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Endophytic Mycobiota in *Aucuba japonica* in Sagami-nada and Its Adjacent Area, Central Japan Based on Molecular Identification

Yousuke Degawa[1], Mikio Nishimura[2], Yumiko Hirayama[3] and Tsuyoshi Hosoya[3]

[1] Laboratory of Mycology, Sugadaira Montane Research Center, University of Tsukuba, 1278–294 Sugadaira Kogen, Ueda-shi, Nagano 386–2204, Japan
E-mail: degawa@sugadaira.tsukuba.ac.jp
[2] Syonandai, Fujisawa-shi, Kanagawa 252–0804, Japan
[3] Department of Botany, National Museum of Nature and Science, 4–1–1 Amakubo, Tsukuba-shi, Ibaraki 305–0005, Japan

Abstract. To elucidate factors affecting the distribution of mycobiota in Sagami sea and its adjacent areas, endophytic mycobiota in *Aucuba japonica* was examined. Leaves of *A. japonica* were collected from seven points in Sagami sea and its adjacent areas, and 141 endophytes were isolated by surface sterilization. Based on ITS-5.8S ribosomal RNA sequence, 36 taxa were recognized. The most frequently detected fungus was *Glomerella cinguilata* (anamorph: *Colletotrichum gloeosporoioides*), followed by *Diaporthe* (anamorph: *Phyllosticta*), sodarialean fungi, xylariaclean fungi. Most of the obtained endophytes belonged to asocmycetes, but few basidiomycetes were included. No clear difference of mycobiota was observed based on part of leaves and isolation media. However, mycobiota were found to be affected by the altitude, suggesting the host condition influenced by the environmental factors surrounding the host.
Key words: altitude, biogeographic distribution, endophyte, environment, fungal diversity.

Introduction

The kingdom Fungi is presently known to embrace some 97,000 species (Kirk *et al*., 2008). However, it is estimated to embrace 1.5 millions of species (Hawksworth, 1991). The Fungi is one of the largest groups of organisms. Due to their diverse ecology, small size of vegetative structures and production of large numbers of propagules, fungi distribute almost everywhere in nature, even in microhabitat. Distribution of fungi is primarily delimited by mode of life (saprophytic, symbiotic, or parasitic). Additionally, host or substrate selectivity (host, habitat, degree of decomposition of the substrates, etc.) are important factors for the distribution of parasitic fungi (Wicklow, 1981). Environmental factors, such as temperature, precipitation, etc. are also important factors.

To elucidate fungal diversity in a given area, conventional collecting based on observations by naked eyes is effective (Rossman *et al*. 1998; Stone *et al*. 2004). However, the procedure is time consuming and costly, and covering all the fungi in the given area may be virtually impossible. More systematic method to estimate mycobiota is required. Detection of mycobiota by isolation from the same substrate using the same method is one of the efficient comparisons. However, several problems arise in such methods.

One of the problems is that identification of the isolate may not be completely possible, because some isolates may not produce spores required for the identification. The problem can be solved by adopting barcoding method using the sequences of the barcoding region. The barcoding method is to distinguish fungal species using the barcoding sequence, namely internal transcribed spacer 1, 2 and 5.8s ribosomal RNA regions (ITS-5.8S) of extracted DNA. By referring the data cumulated in GenBank, it is sometimes possible to identify the given isolate at the specific level. Even if the obtained sequence was not identical with the previously known sequences, it is still possible to

distinguish the isolates to some extent (Stone *et al*., 2004; Seifert, 2009).

Another problem is the concern that all the fungi may not be isolated. Isolation is an artificial process that is affected by isolation media, temperature, sterilization techniques, etc. However, constant results are expected by following the same procedure.

The Sagami Sea and its adjacent areas are known to embrace diverse organisms due to the typical natural environment in central Japan. The authors studied the mycobiota in these areas by conventional method and illustrated remarkable mycobiota (Degawa *et al*., 2006; Hosoya *et al*., 2006). However, to reveal the factors that contribute to fungal community in these areas, more systematic approach is desired.

To delimit the target fungal group, we poured our attention to fungal endophytes. Fungal endophytes here is interpreted in their broad sense: fungi that colonize living plant tissue without causing any immediate, overt negative effect (Hirsch & Braun, 1992). Fungi with parasitic, commensal, or mutualistic relationship to plants are included. A number of studies have been conducted for endophytes of the tree leaves in Japan (e.g. Osono, 2009).

