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SHORT COMMUNICATION

# Temporal variations in endophytic fungal assemblages of *Ginkgo biloba* L.

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**Abstract** We isolated endophytic fungi from living healthy leaves, petioles, and current-year twigs of *Ginkgo biloba* L. from April to November 2004 with the objective of identifying the dominant endophytic fungal taxa, and monitoring their occurrence and frequency. A total of 9 fungal taxa were identified to the genus level. Diversity measures inferred from the Shannon–Wiener, Morisita–Horn, and Sørensen indices indicated that leaves and petioles harbored more diverse endophytic fungal assemblages than twigs, and that fungal taxa involved in twigs shared less with those in leaves and petioles. Among the organs, the occurrence pattern of overall endophytic fungi differed significantly, and two taxa, *Phomopsis* sp. and *Phyllosticta* sp., were the most frequently isolated and thus regarded as the dominant endophytic fungi. *Phomopsis* sp. was isolated frequently from twigs (84.8%) but rather few from leaves (16.1%) and petioles (24.3%). *Phyllosticta* sp. was isolated frequently from leaves (72.9%) and petioles (65.7%) but was never isolated from twigs. Temporal changes in relative frequency of total endophytic fungi tended to differ among sampling dates for all three organs. The occurrence of *Phyllosticta* sp. in both leaves and petioles was first detected in August and peaked in October. *Phomopsis* sp. was detected in twigs throughout the growing season. These results suggest that the distribution of the two dominant endophytic fungi was organ-specific and differed within seasons.

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## Introduction

Endophytic microbes are an intriguing group of organisms associated with a variety of tissues and organs of plants (Stone et al. 2000; Arnold 2007; Saikkonen 2007; Rodriguez et al. 2009). The term *endophyte* refers to any organism that inhabits plant organs and can, at some time in a plant's life, colonize its internal tissues without causing apparent harm to the host (Petrini 1991). Fungal endophytes have been discovered on a broad range of hosts, including ferns and mosses, but sporulate only on one or a few of these (Petrini 1986, 1991). The ubiquitous existence of these fungi has attracted the interest of mycologists, ecologists, physiologists, taxonomists, and applied scientists (Petrini 1991; Rodriguez et al. 2009).

Endophytic fungi have been found in all woody gymnosperms and angiosperms examined (Petrini 1986; Sieber 2007; Rodriguez et al. 2009). Many investigations in Europe and North America have suggested that a few dominant taxa with many fewer groups of fungi form endophytic communities in woody plants (Carroll 1986; Petrini 1986, 1991; Sieber 2007). In Japan, the species composition and frequency of isolation of endophytic fungi have been reported for *Fagus crenata* Blume (Sahashi et al. 1999, 2000; Osono 2002; Kaneko and Kaneko 2004), *Pinus* spp. (Hata and Futai 1996), *Pasania edulis* Makino (Hata et al. 2002), *Neolitsea sericea* Blume (Hata and Sone 2008), *Quercus* spp. (Hashizume et al. 2008), and Ericaceae (Okane et al. 1998). These studies also indicated that some groups of fungi dominated on the host trees examined. Considering the species diversity of

trees, studies of species composition and temporal changes of the endophytic assemblages in deciduous broad-leaved trees are limited (Sahashi et al. 1999; Rodriguez et al. 2009).

*Ginkgo biloba* L. is a deciduous gymnosperm and is possibly the oldest surviving tree species on Earth, dating from more than 200 million years ago. It is the only surviving member of the ginkgo family and is described as a "living fossil" (Tremouillaux-Guiller et al. 2002). *G. biloba* is long-lived and is largely free from disease or attack by insect pests (Liu et al. 2007). Thus, host specificity of fungal associates might be expected to be closer than for trees of recent origin, and if this is the case, co-evolved endophytes may confer on plants beneficial effects such as resistance to equally coevolved pathogens (Scheuer et al. 2008; Gazis and Chaverri 2010). Tree pathogens associated with *G. biloba* have been studied intensively, and ~10 pathogenic fungi have so far been reported (Aoki 1997). In contrast, little is known about the species composition of endophytic fungi in living leaves (Aoki 1997; Kim et al. 1999; Scheuer et al. 2008). Clarification of the species assemblages and temporal variations in endophytic fungal isolates would be an important step toward improving our understanding of the ecophysiological traits of the fungi and of host–endophyte interactions (Sahashi et al. 1999; Rodriguez et al. 2009).

