-	N	0
Acer nikoense	Ac	Ae	A	2	-	-	-	-
Alnus firma	Be	Af	M, A, N	0, 7, 0	-	-	-	-
Actinidia polygama	At	Ag	A	2	-	-	N	1
Acer japonicum	Ac	Aj	A	4	-	-	N	4
Acer micranthum	Ac	Ak	A	1	-	-	-	-
Aralia elata	Ar	Al	A	1	-	-	-	-
Acer mono	Ac	Am	A	2	M	3	N	0
Acer nipponicum	Ac	An	A	3	-	-	-	-

Acanthopanax sciadophylloides	Ar	Ap	A	0	-	-	N	1
Akebia quinata	Lr	Aq	A	1	-	-	-	-
Acer rufinerve	Ac	Ar	A	3	M	4	N	1
Acer sieboldianum	Ac	As	A	4	M	1	N	0
Aesculus turbinata	Hi	At	A	1	M	2	N	0
Acer amoenum var. *matsumurae*	Ac	Ay	-	-	-	-	N	1
Benthamidia cousa	Co	Bc	A	3	-	-	N	3
Betula grossa	Be	Bg	M, A, N	1, 2, 7	M	2	N	1
Boehmeria spicata	Ur	Bs	A	1	-	-	-	-
Clethra barvinervis	Er	Cb	A	3	-	-	M, N	0, 0
Castanea crenata	Fa	Cc	A	1	M	1	N	0
Corylus sieboldiana	Be	Ch	A	0	-	-	-	-
Carpinus tschonoskii	Be	Ci	A	8	-	-	N	2
Cercidiphyllum japonicus	Cr	Cj	A	6	-	-	M, N	0, 1
Carpinus japonica	Be	Ck	A	8	-	-	N	4
Carpinus laxiflora	Be	Cl	M, A, N	0, 1, 4	M	3	N	0
Carpinus cordata	Be	Cs	A	4	-	-	N	0
Clerodendrum trichotomum	Ve	Ct	A	0	-	-	-	-
Cladrastis sikokiana	Fb	Cy	A	3	-	-	N	0
Deutzia crenata	Sx	Dc	A	3	-	-	N	0
Eunomyces alatus f. *subtriflorus*	Ce	Ea	A	3	-	-	N	1

Evodiopanax innovans	Ar	El	A	1	-	-	N	0
Elliottia paniculata	Er	Ep	-	-	-	-	N	0
Fagus crenata	Fa	Fc	A	3	M	2	N	0
Fraxinus sieboldiana	Ol	Fs	A	5	-	-	-	-
Hydrangea hirta	Sx	Hh	A	2	-	-	N	3
Hamamelis japonicus var. *obtusata*	Ha	Hj	A	4	-	-	M, N	0, 3
Hydrangea petiolaris	Sx	Hp	A	1	-	-	M, N	1, 1
Hovenia tomentella	Rh	Ht	-	-	-	-	N	0
Hydrangea paniculata	Sx	Hy	A	4	-	-	-	-
Ilex macropoda	Aq	Im	A	3	-	-	N	3
Juglans mandshurica var. *sachaliensis*	Ju	Jm	A	3	-	-	-	-
Kalopanax pictus	Ar	Kp	-	-	-	-	M, N	2, 0
Lindera erythrocarpa	La	Le	A	3	-	-	Jun, N	0, 0
Lyonia ovalifolia	Er	Lo	A	0	M	3	N	2
Lindera triloba	La	Lt	A	2	-	-	N	0
Lindera umbellata	La	Lu	A	2	-	-	N	0
Mallotus japonicus	Eu	Mj	A	1	-	-	N	6
Meliosma myriantha	Sa	Mm	A	0	-	-	N	5
Magnolia obovata	Ma	Mo	A	3	M	3	N	0
Magnolia salicifolia	Ma	Ms	A	2	-	-	M, N	1, 0
Malus tschonoskii	Ro	Mt	A	2	-	-	N	0

