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Beech cupules share endophytic fungi with leaves and twigs

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ABSTRACT

Endophytic mycobiota on leaves, twigs and cupules of *Fagus crenata* were investigated using a culture-dependent method over a growing season to test the hypothesis that endophytic fungi of cupule (a woody phyllome) share some components of the endophytic fungal assemblages with both leaves and twigs. A total of 14 fungal taxa were isolated, and the most frequent taxon was *Phomopsis* sp., followed by *Xylaria* sp., *Ascochyta fagi* and *Geniculosporium* sp. The compositions of fungal assemblages of leaf laminae and petioles were generally relatively dissimilar to those of current and first-year twigs when compared for each sampling month, and those of cupules and cupule stalks were intermediate between those of leaves and twigs. Permutational multivariate analysis of variance confirmed that month and organ were significant factors of the variation of the composition of endophytic fungal assemblages. *Phomopsis* sp., a common twig endophyte, and *A. fagi*, a common leaf endophyte, were common in cupules and cupule stalks. These results suggested that the endophytic fungal assemblages of cupules shared component taxa with those of both leaves and twigs.

1. Introduction

Endophytic fungi include those that can colonize internal plant tissues at some time in their life without causing apparent harm to their host (Sieber 2007). Beech (*Fagus* spp.) is a dominant tree of cool temperate forests and has been examined for endophytic fungi, with intensive efforts devoted to Japanese beech *F. crenata* (Sahashi et al. 1999, 2000; Kaneko and Kakishima 2001; Osono 2002; Kaneko et al. 2003; Osono and Mori 2003; Kaneko and Kaneko 2004; Fukasawa et al. 2009; Hashizume et al. 2010), European beech *F. sylvatica* (Sieber and Hugentobler 1987; Danti et al. 2002) and American beech *F. grandifolia* (Chapela 1989). Most of these studies investigated endophytic fungi on leaves and twigs; but there have been no published works regarding the endophytic fungi associated with beech cupules. A cupule is a woody phyllome surrounding the seed in a fruit; thus, a cupule shares its origin with that of leaves but is chemically similar to twigs (Osono

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and Takeda 2001; Fukasawa et al. 2009, 2012). We hypothesized that endophytic fungal assemblages of beech cupule shared components of endophytic fungi with both leaf and twig within the shoot. The purpose of the present study was to investigate the endophytic mycobiota on leaves, twigs and cupules of *F. crenata* over a growing season to test our hypothesis.

2. Materials and methods

2.1. Study site and sample collection

Samples were collected in Ashiu Experimental Forest of Kyoto University (35°18′N and 135°43′E), Kyoto, Japan. Details of the study site were described in Osono et al. (2011). In the study site, mass flowering of *F. crenata* and mass production of cupules were observed in 2005, whereas only a few individual trees flowered in 2006. We selected a mature tree (height 16 m) that flowered in 2006, and shoots with flowers were harvested from the canopy at approx. 5–8 m height in Jun, Aug and Oct 2006. Ten shoots carrying current-year leaves, maturing cupules, a current-year twig and a one-year twig (Fig. 1) were arbitrarily selected from the canopy and harvested on each sampling date. Healthy-looking shoots without obvious faunal and/or microbial attacks were selected. The samples were placed in paper bags and taken to the laboratory.

One leaf, one cupule, two current-year twigs (1 cm in length) and two first-year twigs (1 cm in length) were taken from each shoot. The leaf was divided into lamina and petiole, and four leaf disks were punched from the lamina with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. The cupule was cut into four equivalent pieces and one stalk. Thus, a total of 40 disks of leaf lamina, 10 petioles, 40 pieces of cupules, 10 cupule stalks, 20 current-year twigs and 20 first-year twigs were prepared on each sampling date and used for the isolation of fungi.

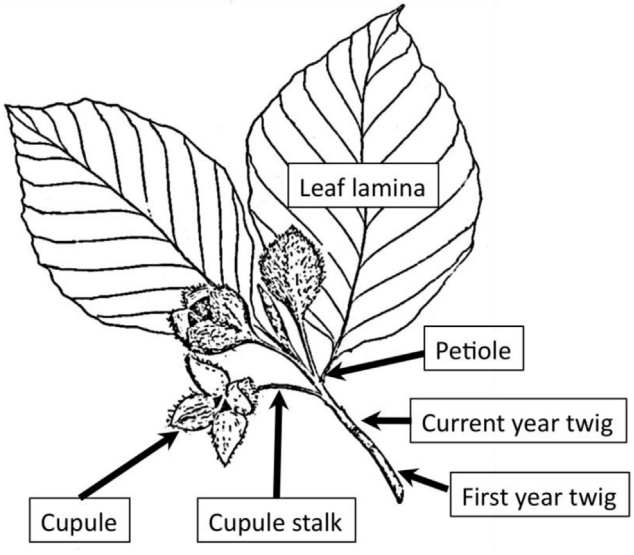

Fig. 1 – A fruiting shoot of *Fagus crenata*.

2.2. Fungal isolation

A surface sterilization method by Osono et al. (2008) was used for the isolation of fungi from beech organs. Fungal isolation was carried out within 24 h of sampling. The plant organ samples were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface-disinfected for 30 s in a solution of 15% hydrogen peroxide, and submerged again for 1 min in 70% ethanol. The samples 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 leaf disks or pieces from cupules and twigs were placed in 9-cm Petri dishes containing malt extract agar (malt extract 2% (w/v), agar 2%; Nacalai Tesque, Kyoto, Japan), with two disks/pieces per plate. Plates were incubated at 20 °C in the dark and observed at 1, 4 and 8 wks after surface sterilization (Osono and Takeda 1999). Identification was primarily based on micromorphological observations, with reference to Gams (2007). Some isolates were then used for molecular analysis as described below. The frequency of an individual taxon was calculated as the percentage of incidences based on the number of plant organs with the taxon relative to the total number of the organ, for each sampling date. Taxa with low frequencies were specifically discussed only if their occurrence was of special interest.

2.3. DNA analysis

Twenty-two isolates of *Phomopsis* sp., *Xylaria* sp. and *Geniculosporium* sp. were used for DNA analysis. Thirteen isolates of *Phomopsis* sp. included seven from cupules, one from cupule stalk, two from twigs and three from lamina. Seven isolates of *Xylaria* sp. included four from cupules, two from cupule stalks and one from leaf lamina. Two isolates of *Geniculosporium* sp. included one from cupule and one from cupule stalk. Before DNA extraction, the isolates were subcultured in 2% malt extract liquid medium. The DNA was extracted from small quantities of mycelia using DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed using a Quick Taq HS DyeMix (Toyobo, Osaka, Japan). Each PCR reaction contained a 50 μl mixture (21 μl distilled water, 25 μl master mix, 3 μl ca. 0.5 ng/μl template DNA and 0.5 μl each primer (final, 0.25 μM)). The primer pair ITS1f (Gardes and Bruns 1993)/LR3 (Vilgalys and Hester 1990) was used to obtain the ITS2 and the D1-D2 domain of the 28S rRNA. Each DNA fragment was amplified using a PCR thermal cycler (DNA Engine, Bio-Rad Laboratories, Hercules, USA) using the following thermal cycling schedule: the first cycle consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C for annealing, 1 min at 68 °C, and a final cycle of 10 min at 68 °C. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). The purified PCR products were sequenced by Macrogen Japan Corp. (Tokyo, Japan). The sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) (AB915934–AB915946, AB918138–AB918140, AB918142–AB918147). The ITS2 and 28S rRNA gene sequences were compared using MEGA5 (Tamura et al. 2011) to determine the sequences identity. All positions containing gaps