The objective of this study was to determine the species composition of endophytic fungi in different organs of *G. biloba* and their relative frequency during an active growth stage. In the study we hypothesized that some dominant fungal species could be detected, as was documented in previous studies, and that their taxonomic identity might differ from those of known examined tree species because of the nature of fossil species.

## Materials and methods

### Study site

The study site was on the campus of Mie University (34°44′N, 136°31′E; 1 m asl), Tsu, Mie, Japan. The mean annual temperature was 15.5°C and mean annual precipitation was 1650.3 mm recorded at the nearest weather station (Tsu Weather Station 34°44′N, 136°31′E; 18 m asl).

### Samples collection

We took samples from three healthy ginkgo trees located at different places on the campus. The average diameter of the trees at breast height was 45.9 cm. On each sampling occasion, seven leaves including petioles and seven twigs were collected from each tree. Healthy leaves, petioles, and current-year twigs were collected at weekly intervals during the period of foliage emergence from 6 April to 2 May 2004. When the leaves were fully expanded, samples were collected at monthly intervals from 3 June to 4 November 2004, when leaf color changed from green to yellow. The samples were brought back to the laboratory and processed for isolation of fungi within 48 h.

### Isolation of fungi

The sampled tissues were surface-sterilized by submerging them in 70% (*v*/*v*) ethanol for 30 s, and then for 3 min into a solution of sodium hypochlorite (1% *w*/*v* available chlorine). The samples were rinsed twice with sterilized distilled water and then dried on sterile filter paper. The surface-sterilized samples were cut into segments 0.2–0.3 cm long and placed on half-strength PDA (1/2 PDA) medium. On each sampling occasion, 30 segments from each organ per tree yielded 90 segments (30 segments × 3 trees). A total of 10 sampling occasions yielded 900 segments of each organ which were examined during the experiment. The plates were incubated at 25°C in the dark and were observed every 3 days for the appearance of fungi. Each colony of fungus appearing from the segments was transferred to fresh 1/2 PDA media as endophytic fungal isolate for further identification.

### Assessment of relative frequency

We identified the fungal isolates to the genus level on the basis of spore morphology and noted whether they belonged to known endophytic taxa. At the genus level, the morphological trait of isolates was identical and thus they were treated as one taxon. Relative frequency of isolates was calculated as the number of isolates of one taxon divided by the total number of isolates, and was expressed as a percentage (Huang et al. 2008).

The relative frequency of the fungal taxa detected and of detected isolates among organs were compared by use of the $\chi^2$ test to determine trends in fungal occurrence. Statistical analysis was conducted with SPSS 11.5J (SPSS, Chicago, IL, USA).

On the basis of the percentage relative frequency in each organ, the Shannon–Wiener index ($H'$) was calculated by use of the formula; $H' = -\Sigma s\ (p_i \log_e p_i)$, where the ratio $p_i$ is the frequency of colonization of the $i$th taxon in the sample and $s$ is number of taxa detected. The similarity of endophytic fungal taxa among different organs was compared on the basis of both frequency of occurrence of a fungus (Morisita–Horn index) and presence/absence data (Sørensen's index). The similarity indices were calculated using EstimateS 8.0.0 (Colwell 2006).

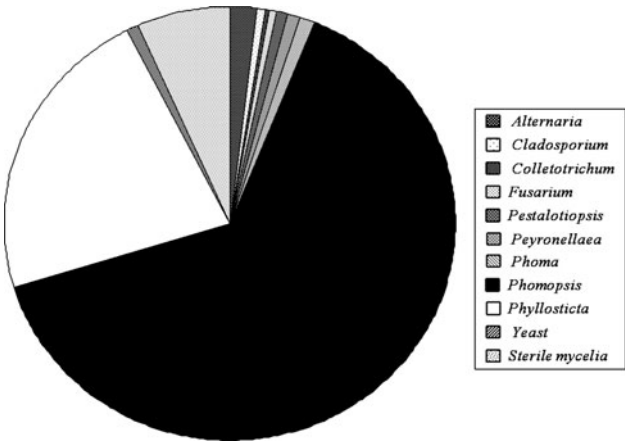


**Fig. 1** Relative frequencies (%) of different endophytic taxa isolated from *Ginkgo biloba*. Data from three organs, leaves, petioles, and twigs, derived from 3 trees were pooled ($n = 1{,}007$)