Prunus grayana	Ro	Pg	A	6	-	-	N	1
Pterostyrax hispida	St	Ph	A	0	-	-	N	0
Pterocarya rhoifolia	Ju	Pr	A	5	-	-	M, N	0, 0
Populus sieboldii	Sl	Ps	A	5	-	-	-	-
Paulownia tomentosa	Pa	Pt	A	1	-	-	-	-
Prunus jamasakura	Ro	Py	A	5	-	-	N	4
Quercus crispula	Fa	Qc	A	4	M	1	N	1
Quercus serrata	Fa	Qs	A	0	M	2	N	2
Rhus javanica var. *roxburghii*	An	Rj	A	2	-	-	-	-
Rhus trichocarpa	An	Rt	A	1	-	-	-	-
Sorbus alnifolia	Ro	Sa	A	1	-	-	M, N	1, 0
Swida controversa	Co	Sc	A	1	-	-	N	1
Schizophragma hydrangeoides	Sx	Sh	A	2	-	-	-	-
Styrax japonica	St	Sj	A	5	-	-	-	-
Sorbus commixta	Ro	Sn	A	1	-	-	N	2
Styrax obassia	St	So	A	1	-	-	N	0
Stachyurus praecox	Sp	Sp	A	3	-	-	N	6
Symplocos chinensis	Sy	Ss	A	3	-	-	N	7
Symplocos coreana	Sy	St	A	2	-	-	-	-
Ulmus parvifolia	Ul	Up	A	3	-	-	N	1
Viburnum furcatum	Ca	Vf	A	3	-	-	-	-

Viburnum plicatum var. *tomentosum*	Ca	Vp	A	3	-	-	M, N	0, 0
Viburnum wrightii	Ca	Vw	A	0	-	-	-	-
Wisteria floribunda	Fb	Wf	A	0	-	-	-	-
Weigela hortensis	Ca	Wh	A	4	-	-	N	1
Zanthoxylum piperitum	Ru	Zp	A	0	-	-	-	-
Zelkova serrata	Ul	Zs	A	0	-	-	N	0
Broad-leaved, evergreen								
Daphniphyllum macropodum var. *humile*	Da	Dm	M	4	-	-	M	0
Eurya japonica	Th	Ej	A	2	-	-	-	-
Ilex pedunculosa	Aq	Ip	M	2	-	-	-	-
Ilex sugerokii	Aq	Is	A	2	-	-	-	-
Pieris japonica	Er	Pj	M	0	-	-	M	0
Quercus acuta	Fa	Qa	-	-	Jul	0	-	-
Quercus salicina	Fa	Ql	A	3	Jul	0	-	-
Trochodendron aralioides	Tr	Ta	M	2	-	-	M	2
Coniferous, deciduous								
Larix gmelinii	Pi	Lg	A	0	-	-	M, N	1, 0
Metasequoia glyptostroboides	Cu	Mg	A	2	-	-	N	0
Coniferous, evergreen							-	-
Abies firma	Pi	Ai	-	-	Jul	0	-	-
Chamaecyparis obtusa	Cu	Co	A	1	-	-	-	-

Cryptomeria japonica	Cu	Cr	A	1	-	-	M, N	1, 3
Picea abies	Pi	Pa	A	1	-	-	M	1
Pinus densiflora	Pi	Pd	A	3	-	-	M	1
Number of tree species examined			94	88		15		63
Number of leaves examined			1840	940		150		750
Number of isolates of xylariaceous fungi			326	216		27		83
Number of tree species from which xylariaceous fungi were isolated			82	74		12		36
(% total number of tree species)			(87%)	(84%)		(80%)		(57%)

[a] Tree family: Ac, Aceraceae; An, Anacardiaceae; Aq, Aquifoliaceae; Ar, Araliaceae; At, Actinidiaceae; Be, Betulaceae; Ca, Caprifoliaceae; Ce, Celastraceae; Co, Cornaceae; Cr, Cercidiphyllaceae; Cu, Cupressaceae; Da, Daphniphyllaceae; Er, Ericaceae; Eu, Euphorbiaceae; Fa, Fagaceae; Fb, Fabaceae; Ha, Hamamelidaceae; Hi, Hippocastanaceae; Ju, Juglandaceae; La, Lauraceae; Lr, Lardizabalaceae; Ol, Oleaceae; Pa, Paulowniaceae; Pi, Pinaceae; Rh, Rhamnaceae; Ro, Rosaceae; Ru, Rutaceae; Sa, Sabiaceae; Sl, Salicaceae; Sx, Saxifragaceae; Sp, Stachyuraceae; St, Styracaceae; Sy, Symplocaceae; Th, Theaceae; Tr, Trochodendraceae; Ul, Ulmaceae; Ur, Urticaceae; Ve Verbenaceae.

[b] Month: M, May; Jun, June; Jul, July; A, August; N, November.

