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FULL PAPER

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Isolation of endophytes from leaves of *Neolitsea sericea* in broadleaf and conifer stands

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Abstract Endophytic fungi were isolated from leaves of *Neolitsea sericea*, a major lauraceous tree in the laurel forests of southern Kyushu, collected from the understory layer of broadleaf and conifer stands. *Cytosphaera* sp. and a species of Ascomycetes in leaf blade segments, plus a xylariaceous species and *Phomopsis* spp. in petiole segments, were isolated at relatively high frequency. In general, isolation frequencies of endophytes were higher in petiole than blade segments. In blade segments, patterns of endophyte isolation were quite different among stands, while relatively similar in petiole segments. Significant effects of sampling sites or canopy vegetation were rarely detected.

Key words Endophytic fungi · *Neolitsea sericea* · Stand-level comparison · Understory

Introduction

In Japan, studies of endophytes in trees have mainly been conducted in trees from families such as Pinaceae, Fagaceae, and Ericaceae (Hata et al. 1998; Okane et al. 1998; Sahashi et al. 1999; Kaneko et al. 2003). In the warm temperate laurel forests of southern Kyushu, the authors have previously reported a case study on isolation of endophytes from the leaves of *Pasania edulis* Makino (Fagaceae) (Hata et al. 2002); however, endophytes in other trees of such forests remain unknown. In this kind of laurel forest, as the name suggests, lauraceous trees constitute a significant part of the vegetation, including several dominant canopy and understory species. On the other hand, research on tree endophytes tends to focus on canopy tree species with relatively few studies on understory species. Thus, this study focused on *Neolitsea sericea* (Bl.) Koidz., a common tree in the understory layer of laurel forests. *Neolitsea sericea* is an evergreen broadleaf lauraceous tree, widely distributed in areas of eastern Asia. In southern Kyushu, it is one of the most common trees, growing both as canopy and understory species. In this study, endophytes were isolated from the leaves of *N. sericea* growing in the understory layer of conifer and broadleaf forest stands to survey the endophytes of *N. sericea* leaves and to examine the effect of the canopy layer on endophytic mycobiota in understory plants.

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Materials and methods

Takakuma Experimental Forest at Kagoshima University (31°31′ N, 130°46′ E) is located in the central mountains of Oosumi Peninsula, the southernmost area of Kyushu mainland. In Takakuma Experimental Forest, two sites were set, separated approximately 200 m from each other by a small valley, each on the boundary of a natural broadleaf forest and an artificial conifer forest. Each site, named site A and site B, contains two subsite stands, one conifer and the other broadleaf (named stands A-co, A-br, B-co, and B-br, for conifer and broadleaf subsite stands in sites A and B, respectively). In site A (about 450 m above sea level; the same site as in Hata et al. 2002), the artificial conifer stand (stand A-co) was that of *Cryptomeria (Cr.) japonica* D. Don of about 40 years. In the natural broadleaf tree stand (stand A-br), *P. edulis* was dominant and accounted for about 40% of the canopy trees. The other dominant tree species in the canopy and subcanopy layer were *Persea thunbergii* (Sieb. & Zucc.) Kosterm., *Castanopsis cuspidata* (Thunb.) Schottky var. *sieboldii* (Makino) Nakai, *Quercus acuta* Thunb., and *Quercus salicina* Bl. among with some *N. sericea* trees. The understory layers of these stands were similar, with a number of broadleaf tree species including *N. sericea* (other tree species include *Aucuba japonica* Thunb., *Ligustrum japonicum* Thunb., *Cleyera japonica* Thunb., *Symplocos lucida* Sieb. & Zucc., *Camellia (Ca.) japonica* L., *Illicium anisatum* L., and several lauraceous species such as

Cinnamomum japonicum Sieb., *P. thunbergii*, *Persea japonica* Sieb., *Actinodaphne longifolia* Nakai, and *Neolitsea aciculata* Koidz.). In site B (about 550 m above sea level), the artificial conifer stand (stand B-co) was that of *Cr. japonica* of about 40 years as in stand A-co. In the natural broadleaf tree stand (stand B-br), trees such as *Pasania edulis*, *Persea thunbergii*, *Castanopsis cuspidata* var. *sieboldii*, and *Cinnamomum japonicum* were dominant along with *N. sericea* in the canopy and subcanopy layer. The understory layers of these stands also seem similar both in vegetation and in density, with such trees as *A. japonica*, *L. japonicum*, *Maesa japonica* (Thunb.) Moritzi, and *Ca. japonica* as well as *N. sericea* and similar lauraceous trees that frequently grow in A-br. There were some differences: *Distylium racemosum* Sieb. & Zucc. and *Damnacanthus indicus* Gaertn. f. were relatively frequent only in B-br, while *Hydrangea macrophylla* (Thunb. ex Murray) Ser. var. *acuminata* (Sieb. & Zucc.) Makino, *Hydrangea luteo-venosa* Koidz., and *Morus bombycis* Koidz. were largely found in B-co, but such differences were not conspicuous. On January 12, 2004, five trees of *N. sericea* (about 1–3 m tall) in each stand were selected, and five healthy current-year leaves were collected from twigs of each tree at 0.5–2 m height. The collected leaves were transported to the laboratory, and endophyte isolation was then performed.