## Results

### Endophytic fungi isolated

Endophytic fungi were detected in all trees and in every organ examined. The isolated fungi were identified to genus level and grouped into 9 taxa (Fig. 1). Some yeast and a few sterile mycelia were also detected. Relative frequency among the taxa was significantly different ($\chi^2 = 1{,}026.4$, df = 10, $P < 0.001$). Among them, *Phomopsis* sp. was the most frequent endophytic fungi (64.1%) and *Phyllosticta* sp. (22.2%) was the second most frequent. Other isolates including *Alternaria*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Pestalotiopsis*, *Peyronellaea* and *Phoma* were detected at frequencies <2%. The Shannon–Wiener diversity index for leaves and petioles was 1.34 and 1.37, respectively, and both the values were rather higher than that for twigs, 0.95.

### Tissue specificity of endophytic fungi

Composition of fungal taxa among organs tended to vary (Table 1). Except for yeast and sterile mycelia, five fungal taxa were isolated from leaves, three from petioles, and six from current-year twigs. The number of detected isolates among organs was significantly different ($\chi^2 = 539.4$, df = 2, $P < 0.001$), and the twig tissues harbored more endophytic fungi (683) than leaves (155) and petioles (169). The values of the Morisita–Horn and Sørensen similarity indices for the combination leaves and petioles were 0.988 and 0.833, respectively (Table 2). However, values resulting from pairs including twigs tended to be lower for both indices.

From twigs, *Phomopsis* sp. was isolated most frequently (84.8%); all the other taxa were isolated much less frequently. *Phyllosticta* sp. was the most frequently isolated taxon from leaves (72.9%) and petioles (65.7%), but was never isolated from twigs. *Phomopsis* sp. was the most frequent taxon, with nearly ubiquitous distribution in all three organs, whereas *Colletotrichum* sp. was found in leaves only. The four fungal taxa *Alternaria*, *Cladosporium*, *Fusarium*, and *Pestalotiopsis* were detected in twigs only. In contrast, 2 taxa, *Phyllosticta* and *Phoma*, were found in leaves and petioles but were not detected in twigs. *Peyronellaea* was found in leaves and twigs but was not detected in petioles (Table 1).

**Table 1** Relative frequencies (RF, %) of endophytic fungi detected from 3 living organs of *Ginkgo biloba*

| Endophytic fungi | RF (%) of endophytic fungi | | |
|---|---|---|---|
| | Leaves ($n = 155$)[a] | Petioles ($n = 169$)[a] | Current-year twigs ($n = 683$)[a] |
| *Alternaria* sp. | 0.0 | 0.0 | 3.1 |
| *Cladosporium* sp. | 0.0 | 0.0 | 0.7 |
| *Colletotrichum* sp. | 2.6 | 0.0 | 0.0 |
| *Fusarium* sp. | 0.0 | 0.0 | 0.7 |
| *Pestalotiopsis* sp. | 0.0 | 0.0 | 1.2 |
| *Peyronellaea* sp. | 0.6 | 0.0 | 1.0 |
| *Phoma* sp. | 1.9 | 4.1 | 0.0 |
| *Phomopsis* sp. | 16.1 | 24.3 | 84.8 |
| *Phyllosticta* sp. | 72.9 | 65.7 | 0.0 |
| Yeast | 1.9 | 1.2 | 0.9 |
| Sterile mycelia | 3.9 | 4.7 | 7.6 |