Aucuba japonica (Cornaceae) is one of the well known and widely distributed plants in these areas. It is an evergreen shrub distributed in lowland to mountains mainly in south-west, but almost all over Japan (Satake *et al*., 1989). *Aucuba japonica* is also known to be endemic to Japan, and the genus is endemic to east Asia. In Sagami Sea and its adjacent areas, *A. japonica* is distributed at high frequency from evergreen broadleaf forest in lowland to deciduous forest in Hakone and Tanzawa mountains at > 500 m altitude. *Aucuba japonica* is one of the suitable plants to compare endophytic mycobiota in different environments.

To elucidate the factors affecting mycobiota in Sagami Sea and its adjacent areas, comparisons of the endophytic mycobiota in *A. japonica* in various site with different altitude was conducted.

Materials and Methods

Collection of the materials

The leaves of *A. japonica* were collected in March of 2010 by altering the altitude from the following seven sites (Figs. 1, 2). 1) Manazuru, Manazuru-machi (alt. 5 m). Population of *A. japonica* distributed along the shore, accompanied with evergreen broadleaf forest. The population facing the shore had few leaves, and healthy looking leaves were even more rare. However, more rich populations were found apart from the shore. 2) Shiroyama, Odawara-shi (alt. 50 m). Population of *A. japonica* from the urban area accompanied with evergreen broad leaved trees. The population was rich in healthy looking leaves, but a number of unhealthy looking leaves were also found. 3) Miyashita, Yugawara-machi (alt. 50 m). The population was well preserved because of suburban shrine forest. The population was rich in healthy looking leaves. 4) Hatajuku, Hakone-machi (alt. 560 m). The population was along with walking trail in broad leaved deciduous forest. The majority of the leaves were healthy looking. 5) Miyagami, Yugawara-machi (alt. 550 m). Major population with healthy leaves was accompanied with broad leaved deciduous forest along with Tsubaki line (motor road). 6) Ubako, Hakone-machi, (alt. 880 m). The population with many variegated leaves was accompanied with broad leaved deciduous forest. *Aucuba japonica* occured in clumps. 7) Komagatake, Hakone-machi (alt. 890 m). The population was found at the margin of *Cryptomeria japonica* plantation, along with motor road. The population was minor.

Isolation

In each site, four healthy looking leaves were collected and named A, B, C, and D for practicality. The leaves were washed by running tap water to remove surface contaminants, and cut into three parts (apical, middle, and basal). Each part was immersed in 70% ethanol for 1 min, followed by 2 min. in 1% Sodium hypochlorite for surface sterilization. The leaf pieces were rinsed by steril-

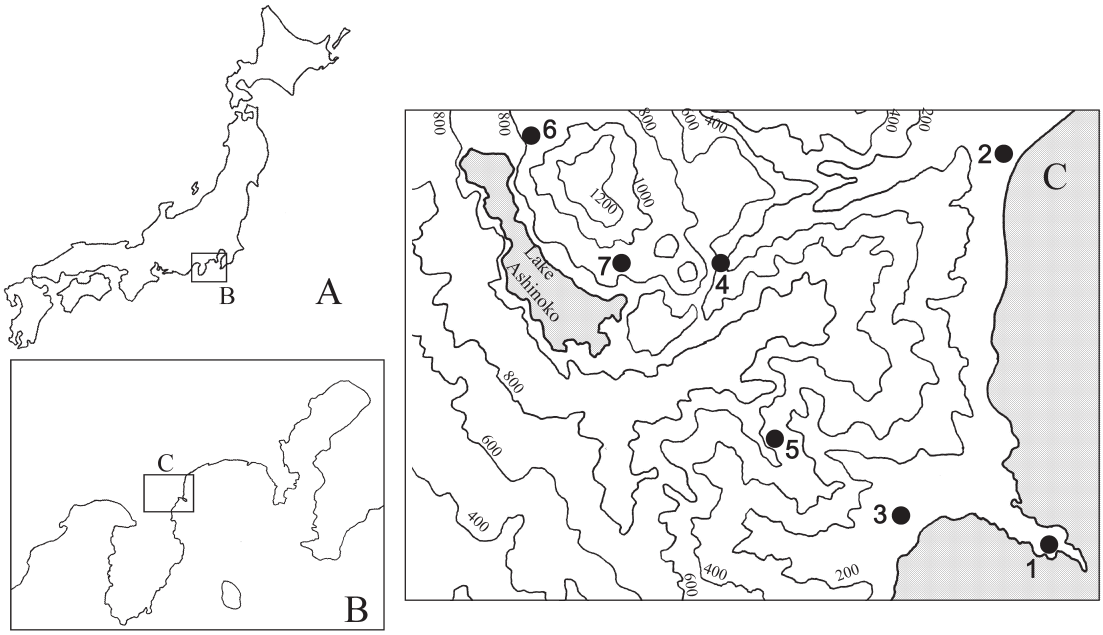


Fig. 1. Collection sites. A, map of Japan showing the Sagami Sea and its adjacent areas studied in the present study (B); B, the Sagami Sea and its adjacent areas including the collection sites (C); C-1, Manazuru; C-2, Shiroyama; C-3, Miyashita; C-4, Hatajuku; C-5, Miyagami; C-6, Ubako; C-7, Komagatake.