Endophyte isolation was conducted using a surface-sterilization method, essentially following Hata et al. (2002). From each leaf, one segment (approx. 2 × 2 mm) from the leaf blade and one segment (approx. 2 mm long) from the petiole was cut using sterile scissors. The segments were surface-sterilized with successive treatments of 70% ethanol for 1 min, 15% hydrogen peroxide for 15 min, and 70% ethanol for 1 min. Surface-sterilized samples were placed on 2% malt extract agar medium plates (MEA), then incubated for approximately 1 month at 20°C. Fungi growing from the segments during the incubation period were recognized as endophytes and isolated for morphological identification.

Isolation frequencies (IF) for the endophytes were calculated using the following formula:

$$IF = Ni/Nt \times 100$$

where Ni is the number of segments from which the fungus was isolated, and Nt is the total number of segments. Differences in IF of the endophytes were tested using Fisher's exact probability test. Effects of sampling site and canopy vegetation were examined by comparing differences in IF of the endophytes between sites A and B, and between conifer and broadleaf stands, respectively.

Results

Table 1 shows the frequency of endophytes isolated from leaf blade and petiole segments. From leaf blade segments of *N. sericea*, *Cytosphaera* sp., and an unidentified ascomycete "Ascomycete sp. 1" (the same species as in Hata et al. 2002, characterized by slow-growing off-white colonies on MEA, rarely producing ascospores, which we could not observe carefully enough for identification) were isolated at relatively high frequency (11.0% and 10.0% on average, respectively). From petiole segments, Xylariaceae sp. 1 (the same species as in Hata et al. 2002, characterized by fast-growing white colonies with white aerial mycelium on MEA, rarely producing a *Geniculosporium*-like anamorph, which we could not observe well enough for genus-level identification) was isolated most frequently, and *Phomopsis* sp. 1 was second most frequently isolated (43.0% and 21.0% on average, respectively). Other isolates from blade and

Table 1. Isolation frequencies (%) of the endophytes from the leaves of *N. sericea* for each stand

	Leaf blade segments					Petiole segments				
	Sampling stands				Average	Sampling stands				Average
	A-co	A-br	B-co	B-br		A-co	A-br	B-co	B-br	
Xylariaceae sp. 1	4.0	4.0	8.0	–	4.0	28.0	56.0	60.0	28.0	43.0
Phomopsis sp. 1	4.0	8.0	8.0	–	5.0	24.0	24.0	28.0	8.0	21.0
Cytosphaera sp.	–	28.0	12.0	4.0	11.0	–	8.0	–	16.0	6.0
Ascomycete sp. 1	–	–	12.0	28.0	10.0	4.0	–	–	4.0	2.0
Phomopsis sp. 2	–	–	–	–	–	4.0	12.0	–	12.0	7.0
Colletotrichum gloeosporioides	–	–	–	4.0	1.0	8.0	–	–	8.0	4.0
Sterile WS1	4.0	–	4.0	–	2.0	8.0	–	4.0	–	3.0
Phyllosticta sp.	–	–	8.0	4.0	3.0	–	–	–	–	–
Sterile WS2	–	–	–	–	–	4.0	–	–	8.0	3.0
Cryptosporiopsis sp.	–	–	–	–	–	8.0	–	–	–	2.0
Sterile BrS1	–	4.0	–	–	1.0	–	4.0	–	–	1.0
Sterile BrS3	–	–	–	–	–	–	–	8.0	–	2.0
Colletotrichum acutatum	–	–	–	–	–	4.0	–	–	–	1.0
Didymosporium sp.	–	–	–	–	–	–	–	–	4.0	1.0
Acremonium sp.	–	–	–	–	–	–	–	–	4.0	1.0
Sterile BrS2	4.0	–	–	–	1.0	–	–	–	–	–
Unidentified	–	–	4.0	–	1.0	–	–	–	–	–