[a] Numbers in parentheses indicate the total number of fungal isolates

**Table 2** Values of Morisita–Horn and Sørensen indices for endophytic fungi among different organs from *Ginkgo biloba*

| | Organ types | Morisita–Horn index | | |
|---|---|---|---|---|
| | | Leaf | Petiole | Twig |
| Sørensen index | Leaf | | 0.988 | 0.218 |
| | Petiole | 0.833 | | 0.343 |
| | Twig | 0.533 | 0.462 | |

Morisita–Horn index (upper half of the matrix) and Sørensen index (lower half of the matrix) for taxon similarity among pairs of different organs of *G. biloba*

### Relative frequency of dominant endophytic fungi during the growing seasons

The relative frequencies of the endophytic fungi seemed to be different among sampling dates in leaves, petioles, and twigs (Fig. 2). The dominant taxon *Phyllosticta* sp. was first detected in both leaves and petioles in August, with a peak in frequency in October. This taxon was still present

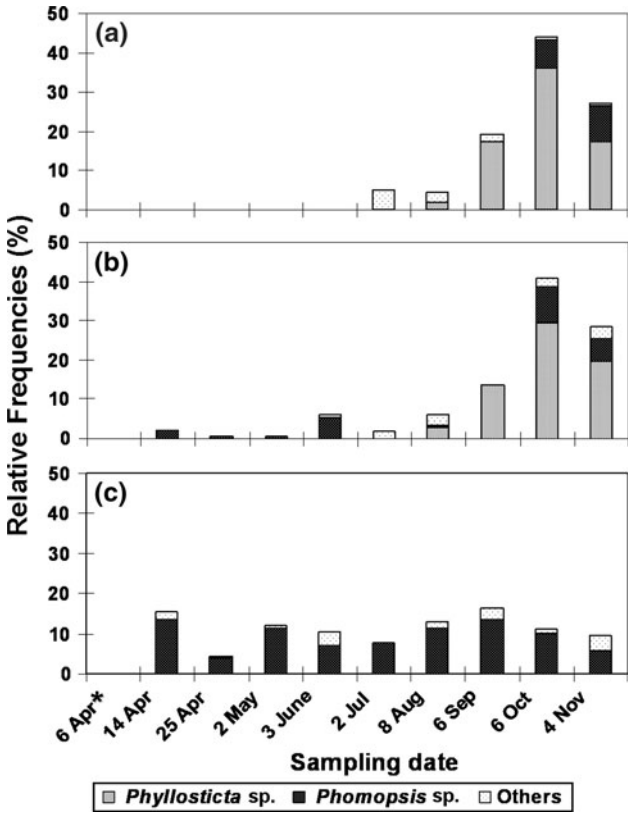


**Fig. 2** Relative frequencies (%) of two dominant endophytic fungi, *Phyllosticta* sp. and *Phomopsis* sp. isolated from **a** leaves, **b** petioles, and **c** twigs during the vegetation period of *Ginkgo biloba*. The *asterisk* indicates that on the first sampling occasion fungi were isolated from new leaf tissue inside buds. Twigs were not sampled

at the end of the growing season in November. Another frequent taxon, *Phomopsis* sp. was isolated from twigs and petioles on 14 April and continued to be isolated over the growing season at different frequencies that had no clear pattern. For leaves, this taxon was first detected in 6 October.

## Discussion

A total of 9 endophytic fungi were detected and there were two dominant taxa, *Phomopsis* sp. and *Phyllosticta* sp. These dominant fungi have frequently been isolated from many plants (Hata et al. 2002; Shankar et al. 2008). Petrini (1991) suggested that only one or a few fungal taxa dominated endophytic communities in a single host species. Such a pattern of dominance of endophytic fungi has been reported in many species of broad-leaf trees (Wilson and Carroll 1994; Sahashi et al. 1999; Hata et al. 2002; Hashizume et al. 2008; Hata and Sone 2008). Thus this study, also, provided more evidence that a few dominant fungal species are associated with ginkgo trees. Apart from the dominant taxa, our study confirmed that *Alternaria*, *Colletotrichum*, *Cladosporium*, *Fusarium*, *Pestalotiopsis*, *Peyronellaea*, and *Phoma* were also present as endophytes. In addition, 5 fungal taxa; *Alternaria*, *Cladosporium*, *Fusarium*, *Pestalotiopsis*, and *Phoma* were also known to be endophytic fungi of ginkgo (Kim et al. 1999). However, these fungal groups have frequently been reported as endophytic fungi from a variety of plant species (Huang et al. 2008; Gazis and Chaverri 2010) and thus were considered taxonomically as common groups rather than unique ones. Therefore, although a ginkgo itself is a fossil species, individuals of the species can be associated with fungi that potentially inhabit their surroundings.