ized DW, and dried over night on sterilized filter paper. Before dried, two sets of a 5 mm leaf pieces from apical and basal parts and two from middle leaf blade were dissected by a sterilized knife so that costa were included (four pieces in total from a single leaf). The leaf pieces were inoculated onto two plates of half diluted potato dextrose agar (1/2PDA, Nissui) and cornmeal agar (CMA, Nissui), respectively, so that each plate contained all the four pieces from the leaf. The plates were sealed with parafilm, and incubated at room temperature. The occurring hyphae was isolated during the following 6 weeks of observation. The isolates were kept in PDA slants.

Molecular anaysis

The isolates were cultivated in 2 ml of 2% malt extract for 2 weeks, and the mycelia was harvested and frozen at –80ºC. About 50 mg of mycelium was mechanically lysed by a Quagen TissueLyser, using ceramic beads. DNA was extracted using a Dneasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. To identify the obtained isolates by molecular technique, internal transcribed spacer 1, 2 and 5.8s ribosomal RNA regions (ITS-5.8S) were sequenced. Primers ITS1 and ITS4 (White *et al*. 1990) were used to amplify the ITS-5.8S region. DNA was amplified using 40 ml PCR reactions, containing 0.2 mM each primer, 1 U TaKaRa Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan), and a deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM each dNTP and ExTaq buffer containing 2 mM Mg^{2+}. PCR was carried out using a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). DNA was denatured for 3 min at 95 ºC, followed by 30 cycles of denaturation at 95 ºC for 30 s, annealing at 50 ºC for 30 s, and extension at 72 ºC for 2 min, followed by final extension at 72 ºC for 10 min.

PCR products were purified using an ExoSAP-IT purification kit (USB, Cleveland, OH, USA). Total DNA samples were deposited in the Molecular Biodiversity Research Center in the National Museum of Nature and Science and are available

Fig. 2. Landscape of the field. A, Manazuru; B, Ubako; C. *A. japonica* leaves with symptoms (these leaves were not collected); D, healthy *A. japonica* leaves collected for the present study.

for research upon request. Sequencing was carried out using a BigDye Terminator v 3.1 Cycle Sequencing Kit on a DNA auto sequencer 3130x (Applied Biosystems) following the manufacturer's instructions. The obtained sequence was assembled and edited by SeqMan (Lasergen v6 DNAstar), and congruent sequences obtained from both strands were saved. Ambiguously aligned sites were excluded from the analysis.

Molecular identification

The obtained DNA sequences were aligned by Clustal W (Thompson *et al*., 1994), and edited manually when necessary using BioEdit v. 7.0.5.2 (Hall, 1999). The obtained alignments were analyzed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) on MEGA4 (Tamura *et al*., 2007) to know the identity between the isolates. Based on the UPGMA analysis, isolates with different sequences (representative isolates) were selected. Best matched sequences for each representative isolate was BLAST searched from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When the best and second best matched sequences did no differ in coverage and max identity, both sequences were selected.

The selected sequences were added to those from the representative isolates, aligned, and neighbor joining (NJ) analysis was carried out. When fungal species with identical sequence were found, the given representative isolate was identified as that species, and other isolates with the identical sequences were also identified as the same species. When highly similar sequence was found in the database, the given representative isolate was tentatively identified to that species (indicated with parenthesis). When highly similar sequence had genus or higher level of identification, generic or higher identification of the isolate were given to the isolate referring to the phyloge-

netic analysis (indicated with parenthesis). Isolates having sequences identical or highly similar with uncertainly identified reference such as "Fungal endophytes", "Fungal sp.", were all treated as "unidentified".

Results and Discussion

General tendency of the isolates

In total, 174 isolates were obtained. After PCR, samples with no or smear band were excluded. Sequences containing double peaks (possible contaminants or heterogeneous nuclei) were excluded from the analysis. In total, 141 isolates were sequenced and identified (Table 1).

The majority of the isolates (94%) belonged to Ascomycota, and the rest belonged to Basidiomycota. No phycomycetous isolate was obtained, suggesting that the surface sterilization was successful. The successful surface sterilization was also indicated by the fact that the majority of the sequences of the unidentified isolates agreed with "fungal endophytes" or isolates from plants. Sooty molds, common fungi on *Aucuba* were not isolated, also supporting the successful surface sterilization, because they are known to invade only epidermal cells (Agrios, 2005).

