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FULL PAPER

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Extensive host range of an endophytic fungus, *Guignardia endophyllicola* (anamorph: *Phyllosticta capitalensis*)

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Abstract Isolation of endophytic species of *Guignardia* (anamorph: *Phyllosticta*) from healthy leaves of 94 plants (91 species and 3 varieties) in 69 genera, 42 families, was carried out in a test site (Kyoto Herbal Garden) to investigate the host range of *Guignardia endophyllicola* (anamorph: *Phyllosticta capitalensis*). Species of *Guignardia* and *Phyllosticta* were isolated from the leaves of 67 plants (66 species and 1 variety) belonging to 54 genera, 38 families. Among them, 53 isolates from different plants belonging to 43 genera in 36 families were similar in morphology, and sequence analysis of internal transcribed spacer (ITS) regions of ribosome DNA revealed these isolates to be conspecific with *G. endophyllicola*. In addition, this fungus was isolated from leaves of various plants collected in different places in Japan and Thailand. Thus, this endophytic fungus has been revealed to live within various vascular plants, angiosperms, gymnosperms, and pteridophytes.

Key words Endophytic fungus · *Guignardia endophyllicola* · Host range · *Phyllosticta capitalensis*

Introduction

Guignardia endophyllicola Okane, Nakagiri et Tad. Ito has been described as one of major endophytic fungi of ericaceous plants (Okane et al. 2001). Morphology and sequence analysis of ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2, including 5.8S rDNA) revealed this fungus to coincide with *Phyllosticta capitalensis* P. Henn. found from *Stanhopea* sp., Orchidaceae (Hennings 1908). The fungus has been known to be parasitic on orchids (van der Aa 1973). Although *P. capitalensis* is not recorded in a census lists of plant pathogens in Japan (Phytopathological Society of Japan 2000), its synonymous species, *Phyllostictina pyriformis* Cash & Watson, was found from orchids, *Cypripedium* sp. (Cash and Watson 1955) and *Dendrobium moniliforme* (L.) Sw. (Hino and Katumoto 1957), collected in Japan. Species of *Phyllosticta* have been considered to be weak and restrictive parasites on host plants (van der Aa 1973; Petrini et al. 1991). However, a discovery by Okane et al. (2001) that *G. endophyllicola* is harbored in phylogenetically quite different host plants, Ericaceae (Dicotyledones) and Orchidaceae (Monocotyledones), suggested that its host range is considerably wide.

As an example of endophytic fungus living within various vascular plants, *Hypoxylon fragiforme* (Pers.: Fr.) Kickx has been known (Petrini and Petrini 1985). This fungus, which has been known as a saprobe of dead angiosperms, especially *Fagus*, was subsequently found to be harbored by not only *Fagus* but also other plants of several different families, i.e., Araceae, Bromeliaceae, Coniferae, Ericaceae, Orchidaceae, Poaceae, and Pteridophyta, as their endophyte. Thus, endophytes include fungi with an extremely wide host range, which may be important in considering host–parasite coevolutions. *Guignardia endophyllicola* may be another example of such an endophyte with an extensive host range.

In this study, we first carried out isolation of *G. endophyllicola* from healthy leaves of various vascular plants in a single test site (Kyoto Herbal Garden) to clarify its range of host plants. Second, to study geographical distribution of this endophytic fungus, we examined endophytes of leaves from various sites in Japan and Thailand.

Baayen et al. (2002) has reported nonpathogenic isolates of the citrus black spot fungus *Guignardia citricarpa* Kiely

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and identified it with *Guignardia mangiferae* A.J. Roy. They showed this fungus to be conspecific with *G. endophyllicola* (*P. capitalensis*) and proposed the latter teleomorphic species to be synonymized. In this article, however, we accept *G. endophyllicola* and discuss its taxonomy.

Materials and methods

Fungal isolation

Healthy leaves of various plants were used to isolate *Guignardia* and *Phyllosticta* species. In Kyoto Herbal Garden, Takeda Chemical Industries Ltd., the leaves of 94 plants (91 species and 3 varieties) in 69 genera, 42 families, were examined (Table 1). Leaves of various plants collected from other sites in Japan and Thailand were also examined.

The leaves tested were immersed in 70% ethanol solution for 1 min, sodium hypochlorite solution (1% available chlorine) for 2 min, rinsed in sterile distilled water, and blotted dry in sterile paper towels for 3 h. After sterilization and drying, the leaves were divided into several segments, and then they were placed on the surface of half-strength malt extract agar medium in plates. In the test for halophilic plant materials, cornmeal seawater agar (CMSWA, commercial cornmeal agar; Nissui, Tokyo, Japan) dissolved in 15 ppt salinity seawater (Jamarin S; Jamarin Lab., Osaka, Japan) was used. The plates were incubated at 17°C for 2 months. The mycelia growing from leaf segments and spores formed on and around the segment were isolated and cultured.

Table 1. List of plants examined in Kyoto Herbal Garden and the result of the isolation of *Guignardia* (*Phyllosticta*) species