Note: Isolation frequency (%) = number of segments from which the fungus was isolated / total number of segments × 100

Table 2. Statistical comparison of the isolation frequencies of the endophytes by Fisher's exact probability test

	Lb vs. Pe	co vs. br		A vs. B	
		Lb	Pe	Lb	Pe
Xylariaceae sp. 1	2.08×10^{-11}	0.617	1.00	1.00	1.00
Phomopsis sp. 1	1.23×10^{-3}	1.00	0.326	1.00	0.624
Cytosphaera sp.	0.311	0.200	0.0267	0.525	0.678
Ascomycete sp. 1	0.0330	0.318	1.00	1.19×10^{-3}	1.00
Phomopsis sp. 2	0.0140	–	0.112	–	1.00
Colletotrichum gloeosporioides	0.369	1.00	1.00	1.00	1.00
Sterile WS1	1.00	0.495	0.242	1.00	1.00
Phyllosticta sp.	0.246	1.00	–	0.242	–
Sterile WS2	0.246	–	1.00	–	1.00
Cryptosporiopsis sp.	0.497	–	0.495	–	0.495
Sterile BrS1	1.00	1.00	1.00	1.00	1.00
Sterile BrS3	0.497	–	0.495	–	0.495
Colletotrichum acutatum	1.00	–	1.00	–	1.00
Didymosporium sp.	1.00	–	1.00	–	1.00
Acremonium sp.	1.00	–	1.00	–	1.00
Sterile BrS2	1.00	1.00	–	1.00	–
Unidentified	1.00	1.00	–	1.00	–

Lb, leaf blade segments; Pe, petiole segments; co, stands with conifer crown (stands A-co and B-co); br, stands with broadleaf tree crown (stands A-br and B-br); A, site A; B, site B; –, no isolation
Note: Values are *P* values directly calculated in Fisher's exact probability test

petiole segments include another species of *Phomopsis*, two species of *Colletotrichum*, species of *Phyllosticta*, *Cryptosporiopsis*, *Didymosporium*, and *Acremonium*, and several sterile or unidentified strains, but at frequencies of less than 10%.

Overall, isolation frequencies of endophytes were higher in petiole than blade segments. Among endophyte species, Xylariaceae sp. 1, *Phomopsis* sp. 1, and *Phomopsis* sp. 2 were significantly more frequently isolated from petiole than blade segments ($P = 2.08 \times 10^{-11}$, $P = 1.23 \times 10^{-3}$, and $P = 0.0140$, respectively, by Fisher's exact probability test; see Tables 1 and 2). In contrast, Ascomycete sp. 1 was significantly more frequently isolated from blade than petiole segments ($P = 0.0330$ by Fisher's exact probability test; see Tables 1 and 2).

In blade segments, patterns of endophyte isolation were markedly different among the subsite stands. In stand A-co, isolation frequencies of the endophytes were generally low (only four species, all 4.0%); in stand A-br, *Cytosphaera* sp. dominated (IF = 28.0%); in stand B-co, five species were isolated at approximately 10% (8.0 or 12.0%); and in stand B-br, Ascomycete sp. 1 dominated (IF = 28.0%). Thus, there were different tendencies for isolated endophytes in all stands. However, endophyte isolation patterns in petiole segments were quite similar among subsite stands; e.g., Xylariaceae sp. 1 was most frequently isolated (28.0%–60.0%), then *Phomopsis* sp. 1 and/or sp. 2 were also frequently isolated from all stands.

No significant effects of sampling site and canopy vegetation were detected for most endophytes (see Table 2). Exceptions were Ascomycete sp. 1, significantly more frequently isolated from site B than site A in blade segments ($P = 1.19 \times 10^{-3}$ in Fisher's exact probability test), and *Cytosphaera* sp., more frequently isolated from broadleaf stands than conifer stands in petiole segments ($P = 0.0267$ in Fisher's exact probability test).