The most frequently occurred fungi were *Glomerella cingulata* (anamorph: *Colletotrichum gloeosporioides*, 39 isolates), followed by *Diaporthe* (anamorph: *Phomopsis*) (25 isolates), *Guignardia* (anamorph: *Phyllosticta*) (15 isolates). Xylarialean isolates (*Muscodor* sp., *Nemania diffusa*, *Nodulisoprium*, and Xylariales and Xylariaceae including tentative identification, 20 isolates) also comprised of the major part of the isolates. These four groups of isolates consisted of 70% of the isolates. These fungal groups are known to be the most popular endophytes in broad leaved trees in Japan (Osono, 2009).

Media dependency of the isolates

More isolates were obtained on 1/2PDA (82 isolates) than CMA (59 isolates) (Table 2). However, the number of species did not differ much (24 spp. in 1/2PDA, 25 spp. in CMA, excluding the unidentified). Thirteen species (101 isolates, excluding the unidentified) occurred on both media, consisting of 71.6% of the total number of the isolates. *Glomerella cingulata, Guignardia mangiferae* and unidentified Xylariaceae were clearly more frequently isolated on 1/2PDA, showing the media preference. The rest did not show clear tendency, suggesting that using a sinlge medium may be enough to cover the majority of the fungal endophytes. On the other hand, 11 species (12 isolates), 12 species (14 isolates) occurred only on 1/2PDA and CMA, respectively, suggesting that using more than one media may be complementary in detecting minor species. Influences of the nutrient media to the isolation of endophytes has been discussed by various authors (e .g. Bills and Polishook, 1991). However, the majority of the endophytes obtained in the present study did not differ considerably in the two method. Whether isolation frequency of the minor endophytes was influenced by the media or not requires more attempts.

Altitude dependency of the isolates

Both the numbers of isolates and species tended to be decreased at low and high altitude (5 m or >880 m, Table 2). This tendency did not depend on parts of the leaves (data not shown). Sixty-six percent of the species (excluding the unidentified) were unique to the given altitude. Numbers of singleton also decreased at both high and low altitude. The most frequently isolated fungi was *Glomerella cingulata,* occurring in all the altitude, followed by *Guignardia mangiferae*, occurring in three out of six sites.

The endophytic mycobiota may be determined by the host condition and fungal physiology. Although *A. japonica* is distributed in high elevation, its primary habitat is fertile marshy ground in warm-temperate forest (Satake *et al*., 1989). It is known that the endophytes in host plant under stress differ from those in host under no stress (Hata & Futai, 1995). In the present study, *A. japonica* at Manazuru (5 m) and Miyagami (550 m) may be exposed to stress because they were close to the seashore and exhaust gas from the

Table 1. Endophytic fungi isolated from leaves of *Aucuba japonica* identified by molecular phylogenetic analysis