Species	Family	*Guignardia/Phyllosticta*	*G. endophyllicola*
Equisetum hyemale L.	Equisetaceae	Undetected	Undetected
Chamaecyparis obtusa Sieb. & Zucc.	Cupressaceae	Undetected	Undetected
Juniperus chinensis L. var. *procumbens* (Sieb.) Endl.	Cupressaceae	Undetected	Undetected
J. virginiana L.	Cupressaceae	Undetected	Undetected
Thujopsis dolabrata Sieb. & Zucc.	Cupressaceae	Undetected	Undetected
T. dolabrata var. *hondai* Makino	Cupressaceae	Isolated	Isolated
Pinus densiflora Sieb. & Zucc.	Pinaceae	Undetected	Undetected
P. thunbergii Parlat.	Pinaceae	Undetected	Undetected
Tsuga sieboldii Carr.	Pinaceae	Isolated	Isolated
Podocarpus macrophyllus (Thunb.) D. Don	Podocarpaceae	Isolated	Isolated
P. nagi (Thunb.) Zoll. & Moritz.	Podocarpaceae	Isolated	Another species
Taxus cuspidata Sieb. & Zucc.	Taxaceae	Undetected	Undetected
Acer rubrum L.	Aceraceae	Isolated	Isolated
Cotinus coggygria Scop. var. *cinerea* Engl.	Anacardiaceae	Isolated	Isolated
Ilex integra Thunb.	Aquifoliaceae	Undetected	Undetected
I. rotunda Thunb.	Aquifoliaceae	Isolated	Isolated
I. serrata Thunb.	Aquifoliaceae	Isolated	Isolated
Berberis thunbergii DC.	Berberidaceae	Isolated	Isolated
Nandina domestica Thunb.	Berberidaceae	Isolated	Isolated
N. domestica var. *leucocarpa* Makino	Berberidaceae	Isolated	Isolated
Alnus serrulatoides Call.	Betulaceae	Isolated	Isolated
Corylus sieboldiana Blume	Betulaceae	Isolated	Isolated
Lonicera morrowii A. Gray	Caprifoliaceae	Isolated	Isolated
Sambucus nigra L.	Caprifoliaceae	Isolated	Isolated
Euonymus alatus (Thunb.) Sieb.	Celastraceae	Isolated	Isolated
Clethra barbinervis Sieb. & Zucc.	Clethraceae	Isolated	Isolated
Coriaria terminalis Hemsl.	Coriariaceae	Isolated	Isolated
Aucuba japonica Thunb.	Cornaceae	Undetected	Undetected
Cornus kousa Buerger ex Hance	Cornaceae	Isolated	Isolated
Daphniphyllum macropodum Miq.	Daphniphyllaceae	Undetected	Undetected
D. teijsmannii Zoll. ex Kurz	Daphniphyllaceae	Isolated	Isolated
Davidia involucrata Baill.	Davidiaceae	Isolated	Isolated
Leucothoe grayana Maxim.	Ericaceae	Isolated	NT
Rhododendron ponticum L.	Ericaceae	Undetected	Undetected
Vaccinium bracteatum Thunb.	Ericaceae	Isolated	Another species
V. oldhamii Miq.	Ericaceae	Isolated	NT
Eucommia ulmoides Oliv.	Eucommiaceae	Undetected	Undetected
Securinega suffrutiosa (Pallas) Rehd. var. *japonica* Hurusawa	Euphorbiaceae	Isolated	NT
Fagus crenata Blume	Fagaceae	Isolated	Isolated
F. japonica Maxim.	Fagaceae	Isolated	Isolated
Lithocarpus edulis (Makino) Nakai	Fagaceae	Undetected	Undetected
Quercus dentata Thunb. ex Murray	Fagaceae	Isolated	Isolated
Q. variabilis Blume	Fagaceae	Isolated	Isolated
Hypericum androsaemum L.	Guttiferae	Isolated	Isolated
Corylopsis sinensis Hemsl.	Hamamelidaceae	Isolated	Isolated
Illicium anisatum L.	Illiciaceae	Undetected	Undetected
Juglans mandshurica Maxim.	Juglandaceae	Isolated	Isolated

Table 1. Continued

Species	Family	*Guignardia/Phyllosticta*	*G. endophyllicola*
Cinnamomum camphora (L.) Presl	Lauraceae	Isolated	Isolated
C. sieboldii Meissn.	Lauraceae	Undetected	Undetected
Laurus nobilis L.	Lauraceae	Isolated	Another species
Lindera strychnifolia (Sieb. & Zucc.) F. Vill.	Lauraceae	Isolated	Isolated
L. umbellata Thunb.	Lauraceae	Isolated	Isolated
Machilus thunbergii Sieb. & Zucc.	Lauraceae	Undetected	Undetected
Sophora japonica L.	Leguminosae	Isolated	Isolated
Magnolia praecocissima Koidz.	Magnoliaceae	Isolated	Isolated
M. salicifolia (Sieb. & Zucc.) Maxim.	Magnoliaceae	Isolated	Isolated
Micheria fuscata Blume	Magnoliaceae	Isolated	Isolated
Morus alba L.	Moraceae	Isolated	Isolated
M. latifolia Poir.	Moraceae	Isolated	Isolated
Myrica rubra Sieb. & Zucc.	Myricaceae	Isolated	Isolated
Chionanthus retusa Lindl. & Paxton	Oleaceae	Isolated	Another species
Forsythia koreana Nakai	Oleaceae	Isolated	Isolated
F. viridissima Lindl.	Oleaceae	Isolated	Isolated
Ligustrum obtusifolium Sieb. & Zucc.	Oleaceae	Undetected	Undetected
L. quihoui Carr.	Oleaceae	Undetected	Undetected
L. tschonoskii Decne.	Oleaceae	Isolated	Another species
Olea europaea L.	Oleaceae	Undetected	Undetected
Osmanthus heterophyllus (G. Don) P. S. Green	Oleaceae	Isolated	Another species
Punica granatum L.	Punicaceae	Isolated	Isolated
P. granatum var. *nana* Pers.	Punicaceae	Isolated	Isolated
Eriobotrya japonica (Thunb.) Lindl.	Rosaceae	Undetected	Undetected
Prunus laurocerasus L. var. *angustifolia* Nichols.	Rosaceae	Isolated	Isolated
P. mume (Sieb.) Sieb. & Zucc.	Rosaceae	Isolated	Another species
P. pendula Maxim.	Rosaceae	Undetected	Undetected
Photinia serratifolia (Desf.) Kalkm.	Rosaceae	Isolated	Another species
Sorbus commixta Hedl.	Rosaceae	Isolated	Isolated
Gardenia jasminoides Ellis	Rubiaceae	Undetected	Undetected
G. jasminoides var. *radicans* Makino	Rubiaceae	Isolated	Isolated
Citrus aurantium L.	Rutaceae	Undetected	Undetected
C. natsudaidai Hayata	Rutaceae	Undetected	Undetected
Orixa japonica Thunb.	Rutaceae	Isolated	Isolated
Phellodendron amurense Rupr.	Rutaceae	Isolated	Isolated
Poncirus trifoliata (L.) Rafin.	Rutaceae	Isolated	NT
Koelreuteria paniculata Laxm.	Sapindaceae	Isolated	Isolated
Picrasma quassioides (D. Don) Benn.	Simaroubaceae	Isolated	Isolated
Camellia japonica L.	Theaceae	Undetected	Undetected
C. sasanqua Thunb. ex Murray	Theaceae	Undetected	Undetected
Eurya japonica Thunb.	Theaceae	Isolated	Isolated
Edgeworthia chrysantha Lindl.	Thymelaeaceae	Isolated	Another species
Tilia miqueliana Maxim	Tiliaceae	Isolated	Isolated
Vitex aganus-castus L.	Verbenaceae	Isolated	Isolated
V. cannabifolia Sieb. et Zucc.	Verbenaceae	Isolated	Isolated
Broad leaf tree	Unknown	Isolated	Another species
Smilax china L.	Liliaceae	Isolated	Isolated

NT, isolates that were not examined because of nonpurification

Morphological observations

To observe both ascigerous and conidiogenous states, subcultures were incubated on cornmeal agar (CMA) and potato dextrose agar (PDA) (both media, Nissui) on which autoclaved leaves of *Rhododendron pulchrum* Sweet were set. These materials were incubated at 24°C. Fungal materials were mounted in 1 drop of lactophenol solution on glass slides for light microscopic observation and measurement of their dimensions.