Discussion

Differences in isolation frequencies of endophytes from leaf blade and petiole segments, quite evident in the present study, have been observed as a universal tendency for endophyte distributions in tree leaves (Carroll 1995). There are several reasons for this tendency. The most likely reason in this case is differences in modes of infection of different endophyte species. One of the endophytes frequently isolated from petioles in the present study belongs to the family Xylariaceae. Endophytes belonging to this family have been isolated from a diverse array of tree species (Petrini et al. 1995). The family Xylariaceae was originally known as wood-rotting fungi; however, some members have often been isolated as endophytes, not only from stems and twigs but also from leaves. Even when they were isolated from leaves, it is likely that they had colonized from twigs, as species of Xylariaceae have not been observed to produce reproductive structures on plant leaves (Petrini et al. 1995). In the present study, if we hypothesize that Xylariaceae sp. 1 infected leaves from twigs via petioles, the isolation pattern of this species, more frequent from petiole than blade segments, seems reasonable. The other dominant endophyte genus in petioles, *Phomopsis* spp., has often been reported as a major endophyte in twigs and leaves, particularly twigs (Sahashi et al. 1999; Hata et al. 2002). Species of this genus also appear to infect leaves from twigs (see similar discussion in Hata et al. 2002). In contrast, dominant endophytes in leaf blade but infrequent in petioles, such as *Cytosphaera* sp. and Ascomycete sp. 1 found in the present study, are likely to infect leaves directly from aerial spores. This difference in mode of infection in endophyte species is a likely cause of difference in the endophytic mycobiota of leaf blades and petioles, seen in this study.

Effects of sampling site and vegetation of the canopy layer on the IF of the endophytes were revealed to be not significant by statistical analysis except for one endophyte species for each factor (Ascomycete sp. 1 in leaf blade segments for the former and *Cytosphaera* sp. in petiole segments for the latter). This result suggests that such stand-level factors have less effect on endophytic mycobiota compared to the effects of other factors such as position in a leaf. The explanation for this relative lack of effect of stand-level factors appears to be different between petiole and blade segments. In petiole segments, isolated endophytes and frequencies were similar for all stands, but markedly differed for all stands in blade segments. Thus, the lack of stand-level effect in the former seems to reflect the stability of the mycobiota, while that in the latter to reflect the instability of the mycobiota. The reason for this difference in stand-level stability of endophytic mycobiota between the different segments is unclear; however, differences in infection modes of major endophytes as already discussed are likely to be involved as one of the reasons.

In Hata et al. (2002), in which endophytic mycobiota of *Pasania edulis*, one of the dominant canopy trees, was surveyed in the same region, *Phomopsis* spp. and Ascomycete sp. 1 were isolated frequently from *P. edulis*, whereas *Cytosphaera* sp. was not isolated. Xylariaceae sp. 1 was also isolated from *P. edulis*, but the isolation frequency was much higher in *N. sericea* in the present study. Fungi such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Phyllosticta* sp., among the dominant endophytes in *P. edulis* in Hata et al. (2002), were also isolated from *N. sericea* in this study, but at low frequency. Thus, endophytic mycobiota of *P. edulis* and *N. sericea* were partly common and partly different. On the other hand, there is only limited information on the endophytes in *Cr. japonica* considering the importance of the tree. Akiba et al. (2005) reported two species, *Plectosphaera cryptomeriae* (Hara) Kobayashi and *Phyllosticta cryptomeriae* Kawamura, as dominant endophytes in healthy needles of *Cr. japonica* in Kyushu district, both of which were absent or at least rare (even when the latter was hypothesized to be identical to *Phyllostica* sp.) in *N. sericea*. Thus, the endophytic mycobiota of the canopy layer seems different between artificial coniferous forest and broadleaf forest of southern Kyushu in which *Cr. japonica* and *P. edulis*, respectively, are dominant. However, such differences had only a small effect on the endophytic mycobiota in *N. sericea* in the understory layer.

Arnold and Herre (2003) showed that endophytes colonized leaves of *Theobroma cacao* L., which is an understory plant, more rapidly beneath the forest canopy than in cleared sites. Thus, although the effect of canopy vegetation was not clear in the present study, the presence of the canopy layer itself seems to have significant effects on endophyte colonization in understory plants. Effects of canopy layer on the endophytes colonizing in understory plants may include physical and biological factors. The former include effects by canopy cover such as protection for understory plants from the outer environment and maintenance of humidity, thus favoring endophyte propagules around such plants. Petrini (1991) pointed out that "higher colonization rates by endophytic fungi can be observed for samples from homogeneous stands with a closed canopy," which may also be attributed to similar physical effects of the canopy. The latter include effects from the differences in inoculum sources of the endophytes. For example, in the litter layer of the stand, endophyte propagules are probably different, depending on stands with different canopy vegetation. However, such differences of propagules caused by species composition of canopy trees are likely to have only small effects on the occurrence of endophytes in understory plants in cases such as the present study. Consequently, it is considered that the canopy layer has mainly physical effects on endophytic assemblages of understory plants in such cases.

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