FC	Identification[1)]	Family	Order	Phyllum[2)]	Site	Altitude (m)	Isolation Medium	Leaves[3)]	Part[14)]
3037	(*Bjerkandera fumosa*)	Meruliaceae	Polyporaels	B	Shiroyama	50	CMA	C	M
3161	(Cf. *Ramichloridium* sp.)	Mycosphaerellaceae	Capnodiales	A	Hatajuku	560	1/2PDA	C	M
3048	(*Cladonia coniocraea*)	Cladoniaceae	Lecanorales	A	Hatajuku	560	1/2PDA	A	A
3114	(*Glomerella cingulata*)	Glomerellaceae	incertae sedis	A	Komagatake	890	CMA	D	M
3157	(*Glomerella cingulata*)	Glomerellaceae	incertae sedis	A	Hatajuku	560	1/2PDA	B	M
3170	(*Fomitopsis* sp.)	Fomitopsidaceae	Polyporaels	B	Ubako	880	CMA	C	M
3164	(*Guignardia* sp.)	Botryosphaeriaceae	Botryosphaeriales	A	Miyagami	550	CMA	A	M
3171	(*Lachnum* sp.)	Lachnaceae	Helotiales	A	Komagatake	890	1/2PDA	A	A
3068	(*Lophodermium minor*)	Rhytismataceae	Polyporaels	A	Miyagami	550	CMA	A	B
3073	(*Lophodermium minor*)	Rhytismataceae	Rhytismatales	A	Miyagami	550	1/2PDA	B	M
3107	(*Muscodor* sp.)	Xylariaceae	Xyalriales	A	Komagatake	890	CMA	C	B
3108	(*Muscodor* sp.)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	D	B
3109	(*Muscodor* sp.)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	D	B
3110	(*Muscodor* sp.)	Xylariaceae	Xyalriales	A	Komagatake	890	CMA	D	B
3053	(*Nodulisporium* sp.)	Xylariaceae	Xyalriales	A	Hatajuku	560	1/2PDA	B	A
3012	(*Phomopsis liquidambari)*	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	C	A
3013	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	C	A
3010	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	B	M
3011	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	B	M
3015	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	C	M
3017	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	C	M
3018	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	C	M
3020	(*Phomopsis liquidambari*)	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	D	M
3047	(*Phomopsis* sp.)	Diaporthaceae	Diaporthales	A	Ubako	880	1/2PDA	B	M
3067	(Sordariomycetes)	−	−	A	Miyagami	550	1/2PDA	A	B
3129	(Xylariaceae)	Xylariaceae	Xyalriales	A	Ubako	880	1/2PDA	A	A
3096	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	CMA	B	A
3104	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	CMA	C	A
3097	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	B	B
3099	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	B	B
3094	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	A	M
3103	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	B	M
3141	(Xylariaceae)	Xylariaceae	Xyalriales	A	Komagatake	890	1/2PDA	C	M
3064	(Xylariales)	−	Xyalriales	A	Hatajuku	560	CMA	D	B
3089	*Alternaria arborescens*	Pleospoaceae	Pleosporales	A	Manazuru	5	CMA	D	B
3090	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	CMA	A	A
3091	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	CMA	A	B
3139	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	1/2PDA	A	B
3140	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	1/2PDA	A	B
3092	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	CMA	A	M
3093	*Arthrinium phaeospermum*	Apiosporaceae	incertae sedis	A	Komagatake	890	1/2PDA	A	M
3070	*Bjerkandera fumosa*	Meruliaceae	Polyporaels	B	Miyagami	550	CMA	B	A
3069	*Cyphellopsis anomala*	Cyphellaceae	Agaricales	A	Miyagami	550	CMA	A	M
3001	*Diaporthe eres*	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	A	A
3002	*Diaporthe eres*	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	A	A
3117	*Diaporthe eres*	Diaporthaceae	Diaporthales	A	Ubako	880	CMA	B	B
3005	*Diaporthe eres*	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	A	B
3009	*Diaporthe eres*	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	B	B
3045	*Fomes fomentarius*	Polyporaceae	Polyporaels	B	Shiroyama	50	CMA	D	M
3120	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	CMA	C	A

1)For detail, see the text. 2) A: Ascomycota, B: Basidiomycota. 3) Four collected leaves were named as A-D for practicality (see text). 4) A: apical, B: basal, M: middle part of the leaves.

Table 1. (Cont.)

FC	Identification[1)]	Family	Order	Phyllum[2)]	Site	Altitude (m)	Isolation Medium	Leaves[3)]	Part[14)]
3121	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	C	A
3125	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	D	A
3031	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Shiroyama	50	1/2PDA	B	A
3055	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	CMA	B	A
3078	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	B	A
3079	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	CMA	B	A
3149	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	A	A
3122	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	CMA	C	B
3042	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Shiroyama	50	1/2PDA	D	B
3043	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Shiroyama	50	1/2PDA	D	B
3144	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Shiroyama	50	1/2PDA	B	B
3052	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	CMA	A	B
3165	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	CMA	C	B
3080	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	B	B
3083	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	C	B
3084	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	CMA	C	B
3169	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	D	B
3135	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Miyashita	50	CMA	C	B
3046	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	B	M
3115	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	A	M
3119	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	B	M
3124	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	C	M
3128	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Ubako	880	1/2PDA	D	M
3112	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Komagatake	890	CMA	D	M
3113	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Komagatake	890	1/2PDA	D	M
3142	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Komagatake	890	CMA	A	M
3057	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	CMA	B	M
3058	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	1/2PDA	B	M
3059	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Hatajuku	560	1/2PDA	B	M
3076	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	A	M
3077	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	A	M
3085	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	C	M
3150	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Manazuru	5	1/2PDA	B	M
3155	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Miyagami	550	1/2PDA	A	M
3021	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Miyashita	50	1/2PDA	D	M
3022	*Glomerella cingulata*	Glomerellaceae	incertae sedis	A	Miyashita	50	CMA	D	M
3145	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Shiroyama	50	1/2PDA	C	A
3159	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Hatajuku	560	1/2PDA	C	A
3087	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Manazuru	5	1/2PDA	D	A
3132	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	B	A
3137	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	D	A
3056	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Hatajuku	560	CMA	B	B
3133	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	B	B
3136	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	D	B
3006	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	CMA	A	M
3130	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	A	M
3131	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	A	M
3134	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	B	M
3138	*Guignardia mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	1/2PDA	D	M
3172	*Guignardia* sp.	Botryosphaeriaceae	Botryosphaeriales	A	Miyagami	550	1/2PDA	A	A

1)For detail, see the text. 2) A: Ascomycota, B: Basidiomycota. 3) Four collected leaves were named as A-D for practicality (see text). 4) A: apical, B: basal, M: middle part of the leaves.