DNA isolation

Fungal strains were incubated for 3–4 weeks at 24°C on a half-strength malt extract medium solution. The mycelium was harvested by vacuum filtration, washed with sterilized distilled water, and frozen at −20°C.

DNA was extracted by the method of Marmur (1961) and Saito and Miura (1963), with some modifications. To extract total genomic DNA, 0.7–1.5g (fresh weight) of mycelium was placed with liquid nitrogen in a mortar and ground with a pestle into a fine powder. The mycelium powder was suspended in 7ml 10mM Tris-HCl–0.1M NaCl–1mM EDTA (TNE) buffer (pH 7.5) and transferred to a 30-ml centrifuge tube. Then, 350μl 10% sodium dodecyl sulfate (SDS) and 70μl proteinase K solution (20mg/ml) were added, and the mixture was incubated at 60°C for 30min. The lysate was extracted with 7ml phenol-chloroform-isoamylalchohol (25:24:1, v/v). The same volume of ice-cold isopropanol was added to the aqueous layer to precipitate DNA. The precipitate was rinsed with 70% ethanol, dried, and dissolved in 900 μl sterile distilled water in a microtube.

The DNA was purified by treatment with 4 μl RNase solution at 37°C for 30 min; then, 40 μl 10% SDS and 4 μl proteinase K solution were added, and the mixture was incubated at 37°C for 1 h. The solution was extracted twice or more with the same volume of phenol-chloroform-isoamylalchohol. The DNA was isopropanol precipitated from aqueous layers in the presence of 60 μl 3 M ammonium acetate, washed in 70% ethanol, dried, and dissolved in 300 μl sterile distilled water. The concentration of DNA solution was measured by using a photometer (Beckman DU-65; Beckman Coulter, Tokyo, Japan). DNA samples having an A260/A280 ratio of approximately 1.8 were used. As another method, a Nucleon PhytoPure DNA extraction kit (Amersham Biosciences, Piscataway, NJ, USA) was also applied according to the manufacturer's instructions.

Sequence analysis of the internal transcribed spacer regions (ITS1 and ITS2, including 5.8S rDNA)

The ITS regions were amplified by polymerase chain reaction (PCR) using TaKaRa Taq (TaKaRa Shuzo, Kyoto, Japan) as a single fragment with the standard primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), or Ge-F (5′-GAGCCGGAAAGTTCGTCAAA-3′) and Ge-R (5′-CGCTTCACTCGCCGTTACTG-3′) designed in this study. Amplification of the desired fragment was performed with a Perkin-Elmer GenAmp PCR System 7000 thermal cycler (Perkin-Elmer, Foster City, CA, USA) with the following program: 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, extension for 2 min at 72°C, incubation for 5 min at 72°C, and soaking at 4°C.

Amplified DNA was sequenced with the Applied Biosystems PRISM dye terminator cycle sequencing reaction kit (Perkin-Elmer) in a thermal cycler employing the following ramp: 25 cycles of 15 s at 96°C and 4 min at 55°C, followed by a 4°C soak. Nucleotide sequences were determined in both directions using the primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGGAGC-3′), ITS4, and ITS5 (White et al. 1990), or Ge-F and Ge-R were employed in place of the latter two primers. Sequences were analyzed with an Applied Biosystems PRISMTM 310 Genetic Analyzer. The CLUSTAL W ver. 1.7 software (Thompson et al. 1994) package was used to generate the evolutionary distances (the K_{nuc} value of Kimura; Kimura 1980), the similarity values, the neighbor-joining (NJ) analysis (Saitou and Nei 1987) from K_{nuc} values, and the bootstrap resampling method of Felsenstein (1985) with 1000 replicates for evaluation of the topology of the phylogenetic tree. The NJ plot (Perrière and Gouy 1996) was used for plotting the phylogenetic tree.

Two isolates named *Guignardia philoprina* (Berkeley & Curtis) van der Aa NBRC 32908 and *G. laricina* (Sawada) Yamamoto & K. Ito NBRC 7888 were specified as an outgroup because they have been found to be neighboring the *Guignardia* and *Phyllosticta* clade by sequence analysis of 18S rDNA (unpublished data). However, taxonomy of these two strains is disputed.

In this study, 97 isolates were examined, and as other operational taxonomic units (OTUs), sequence data of ITS regions of 19 known species deposited in the DDBJ/EMBL/GenBank nucleotide sequence database were employed. Among them, 8 strains were analyzed in this study; accession numbers are shown in Table 2.

Table 2. Investigated strains of known species of *Guignardia* and *Phyllosticta*

Species	Host plant	Strain no.[b]	Accession no.
G. aesculi (Peck) Steward	*Aesculus hippocastanum*	NBRC 32905	AB095504
G. bidwellii (Ellis) Viala & Ravas	*Parthenocissus tricuspodata*	NBRC 32906	AB095505
G. citricarpa Kiely	*Citrus aurantium*	IMI 304799	AY042917, AY042918
G. endophyllicola Okane et al.	*Rhododendron phulchrum* cv. ohmurasaki	NBRC 32119	AB041233
G. gaultheriae van der Aa	*Gaultheria humifusa*	NBRC 32907	AB095506
G. laricina (Sawada) Yamamoto & K. Ito[a]	Unknown	NBRC 7888	AB041245
G. philoprina (Berk. & Curtis) van der Aa	*Cryptomeria japonica*	NBRC 32909	AB095507
G. philoprina	*Taxus baccata*	CBS 447.68	AF312014
G. philoprina[a]	*Rhododendron* sp.	NBRC 32908	AB041243
G. vaccinii Shear	*Oxycoccus macrocarpos*	NBRC 32911	AB095508
P. ampelicida (Engel.) van der Aa	*Parthenocissus tricuspodata*	NBRC 9466	AB095509
P. ampelicida	*Parthenocissus tricuspodata*	NBRC 9757	AB095510
P. ampelicida	*Parthenocissus tricuspodata*	NBRC 9903	AB095511
P. beaumarisii A. R. Paul & Blackburn	*Muehlenbeckia adpressa*	CBS 535.87	AY042927, AY042928
P. eugeniae Young	*Eugenia aromatica*	CBS 445.82	AY042925, AY042926
P. hypoglossi (Montagne) Allescher	*Ruscus aculeatus*	CBS 434.92	AY042923, AY042924
P. pyrolae Ellis & Everhart	*Erica carnea*	NBRC 32652	AB041242
P. spinarum (Diedicke) Nag Raj	Unknown	IMI 070028	AY042907, AY042908
P. telopeae Yip	*Telopea speciosissima*	DAR 60749	AY042909, AY042910