The composition and abundance of endophytic fungi varied according to the organs of the host. In fact, the diversity measures Shannon–Wiener, Morisita–Horn, and Sørensen indices indicated that the leaves and petioles harbored more diverse endophytic fungal assemblages than the twigs, and that fungal species in the twigs were less likely to be present in the leaves and petioles. The relative frequency of total endophytic fungi was significantly different among organs, and the fungi from twigs occurred more frequently than those from leaves and petioles. Ragazzi et al. (2001) reported that endophytic fungi were more abundant in current-year twigs than in leaves or petioles of *Q. cerris* L. These phenomena might be related to the close proximity of current-year twigs to older twigs that had been colonized by abundant endophytic fungi over previous years; the endophytes can then move to the current-year twigs (Ragazzi et al. 1999).

More specifically, frequent occurrences of some endophytic fungi in specific organs have been amply demonstrated for Norway spruce and silver fir (Sieber 1989). Our study showed that *Phomopsis* sp. was much more frequent in twigs, whereas *Phyllosticta* sp. was most frequently isolated from both leaves and petioles. Other studies have also shown that *Phomopsis* sp. was commonly the most frequent endophytic fungus isolated from twig tissues (Sahashi et al. 1999; Hata et al. 2002; Osono and Mori 2003; Hata and Sone 2008), and *Phyllosticta* sp. was isolated more frequently from leaves than from other organs (Hata and Sone 2008). These results indicate that endophytic fungi have organ preference or specificity to host plants (Carroll and Carroll 1978). Thus the mechanism of dominance of the two dominant fungi should be clarified in relation to the organ structure of the host trees.

No fungi were detected during the period of foliage emergence, although living ginkgo leaves were collected from the beginning of bud formation. Similar observations have been reported for conifers (Petrini and Carroll 1981), palms (Rodrigues 1994), and oaks (Wilson and Carroll 1994). For deciduous trees, few fungal species have been shown to colonize leaves during bud formation or foliage

emergence. In fact, fungal colonization of *F. crenata* was detected only after leaves had fully expanded, and isolation frequencies increased over the growing season (Sahashi et al. 1999, 2000; Osono and Mori 2005). These results indicated that fungi such as foliar endophytes might colonize plant tissues via horizontal transmission.

For the dominant fungi, *Phomopsis* sp. and *Phyllosticta* sp., temporal variations were observed over the growing season. *Phyllosticta* sp. peaked in October whereas *Phomopsis* sp. was detected throughout the growing season and had no obvious occurrence pattern. The different occurrence patterns of the two dominant endophytic fungi may be related to different forms of infection and time, and in the preference of host plant tissues (Petrini 1991; Carroll 1995). Considering the continuous isolation of *Phomopsis* sp. from twigs, this species might be saprotrophic in nature during life cycles. On the other hand, *Phyllosticta* sp. occurred in leaves and petioles only late during the growing stage, indicating that infection with spores could be occurring. However, the species decreased before leaves senesce, and thus colonization might be intimately related with leaf status. In further research, the fates of these dominant fungi after leaf fall should be studied to clarify the functional role of the fungi.

In this study we sampled living *G. biloba* organs to investigate endophytic fungi. Two dominant fungi, *Phomopsis* sp. and *Phyllosticta* sp. were isolated from living organs, and had organ-specific distributions. The different patterns of occurrence and the preference of endophyte assemblages for specific tissues indicated that some fungal endophytes have an affinity for different tissue types. This may reflect their capability of utilizing or surviving within a specific substrate (Rodrigues 1994; Huang et al. 2008). Because the two dominant fungi were taxonomically common as major endophytic groups in general plants, further studies are needed to clarify whether they have selective affinity for *G. biloba* at species or strain levels.

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