Table 1. (Cont.)

FC	Identification[1)]	Family	Order	Phyllum[2)]	Site	Altitude (m)	Isolation Medium	Leaves[3)]	Part[14)]
3054	*Laetiporus versisporus*	Fomitopsidaceae	Polyporaels	B	Hatajuku	560	1/2PDA	B	A
3044	*Malassezia restricta*	−	Malasseziales	B	Shiroyama	50	CMA	D	M
3160	*Mycosphaerella heimii*	Mycosphaerellaceae	Capnodiales	A	Hatajuku	560	1/2PDA	C	B
3158	*Mycosphaerella heimii*	Mycosphaerellaceae	Capnodiales	A	Hatajuku	560	1/2PDA	B	B
3060	*Mycosphaerella heimii*	Mycosphaerellaceae	Capnodiales	A	Hatajuku	560	CMA	B	M
3151	*Mycosphaerella* sp.	Mycosphaerellaceae	Capnodiales	A	Manazuru	5	CMA	C	M
3152	*Mycosphaerella* sp.	Mycosphaerellaceae	Capnodiales	A	Manazuru	5	CMA	C	M
3049	*Nemania diffusa*	Xylariaceae	Xyalriales	A	Hatajuku	560	1/2PDA	A	B
3028	*Nemania diffusa*	Xylariaceae	Xyalriales	A	Shiroyama	50	1/2PDA	A	M
3007	*Neofusicoccum mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	CMA	A	M
3008	*Neofusicoccum mangiferae*	Botryosphaeriaceae	Botryosphaeriales	A	Miyashita	50	CMA	A	M
3035	*Phomopsis amygdali*	Diaporthaceae	Diaporthales	A	Shiroyama	50	CMA	C	A
3036	*Phomopsis amygdali*	Diaporthaceae	Diaporthales	A	Shiroyama	50	CMA	C	A
3003	*Phomopsis amygdali*	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	A	A
3074	*Phomopsis amygdali*	Diaporthaceae	Diaporthales	A	Miyagami	550	1/2PDA	C	B
3075	*Phomopsis amygdali*	Diaporthaceae	Diaporthales	A	Miyagami	550	1/2PDA	C	B
3019	*Phomopsis liquidambari*	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	D	A
3004	*Phomopsis liquidambari*	Diaporthaceae	Diaporthales	A	Miyashita	50	CMA	A	B
3014	*Phomopsis liquidambari*	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	C	B
3016	*Phomopsis liquidambari*	Diaporthaceae	Diaporthales	A	Miyashita	50	1/2PDA	C	M
3156	*Phomopsis* sp.	Diaporthaceae	Diaporthales	A	Miyagami	550	CMA	C	B
3101	*Phyllosticta cryptomeriae*	Botryosphaeriaceae	Botryosphaeriales	A	Komagatake	890	1/2PDA	B	M
3061	*Polyporus tuberaster*	Polyporaceae	Polyporaels	B	Hatajuku	560	1/2PDA	D	A
3071	unidentified			A	Miyagami	550	1/2PDA	B	A
3082	unidentified			A	Manazuru	5	1/2PDA	C	B
3072	unidentified			A	Miyagami	550	1/2PDA	B	B
3118	unidentified			A	Ubako	880	CMA	B	M
3126	unidentified			A	Ubako	880	1/2PDA	D	M
3102	unidentified			A	Komagatake	890	CMA	B	M
3032	unidentified			A	Shiroyama	50	CMA	B	M
3033	unidentified			A	Shiroyama	50	CMA	B	M
3038	unidentified			A	Shiroyama	50	1/2PDA	C	M
3066	unidentified			A	Hatajuku	560	CMA	D	M
3162	unidentified			A	Hatajuku	560	1/2PDA	D	M
3163	unidentified			A	Hatajuku	560	1/2PDA	D	M
3081	unidentified			A	Manazuru	5	1/2PDA	B	M
3154	unidentified			A	Manazuru	5	CMA	D	M
3063	Xylariales	−	Xyalriales	A	Hatajuku	560	CMA	D	A
3123	Xylariales	−	Xyalriales	A	Ubako	880	1/2PDA	C	B
3050	Xylariales	−	Xyalriales	A	Hatajuku	560	1/2PDA	A	B
3027	Xylariales	−	Xyalriales	A	Shiroyama	50	CMA	A	M

1)For detail, see the text. 2) A: Ascomycota, B: Basidiomycota. 3) Four collected leaves were named as A-D for practicality (see text). 4) A: apical, B: basal, M: middle part of the leaves.