[a] These two strains were employed as outgroups, but their taxonomy is disputed
[b] NBRC is an acronym of the corresponding author's organization; IFO (Institute for Fermentation, Osaka) is the former acronym

Results

Kyoto Herbal Garden

Among 94 plant species collected in the Kyoto Herbal Garden, species of *Guignardia* and *Phyllosticta* were isolated from leaves of 67 plants (66 species and 1 variety) belonging to 54 genera in 38 families (including 1 isolate from an unidentified broadleaf tree) (see Table 1). *Phyllosticta* and its teleomorphic *Guignardia* species were detected from plants in the garden with high frequency, approximately 70%. Most of 63 isolates (4 unpurified isolates were omitted) were indistinguishable in colony appearance and morphology. Among them, 27 isolates were holomorphic strains that form ascigerous and conidiogenous states in culture, although 7 were teleomorphic strains and 29 were anamorphic strains (Table 3). They grew slowly on media; the colonies were greenish-gray, becoming near black with abundant submerged mycelium, and their margin was smooth to undulate. Morphological data of each isolate are shown in Table 3.

A sequence analysis based on the ITS regions showed that 53 isolates from the same number of different plants belonging to 43 genera in 36 families were identified as *G. endophyllicola* (see Tables 1, 3). Because no significant difference in ITS sequence data was found between these 53 isolates and the 9 strains of *G. endophyllicola*, which had been previously deposited by the authors (Okane et al. 2001) in the DDBJ/EMBL/GenBank nucleotide sequence database (accession no. AB041233–AB041241), we did not deposit additional sequence data to the database here. The NJ tree revealed a clade consisting of the present isolates and *G. endophyllicola* [NBRC 33119 (ex-type strain), AB041233], and this clade is clearly distinguished from other isolates obtained in this study and other species (Fig. 1). Although some branches were not supported by high confidence limits from a bootstrap analysis, the *G. endophyllicola* clade was supported by over 95% in any rearrangement of OTUs. In this analysis, 1 isolate from an unidentified broadleaf tree (isolate no. 89) was excluded because of reduction of the informative data.

Among the 10 isolates (isolate no. 80 to 89 in Table 3), which were distinguished by the sequence analysis from 53 isolates of *G. endophyllicola* (Fig. 1), some isolates clearly differ from *G. endophyllicola* (*P. capitalensis*) in having longer appendages of conidia [isolates from *Osmanthus hetetophyllus* (no. 84), *Podocarpus nagi* (no. 85), and *Prumus mume* (no. 86)], small conidia [isolates from *Edgeworthia chrysantha* (no. 88), *Vaccinium bracteatum* (no. 80)], and forming compact colony [isolates from *P. nagi* (no. 85), *P. mume* (no. 86), and *V. bracteatum* (no. 80)]. Ascigerous states of these 10 isolates have not been observed on the media used.

Other sites

Besides the isolates from Kyoto Herbal Garden, 26 isolates morphologically similar to *G. endophyllicola* were found from various plants (20 species, 15 genera) collected in Toyama, Osaka, Hiroshima, Kagoshima, Okinawa Prefectures, and Thailand (Table 4). These isolates were also revealed to be conspecific with *G. endophyllicola* by morphology and ITS sequence analysis (see Fig. 1). We investigated 10 isolates (8 from Zingiberaceae, 1 from Rosaceae, and 1 from Musaceae) from Thailand and found 9 isolates to be identical with *G. endophyllicola* (Table 4, Fig. 1). Isolates from *Prunus cerasoides*, which is used as a medicinal plant in Thailand, and *Musa acuminata* produced both teleomorphic and anamorphic states.

Eighteen isolates distinguished from *G. endophyllicola* in Kyoto and the other sites (Table 5) need rigorous taxonomic study for its species-level identification.

Discussion

Endophytic fungi belonging to *Guignardia* and *Phyllosticta* were found from 87 plants in Japan (63 from Kyoto Herbal Garden and 24 from the other sites), and 70 isolates of them were identified as *G. endophyllicola* in morphology and sequence analysis of ITS regions. As the genus *Phyllosticta* has been mentioned as the quintessential endophyte genus (Carroll 1990), they were detected in high frequency.

Although the data are not shown here, 49 of 87 domestic plants from which the fungi were isolated have not been reported as the host plants of *Guignardia* and *Phyllosticta* species according to a census list of plant pathogens, i.e., "Common names of plant disease in Japan" (Phytopathological Society of Japan 2000). Among them, 40 plants were found to harbor *G. endophyllicola*. According to a report of plant pathogenic fungi in the United States (Farr et al. 1989), 11 plants examined here have not been reported as the host of *Guignardia* and *Phyllosticta* species in the United States. Species of *Phyllosticta* have been found from numerous plants, but those developing teleomorphic *Guignardia* species have rarely reported from most of the same plants examined in this study. Although these fungi reported in the United States need to be compared with *G. endophyllicola*, these facts suggest that endophytic fungi such as *G. endophyllicola* are quiescent inside tissues of a very large number of plant species without causing apparent harm to them.

Guignardia endophyllicola was isolated in high frequency and revealed to be one of the major endophytes of several ericaceous plants (Okane et al. 1998, 2001). In the present study, this fungus was often detected from healthy leaves of monocotyledonous plants, i.e., *Arundina chinensis* (Orchidaceae) and another orchid collected in Iriomote Is., Okinawa Pref., *Smilax china* (Liliaceae) in Kyoto, and *Musa acuminata* and eight zingiberaceous plants in Thailand. *Phyllosticta capitalensis* had been known to be specifically parasitic on orchids (van der Aa 1973). Two strains of *P. capitalensis* (NBRC 32914 and NBRC 33062), which had been used for taxonomic study on *G. endophyllicola* found from ericaceous plants (Okane et al. 2001), were those also isolated from orchids in Germany and New Zealand.