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Table 2 BLAST search result of 15 operational taxonomic units (OTUs) of xylariaceous fungi on live leaves and bleached and nonbleached portions of dead leaves, and the number of tree species from which the 15 OTUs were isolated.

				Number of isolates						Number of tree species					
	Accession		Identity	Live [216]		Dead leaves				Live [88]		Dead leaves			
OTU	number	BLAST search result	(%)			Bleached [27]		Nonbleached [83]				Bleached [15]		Nonbleached [63]	
Xylaria sp.1	AB686646	Xylariales cf. JP14-3, Q906954	99	68	(31%)	25	(93%)	42	(51%)	43	(49%)	12	(80%)	22	(35%)
Nemania sp.	AB669031	Fungal sp. mh337.6, GU552551	100	89	(41%)	0		34	(41%)	51	(58%)	0		26	(41%)
Biscogniauxia sp.	AB686647	Xylariaceae sp. , GU048581	98	44	(20%)	1	(4%)	5	(6%)	24	(27%)	1	(7%)	5	(8%)
Annulohypoxylon sp.	AB669044	*Annulohypoxylon moriforme*, DQ840058	99	3	(1.4%)	0		1	(1.2%)	2	(2%)	0		1	(2%)
Daldinia sp.	AB669047	*Daldinia childiae*, EF562505	99	2	(0.9%)	0		0		2	(2%)	0		0	
Xylaria sp.2	AB686648	Fungal sp. mh1111.61, GU552542	99	2	(0.9%)	0		0		2	(2%)	0		0	
Rosellinia sp.	AB686649	*Rosellinia corticium*, DQ840078	99	2	(0.9%)	0		0		2	(2%)	0		0	
Xylaria hypoxyloln	AB686650	*Xylaria hypoxylon*, NG_027599	97	1	(0.5%)	0		0		1	(1%)	0		0	
Nodulisporium sp.	AB686651	*Annulohypoxylon moriforme*, DQ840057	99	0		1	(4%)	0		0		1	(7%)	0	
Hypoxylon cf. *fragiforme*	AB686652	*Hypoxylon fragiforme*, AY083829	99	0		0		1	(1.2%)	0		0		1	(2%)
Xylaria sp.4	AB686653	Fungal endophyte, EU687185	97	1	(0.5%)	0		0		1	(1%)	0		0	
Xylaria sp.3	AB686654	Xylariaceae sp., AB376751	98	1	(0.5%)	0		0		1	(1%)	0		0	
Xylaria sp.5	AB686655	Xylariales cf. JP14-3, GQ906954	94	1	(0.5%)	0		0		1	(1%)	0		0	

Xylaria sp.6	AB669070	*Anthostomella leucospermi*, EU552100	99	1	(0.5%)	0	0	1	(1%)	0	0
Xylariaceae sp.	AB669071	Xylariaceae sp., GU048581	95	1	(0.5%)	0	0	1	(1%)	0	0

Note: Total numbers of isolates or tree species examined are shown in square brackets, and the percentages relative to the total number of tree species are shown in parentheses.

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Table 3 Number of tree species from which the 15 OTUs of xylariaceous fungi and three major OTUs were isolated, as related to the life form of tree species.

	Total number of tree species examined	Xylariaceae, 15 OTUs	*Xylaria* sp.1	*Nemania* sp.	*Biscogniauxia* sp.
Live leaves					
Broad-leaved, deciduous	75	63 (84%)	38 (51%)	43 (57%)	21 (28%)
Broad-leaved, evergreen	7	6 (86%)	3 (43%)	4 (57%)	1 (14%)
Coniferous, deciduous	2	1 (50%)	1 (50%)	1 (50%)	0 (0%)
Coniferous, evergreen	4	4 (100%)	1 (25%)	3 (75%)	0 (0%)
Bleached portions of dead leaves					
Broad-leaved, deciduous	12	12 (100%)	12 (100%)	0 (0%)	1 (8%)
Broad-leaved, evergreen	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Coniferous, evergreen	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Nonbleached portions of dead leaves					
Broad-leaved, deciduous	55	31 (56%)	20 (36%)	25 (45%)	2 (4%)
Broad-leaved, evergreen	3	1 (33%)	1 (33%)	0 (0%)	0 (0%)
Coniferous, deciduous	2	1 (50%)	0 (0%)	0 (0%)	1 (50%)
Coniferous, evergreen	3	3 (100%)	1 (33%)	1 (33%)	2 (67%)

Note: No bleached portions of dead leaves were examined for coniferous deciduous tree species.