Table 2. Number of isolates of the endophytic fungi from *Aucuba japonica* at various altitude with a reference to the difference of the medium for isolation.

	Medium		Altitude (m)						
Fungi	1/2PDA	CMA	5	50	550	560	880	890	Total
Glomerella cingulata	25	12	11	7	1	6	9	3	37
Guignardia mangiferae	11	2	1	10		2			13
(Xylariaceae)	6	2					1	7	8
(*Phomopsis liquidambari*)	3	5		8					8
Arthrinium phaeospermum	3	3						6	6
Phomopsis amygdali	3	2		3	2				5
Diaporthe eres	1	4		4			1		5
Phomopsis liquidambari	2	2		4					4
(*Muscodor* sp.)	2	2						4	4
Xylariales	2	2		1		2	1		4
Mycosphaerella heimii	2	1				3			3
Nemania diffusa	2			1		1			2
Mycosphaerella sp.		2	2						2
(*Glomerella cingulata*)	1	1				1		1	2
Neofusicoccum mangiferae		2		2					2
(*Lophodermium minor*)	1	1			2				2
Alternaria arborescens		1	1						1
Fomes fomentarius		1		1					1
Malassezia restricta		1		1					1
(*Bjerkandera fumosa*)		1		1					1
Bjerkandera fumosa		1			1				1
Cyphellopsis anomala		1			1				1
Guignardia sp.	1				1				1
Phomopsis sp.		1			1				1
(Sordariomycetes)	1				1				1
(*Guignardia* sp.)		1			1				1
Laetiporus versisporus	1					1			1
Polyporus tuberaster	1					1			1
(*Cladonia coniocraea*)	1					1			1
(Cf. *Ramichloridium* sp.)	1					1			1
(Xylariales)		1				1			1
(*Nodulisporium* sp.)	1					1			1
(*Fomitopsis* sp.)		1					1		1
(*Phomopsis* sp.)	1						1		1
Phyllosticta cryptomeriae	1							1	1
(*Lachnum* sp.)	1							1	1
unidentified	8	6	3	3	2	3	2	1	14
Number of isolates	82	59	18	46	13	24	16	24	141
Number of species*	24	25	4	12	9	12	6	7	36

*For numbers of species, numbers of unidentified isolates were excluded from the count.

Table 3. Relative isolation frequency* and total number of isolates of endophytic fungi from various part of the leaves of *Aucuba japonica*

	Isolation frequency by the part of the leaf			
Fungi	Base	Middle	Apical	Total
Glomerella cingulata	26.2	27.7	23.5	26.2
Guignardia mangiferae	7.1	7.7	14.7	9.2
(Xylariaceae)	4.8	4.7	4.6	5.7
Arthrinium phaeospermum	7.1	3.1	2.9	4.3
Phomopsis liquidambari	4.8	1.5	2.9	2.8
Xylariales	4.8	1.5	2.9	2.8
Mycosphaerella heimii	4.8	1.5		2.1
Nemania diffusa	2.4	1.5		1.4
(*Lophodermium minor*)	2.4	1.5		1.4
Diaporthe eres	7.1		5.9	3.5
Phomopsis amygdali	4.8		4.6	2.1
(*Muscodor* sp.)	10.0			2.8
Alternaria arborescens	2.4			1.4
Phomopsis sp.	2.4			1.4
(Sordariomycetes)	2.4			1.4
(Xylariales)	2.4			1.4
(*Phomopsis liquidambari*)		9.2	5.9	6.7
Mycosphaerella sp.		3.1		1.4
Neofusicoccum mangiferae		3.1		1.4
(*Glomerella cingulata*)		3.1		1.4
Cyphellopsis anomala		1.5		0.7
Fomes fomentarius		1.5		0.7
Malassezia restricta		1.5		0.7
Phyllosticta cryptomeriae		1.5		0.7
(*Bjerkandera fumosa*)		1.5		0.7
(*Guignardia* sp.)		1.5		0.7
(*Fomitopsis* sp.)		1.5		0.7
(*Phomopsis* sp.)		1.5		0.7
(Cf. *Ramichloridium* sp.)		1.5		0.7
Bjerkandera fumosa			2.9	0.7
Guignardia sp.			2.9	0.7
Laetiporus versisporus			2.9	0.7
Polyporus tuberaster			2.9	0.7
(*Cladonia coniocraea*)			2.9	0.7
(*Lachnum* sp.)			2.9	0.7
(*Nodulisporium* sp.)			2.9	0.7
unidentified	4.8	16.9	2.9	10.0
Number of isolates	42	65	34	141
Number of species**	16	22	16	36

* Relative isolation frequency = (number of isolates of the given species/total number of the isolates in each part) × 100.