Table 3. Isolates of *Guignardia* (*Phyllosticta*) species found from plants in Kyoto Herbal Garden and their morphology[a]

Isolate no.	Host plant	Family	Morphology State	(μm) (mean in parentheses)[b] Asci	Ascospores	Conidia	Appendages
1	*Thujopsis dolabrata* var. *hondai*	Cupressaceae	Holomorph	51–75 × 11–13 (60.0 × 12.2)	11–17 × 3–6 (14.6 × 5.1)	9–11 × 5–7 (10.0 × 6.4)	3–9 (5.2)
2	*Tsuga sieboldii*	Pinaceae	Anamorph			10–12 × 5–8 (10.7 × 6.3)	2–7 (4.8)
3	*Podocarpus macrophyllus*	Podocarpaceae	Holomorph	NT	NT	9–11 × 5–8 (10.3 × 6.6)	3–10 (5.3)
4	*Acer rubrum*	Aceraceae	Anamorph			9–15 × 6–8 (11.0 × 6.4)	4–15 (7.3)
5	*Cotinus coggygria* var. *cinerea*	Anacardiaceae	Anamorph			10–13 × 5–8 (11.2 × 6.3)	5–13 (8.2)
6	*Ilex rotunda*	Aquifoliaceae	Anamorph			9–12 × 4–6 (10.4 × 5.4)	5–8 (6.1)
7	*I. serrata*	Aquifoliaceae	Holomorph	45–67 × 11–16 (56.8 × 13.7)	12–15 × 4–5 (13.8 × 4.3)	9–12 × 4–6 (10.2 × 5.2)	5–10 (7.6)
8	*Berberis thunbergii*	Berberidaceae	Anamorph			10–13 × 5–8 (11.2 × 6.1)	4–8 (6.0)
9	*Nandina domestica*	Berberidaceae	Holomorph	NT	NT	9–13 × 5–8 (10.5 × 6.2)	5–10 (7.9)
10	*N. domestica* var. *leucocarpa*	Berberidaceae	Anamorph			9–11 × 6–8 (10.1 × 6.7)	2–5 (4.2)
11	*Alnus serrulatoides*	Betulaceae	Anamorph			10–13 × 6–7 (11.7 × 6.6)	2–13 (5.1)
12	*Corylus sieboldiana*	Betulaceae	Holomorph	53–82 × 11–14 (68.5 × 11.9)	12–17 × 4–6 (14.6 × 5.1)	9–13 × 5–7 (10.6 × 6.1)	4–11 (6.5)
13	*Lonicera morrowii*	Caprifoliaceae	Holomorph	47–69 × 10–14 (58.0 × 12.1)	13–15 × 3–5 (14.4 × 4.5)	9–12 × 5–8 (10.2 × 6.1)	2–9 (5.2)
14	*Sambucus nigra*	Caprifoliaceae	Teleomorph	45–76 × 11–14 (60.3 × 12.4)	14–17 × 4–6 (15.4 × 5.2)		
15	*Euonymus alatus*	Celastraceae	Anamorph			9–13 × 6–10 (10.5 × 7.3)	4–10 (6.7)
16	*Clethra barbinervis*	Clethraceae	Teleomorph	53–55 × 13–14 (53.8 × 13.3)	14–15 × 4–5 (14.3 × 4.7)		
17	*Coriaria terminalis*	Coriariaceae	Holomorph	49–73 × 10–15 (59.1 × 12.3)	13–17 × 4–7 (15.0 × 5.5)	NT	NT
18	*Cornus kousa*	Cornaceae	Holomorph	NT	NT	10–13 × 6–9 (11.6 × 7.7)	3–11 (6.5)
19	*Davidia involucrata*	Davidiaceae	Anamorph			9–13 × 6–8 (11.1 × 7.0)	2–4 (2.8)
20	*Daphniphyllum teijsmannii*	Daphniphyllaceae	Holomorph	47–76 × 10–15 (55.6 × 12.3)	12–16 × 5–7 (14.7 × 6.0)	9–13 × 4–7 (10.5 × 5.8)	1–4 (1.3)
21	*Fagus crenata*	Fagaceae	Holomorph	44–68 × 9–13 (53.2 × 11.8)	13–17 × 5–7 (15.2 × 6.0)	9–13 × 6–7 (10.6 × 6.8)	4–8 (5.8)
22	*F. japonica*	Fagaceae	Teleomorph	48–70 × 11–14 (60.6 × 12.4)	13–18 × 3–6 (16.0 × 4.7)		
23	*Quercus dentata*	Fagaceae	Teleomorph	NT	NT		
24	*Q. variabilis*	Fagaceae	Teleomorph	59–81 × 9–14 (66.1 × 11.3)	14–16 × 5–7 (14.9 × 5.4)		
25	*Hypericum androsaemum*	Guttiferae	Holomorph	58–83 × 13–16 (69.4 × 13.7)	14–16 × 5–6 (15.0 × 5.7)	10–12 × 5–6 (10.8 × 5.9)	2–3 (2.1)
26	*Corylopsis sinensis*	Hamamelidaceae	Holomorph	45–66 × 10–16 (52.8 × 11.9)	11–16 × 4–6 (13.7 × 5.0)	9–11 × 6–8 (9.9 × 6.5)	
27	*Juglans mandshurica*	Juglandaceae	Holomorph	53–97 × 11–14 (69.5 × 12.6)	13–17 × 4–7 (14.9 × 5.2)	9–12 × 5–7 (10.4 × 6.1)	3–13 (5.9)
28	*Cinnamomum camphora*	Lauraceae	Holomorph	49–82 × 11–15 (58.1 × 12.5)	12–16 × 4–6 (13.9 × 4.9)	NT	NT
29	*Lindera strychnifolia*	Lauraceae	Holomorph	42–68 × 11–14 (54.1 × 12.7)	12–14 × 4–5 (12.5 × 4.5)	NT	NT
30	*L. umbellata*	Lauraceae	Anamorph			9–10 × 5–8 (9.5 × 6.2)	4–5 (4.6)
31	*Sophora japonica*	Leguminosae	Holomorph	43–50 × 12–13 (46.9 × 12.5)	13–15 × 5–6 (14.0 × 5.2)	NT	NT
32	*Magnolia praecocissima*	Magnoliaceae	Anamorph			10–12 × 6 (10.6 × 6.3)	4–8 (5.3)

33	*M. salicifolia*	Magnoliaceae	Holomorph	45–67 × 11–16 (58.5 × 12.7)	12–16 × 4–6 (14.3 × 5.2)	9–12 × 5–8 (10.2 × 6.6)	3–7 (4.9)
34	*Micheria fuscata*	Magnoliaceae	Anamorph			10–12 × 5–6 (10.5 × 5.9)	5–6 (5.2)
35	*Morus alba*	Moraceae	Holomorph	60–68 × 13–15 (63.9 × 14.2)	12–16 × 3–5 (13.7 × 3.9)	9–13 × 6–7 (10.8 × 6.3)	5–9 (6.4)
36	*M. latifolia*	Moraceae	Holomorph	38–55 × 9–13 (46.9 × 11.5)	11–15 × 4–5 (13.3 × 4.5)	9–11 × 5–8 (9.8 × 6.2)	5–6 (5.5)
37	*Myrica rubra*	Myricaceae	Anamorph			8–11 × 5–6 (9.9 × 6.1)	3–8 (5.2)
38	*Forsythia koreana*	Oleaceae	Teleomorph	60–73 × 13–15 (64.4 × 12.9)	13–15 × 5–6 (14.3 × 5.4)		
39	*F. viridissima*	Oleaceae	Anamorph			10–13 × 6–8 (11.5 × 7.1)	4–8 (5.1)
40	*Punica granatum*	Punicaceae	Holomorph	52–81 × 10–14 (67.0 × 12.1)	13–16 × 3–6 (14.7 × 4.5)	10–11 × 5–7 (10.5 × 6.2)	4–16 (6.8)
41	*P. granatum* var. *nana*	Punicaceae	Holomorph	NT	NT	9–13 × 6 (10.4 × 6.3)	3–6 (4.6)
42	*Prunus laurocerasus* var. *angustifolia*	Rosaceae	Holomorph	35–62 × 12–14 (50.7 × 13.1)	12–16 × 3–5 (13.8 × 4.2)	10–11 × 6–7 (10.4 × 6.1)	4–5 (4.3)
43	*Sorbus commixta*	Rosaceae	Anamorph			11–14 × 5–7 (11.9 × 6.0)	4–15 (8.0)
44	*Gardenia jasminoides* var. *radicans*	Rubiaceae	Anamorph, sperm.		10–12 × 6–7 (10.8 × 6.5)		
45	*Phellodendron amurense*	Rutaceae	Anamorph			9–13 × 4–7 (10.8 × 6.3)	2–9 (5.2)
46	*Orixa japonica*	Rutaceae	Holomorph	NT	NT	NT	NT
47	*Koelreuteria paniculata*	Sapindaceae	Holomorph	NT	NT	NT	NT
48	*Picrasma quassioides*	Simaroubaceae	Holomorph, sperm.	56–71 × 12–15 (64.3 × 13.1)	11–16 × 3–5 (13.7 × 4.0)	NT	NT
49	*Eurya japonica*	Theaceae	Holomorph	40–55 × 11–14 (45.3 × 12.2)	12–16 × 4–7 (14.2 × 5.9)	8–12 × 6–7 (10.2 × 6.3)	3–7 (4.5)
50	*Tilia miqueliana*	Tiliaceae	Teleomorph	45–65 × 11–15 (55.1 × 13.1)	14–16 × 5–6 (14.9 × 5.0)		
51	*Vitex aganus-castus*	Verbenaceae	Anamorph			10–14 × 6–8 (12.2 × 6.9)	4–8 (6.1)
52	*V. cannabifolia*	Verbenaceae	Anamorph			10–13 × 5–6 (10.9 × 5.6)	4–8 (5.7)
53	*Smilax china*	Liliaceae	Holomorph	41–64 × 12–16 (54.9 × 13.6)	12–16 × 5–7 (14.2 × 6.0)	9–12 × 5–8 (10.8 × 6.8)	4–10 (6.2)
80	*Vaccinium bracteatum*	Ericaceae	Anamorph			8–10 × 5–6 (8.5 × 6.0)	6–12 (8.3)
81	*Laurus nobilis*	Lauraceae	Anamorph			9–11 × 5–6 (9.7 × 5.6)	4–13 (7.3)
82	*Chionanthus retusa*	Oleaceae	Anamorph			10–13 × 6–8 (11.8 × 7.0)	5–8 (6.6)
83	*Ligustrum tschonoskii*	Oleaceae	Anamorph			8–11 × 6–8 (9.7 × 6.9)	3–10 (6.2)
84	*Osmanthus heterophyllus*	Oleaceae	Anamorph			9–12 × 6–8 (11.0 × 6.9)	6–22 (10.8)
85	*Podocarpus nagi*	Podocarpaceae	Anamorph			10–14 × 6–8 (11.8 × 7.2)	10–33 (18.4)
86	*Prunus mume*	Rosaceae	Anamorph			10–14 × 5–8 (11.6 × 6.9)	5–22 (9.1)
87	*Photinia serratifolia*	Rosaceae	Anamorph			NT	NT
88	*Edgeworthia chrysantha*	Thymelaeaceae	Anamorph			7–11 × 5–7 (9.2 × 5.9)	4–13 (7.7)
89	Unidentified broad leaf tree	Unknown	Anamorph			10–13 × 7–9 (11.3 × 7.6)	2–4 (2.7)

[a] Isolates 1–53 are those proved to be *G. endophyllicola*; isolates 80–89 are those proved to differ from *G. endophyllicola*

[b] State observed is noted. For all states, isolates that could not be measured because of insufficient materials are marked NT

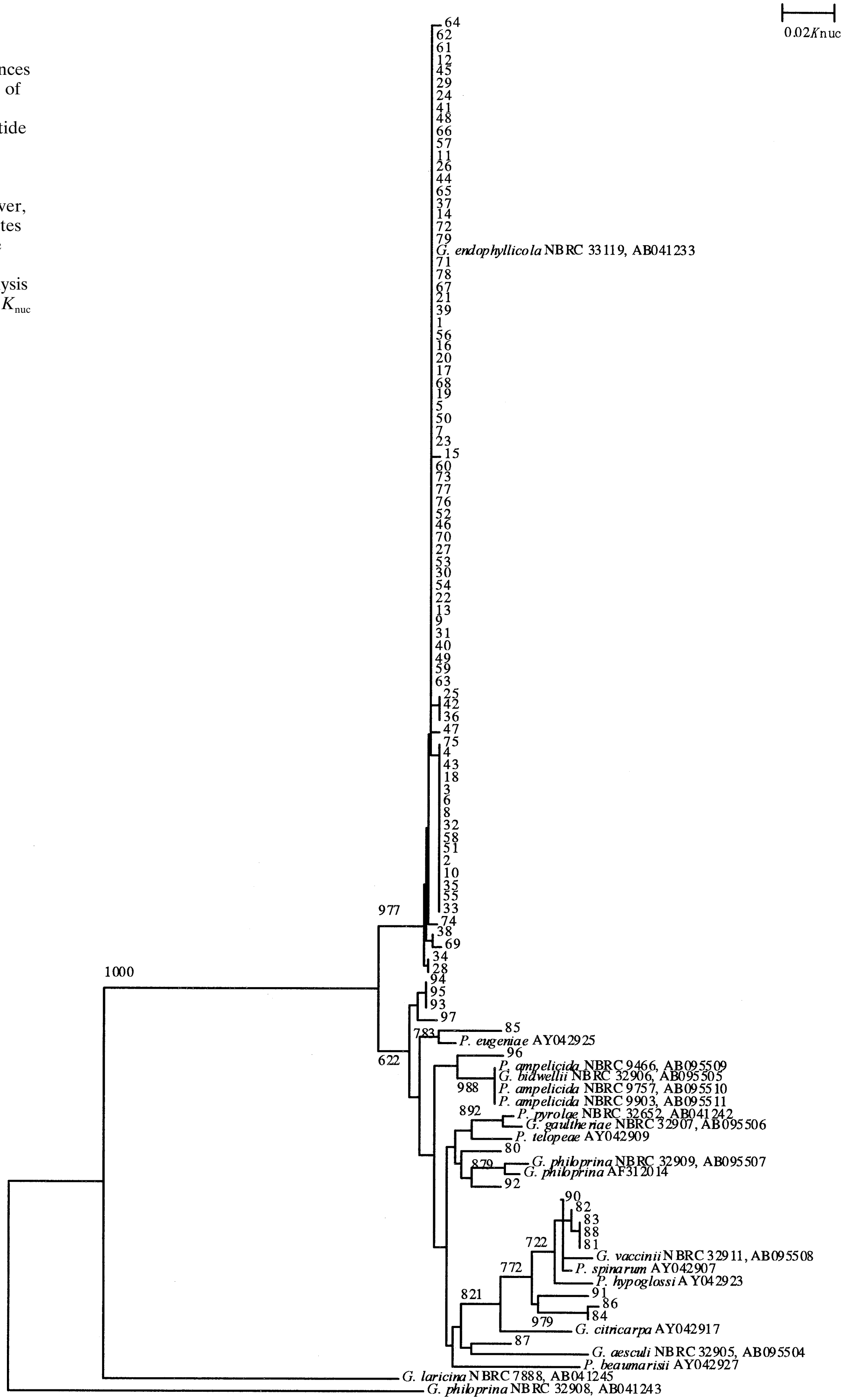

Fig. 1. Neighbor-joining tree derived from the internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA, ITS2) sequences of isolates examined and strains of known species deposited in the DDBJ/EMBL/GenBank nucleotide sequence database. *Guignardia laricina* NBRC 7888 and *G. philoprina* NBRC 32908 were specified as the outgroup; however, the taxonomy of these two isolates is disputed. The *numbers* on the branches are confidence limits estimated from a bootstrap analysis with 1000 replications. *Bar* 0.01 K_{nuc} in nucleotide sequences

Table 4. Isolates of *G. endophyllicola* found from plants in sites other than Kyoto Herbal Garden

Isolate no.	Host plant	Family	Site
54	*Rhododendron indicum* (L.) Sweet	Ericaceae	Takaoka, Toyama
55	*R. indicum*	Ericaceae	Ikeda, Osaka
56	Pteridophyte	Pteridophta	Mt. Hiba, Hiroshima
57	*R. indicum*	Ericaceae	Mt. Hiba, Hiroshima
58	*Zanthoxylum armatum* DC. *subtrifoliatum* (Franch.) Kitamura	Rutaceae	Mt. Hiba, Hiroshima
59	*Ilex rotunda*	Aquifoliaceae	Amami Is., Kagoshima
60	*Cinnamomum japonicum* Sieb. ex Nakai	Lauraceae	Amami Is., Kagoshima
61	*Caesalpinia crista* L.	Leguminosae	Amami Is., Kagoshima
62	*Kandelia candel* (L.) Druce	Rhizophoraceae	Amami Is., Kagoshima
63	*Rubus croceacanthus* Leveille	Rosaceae	Amami Is., Kagoshima
64	*Rhododendron latoucheae* Franch.	Ericaceae	Ishigaki Is., Okinawa
65	*Cerbera manghas* L.	Apocynaceae	Iriomote Is., Okinawa
66	*R. simsii* Planch.	Ericaceae	Iriomote Is., Okinawa
67	*R. simsii*	Ericaceae	Iriomote Is., Okinawa
68	*C. camphora*	Lauraceae	Iriomote Is., Okinawa
69	*Arundina gramminifolia* (Don) Hochr.	Orchidaceae	Iriomote Is., Okinawa
70	Orchid	Orchidaceae	Iriomote Is., Okinawa
71	*Amomum siamense* Criab.	Zingiberaceae	Thailand
72	*A. siamense*	Zingiberaceae	Thailand
73	*Zingiber officinales* (Willd.) Rosc.	Zingiberaceae	Thailand
74	*Z. officinales*	Zingiberaceae	Thailand
75	*Amomum uliginosum* J.G. Konig ex Retz.	Zingiberaceae	Thailand
76	*A. uliginosum*	Zingiberaceae	Thailand
77	*Amomum* sp.	Zingiberaceae	Thailand
78	*Prunus cerasoides* D. Don	Rosaceae	Thailand
79	*Musa acuminata* Colla	Musaceae	Thailand

Table 5. Isolates of *Guignardia* (*Phyllosticta*) species other than *G. endophyllicola*

Isolate no.	Host plant	Family	Site
80	*Vaccinium bracteatum*	Ericaceae	Kyoto
81	*Laurus nobilis*	Lauraveae	Kyoto
82	*Chionanthus retusus*	Oleaceae	Kyoto
83	*Ligustrum compactum* var. *tschonskii*	Oleaceae	Kyoto
84	*Osmanthus heterophyllus*	Oleaceae	Kyoto
85	*Podocarpus nagi*	Podocarpaceae	Kyoto
86	*Prunus mume*	Rosaceae	Kyoto
87	*Photinia serratifolia*	Rosaceae	Kyoto
88	*Edgeworthia chrysatha*	Thymelaeaceae	Kyoto
89	Unidentified broad leaf tree	Unknown	Kyoto
90	*Rododendron dilatatum* Miquel	Ericaceae	Hyogo
91	*Dendropanax trifidus* (Thunb.) Makino	Araliaceae	Amami Is., Kagoshima
92	*Myrsine seguinii* Lev.	Myrsinaceae	Amami Is., Kagoshima
93	*Bruguiera gymnorrhiza* (L.) Lam.	Rhizophoraceae	Amami Is., Kagoshima
94	*Kandelia candel*	Rhizophoraceae	Amami Is., Kagoshima
95	*K. candel*	Rhizophoraceae	Amami Is., Kagoshima
96	*B. gymnorrhiza*	Rhizophoraceae	Iriomote Is., Okinawa
97	*Alpinia malaccensis* (Burm.f.) Roscoe	Zingiberaceae	Thailand

Phyllostictina pyriformis, synonymous with *P. capitalensis*, was found on orchids including *Cypripedium* sp. in Japan (Cash and Watson 1955); subsequently, it has been often found on a leaf of *Dendrobium moniliforme* cultivated in Japan (Hino and Katumoto 1957). However, this fungus is not recognized as a severe pathogen of orchids (Katumoto, personal communication), and in fact this fungus was not recorded in the list (Phytopathological Society of Japan 2000). This finding suggests that orchids and other monocotyledonous plants may also harbor this fungus as an endophyte in the field. In Thailand, there is some possibility that zingiberaceous plants are one of the important hosts of this fungus.

On the other hand, although the colonization frequency of *G. endophyllicola* in each plant was not investigated in this study, the low frequency suggested that this fungus did not colonize preferentially in coniferous plants belonging to Pinaceae and Cupressaceae. Further investigation of the colonization frequency in each host plant may reveal the host preference of *G. endophyllicola*.

Baayen et al. (2002) reported that *G. mangiferae* (including *G. endophyllicola* as a synonym) occurs endophytically on a wide range of woody plants belonging to numerous families. In this study, *G. endophyllicola* was isolated from diverse plants including a pteridophyte collected in Mt. Hiba, Hiroshima Pref., and from some coniferous plants in

Kyoto, i.e., *Thujopsis dolabrata*, *Tsuga sieboldii*, and *Podocarpus macrophyllus*. Thus, this fungus has been revealed to have inoculum potential to diverse vascular plants, pteridophytes, gymnosperms, and angiosperms.

Baayen et al. (2002) studied many isolates from various regions and reported that this fungus was harbored by many woody plants and a cosmopolitan endophyte. In this study, nine isolates from Thailand were found to be conspecific with *G. endophyllicola* (see Table 4, Fig. 1). It is estimated that this fungus is widely distributed in temperate, subtropical, and tropical regions in Asian oceanic regions. *Guignardia endophyllicola*, having diversified host plants and a wide area of distribution, is speculated to situate among primitive species before establishing strict host specificity in the relatives. Further study based on molecular biology is necessary to clarify a phylogeny of the related fungi.

We tried preliminarily to isolate *G. endophyllicola* from healthy leaves of *Rhododendron* spp. (Ericaceae) in the east of Japan (mainly in the Kanto region, middle of the main island of Japan). However, this fungus has not always been detected from the samples collected in several locations in this region. *Phyllosticta capitalensis* has been reported as having a wide distribution, mainly in warmer areas, occurring only on cultivated orchids in glasshouses in the temperate zone of Belgium, Germany, and Netherlands (van der Aa 1973). It is considered that *G. endophyllicola* has not thoroughly dispersed to the cool temperate zone wherein aestatilignosa (temperate deciduous forest) is dominant, or that this fungus is harbored by more suitable host plants other than *Rhododendron* in such regions of Japan. To clarify a geographical distribution of *G. endophyllicola*, we need to conduct investigations on various vascular plants.

Hypoxylon fragiforme has been shown to be harbored by numerous plants (Petrini and Petrini 1985), resembling the present fungus. Petrini (1996) suggested that *H. fragiforme* may change its life cycle (developing teleomorph) according to the host plant, and he called this phenomenon "expression specificity." In the case of *G. endophyllicola*, the ascigerous state had not been reported on isolates from orchids, but many of the isolates from various plants including an orchid from Iriomote Is. developed the ascigerous state in this study. States of each isolates observed are shown in Table 3 with dimensions of ascigerous and conidial states. The spermatial state was also observed in some isolates. Although we did not find expression specificity on *G. endophyllicola*, further studies may reveal the relation between the specialization of parasitism and development of the teleomorph or the pathogenicity to their host plants. These two endophytic species, *H. fragiforme* and the present fungus, being harbored by various host plants, may be key organisms to study the host–parasite interaction and fungal diversity in parasitism, genetics, and other biological subject areas.

We carried out an investigation on the extent of the host range of *G. endophyllicola* by fungal isolation from healthy leaves of various plants. As a result, this fungus has been revealed to live inside tissues of numerous vascular plant species including some pteridophytes. It is probable that *G. endophyllicola* does not cause severe plant disease and forms preferably a symbiotic relationship with numerous plants beyond our expectations, because this fungus was detected from various plants that had not been reported previously as hosts of *Guignardia* and *Phyllosticta* species. Hereafter, studies are required to clarify a host–parasite interaction between this fungus and each host plant with inoculation experiments, because its ecological characteristics, e.g., infection mode, and mutualistic or pathogenic reaction, possibly switch and diversify according to the plant species and their physiological condition.

Taxonomy of the present fungus

Concerning a taxonomic dispute about the present fungus, we consider that both *G. endophyllicola* and an endophytic fungus that was identified with *G. mangiferae* by Baayen et al. (2002) are the same organism, according to their morphology and sequence data of the ITS regions. Baayen et al. (2002) reported the dimensions (in μm) of the endophytic fungus from citrus and other woody plants as follows: ascomata, 250–400 × 175–250; asci, 65–100 × 10–14; ascospores, 15–17.5 × 6.5–7.5; conidia, 9–13.5 × 6–7.5; conidial appendages, 4–10. However, they mentioned nothing about morphological comparison between this endophytic fungus and *G. mangiferae*. No significant differences are found between the morphological data shown by Baayen et al. (2002) and those by the authors (see Table 3) except for ascomata size; i.e., large ascomata were described by the former researchers. On the other hand, in the descriptions of *G. mangiferae* by Roy (1968), Punithalingam (1974), and Sivanesan (1984), the dimensions (μm) of this species are as follows: ascomata, 84–146 × 155–171; asci, 50–65 × 10–13; ascospores, 10–15 × 4–7; conidia, 8–10 × 4–5; conidial appendages, 5–8. Consequently, this endophytic fungus with *P. capitalensis* anamorph seems to produce larger asci, ascospores, and conidia than those of *G. mangiferae*, and to develop the spermogonial state, which was described to be absent in *G. mangiferae* (Roy 1968; Punithalingam 1974; Sivanesan 1984). These are the reason we newly described *G. endophyllicola* as the teleomorphic state of *P. capitalensis* (Okane et al. 2001) and accept the name in this study.

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