** For numbers of species, numbers of unidentified isolates were excluded from the count.

motor road, respectively. The high altitude may also have affected the fungi, resulting the less diverse endophytic mycobiota by low temperature.

Hashizume *et al*. (2008) compared the endophytes in *Quercus acuta* in different altitude, and showed that endophytic mycobiota changed by-altitude. They also suggest that the optimum temperature of the fungi may be the major reason for this difference. This explanation may be also adoptable to the present result.

Distribution of the endophytes in the leaves

Because two pieces were inoculated for the middle part of the leaves while single piece was used for isolation from the basal and apical part, relative isolation frequency (isolates of the given species/total number of the isolates in each part ×100) was used for comparison (Table 3). Five members were isolated from all parts of the leaves: *Glomerella cingulata, Guignardia mangiferae, Arthrinum phaeospermum, Phomopsis liquidambari,* and Xylariales including Xylariaceae. These members distributed in all parts of the leaves, and the relative isolation frequency of them did not differ considerably by part of the leaves. The middle part of the leaf embraced the largest numbers of species, and fewer members were unique to the base or apical part. The present results are similar to those in Sahashi *et al*. (1999) and Kaneko *et al*. (2003) who reported that isolation frequency of the major endophytes did not differ in part of the leaves in *Fagus crenata*. The endophytic mycobiota in leaves of *A. japonica* were found to be homogeneous in the leaves.

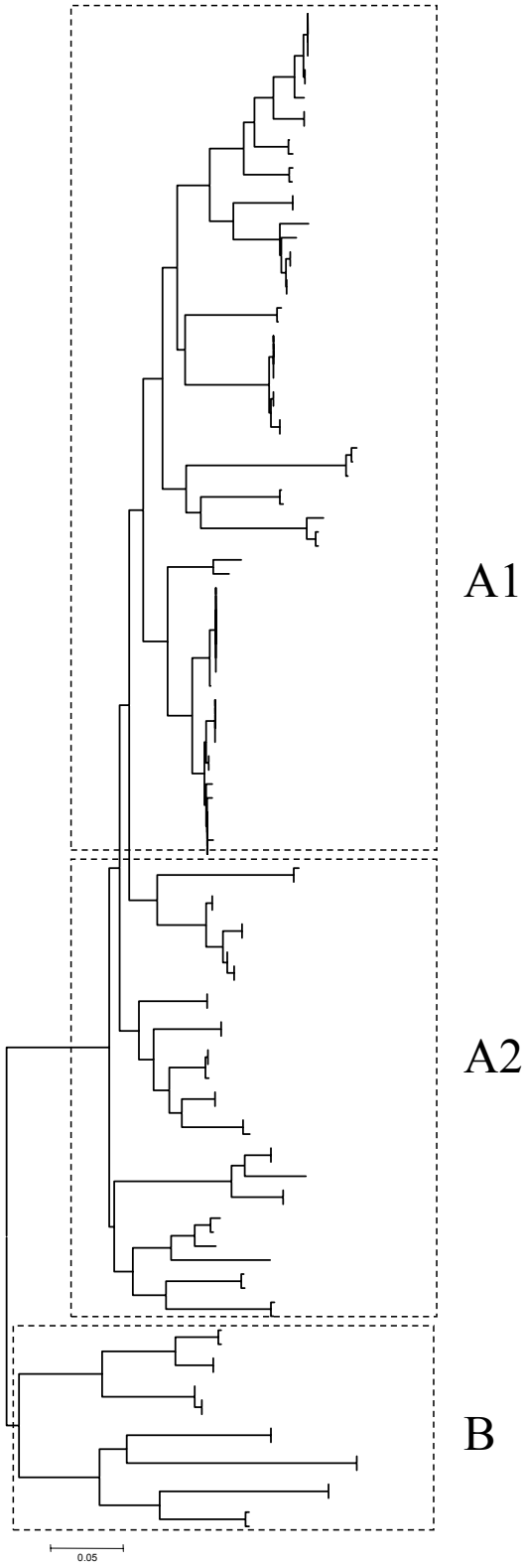
Phylogenetic aspects of the isolates

Two major clades, one for Ascomycota and the other for Basidiomycota were recognized in NJ tree (Figs. 3–6). The Ascomycota clade was subdivided into several major clades, but they were not strongly supported. However, most of the terminal nodes, mostly representing the species or genera, were strongly supported. In some cases, groups higher than genera were strongly supported. They represented Sordariomycetes clade1 (mainly Xylariales), Sordariomycetes clade 2, *Glomerella cingulata* (anamorph: *Glomerella cingulata*), *Diaporthe* spp. (anamorph: *Phomopsis* spp.), *Guignardia* spp. (anamorph: *Phyllosticta* spp.), Mycosphaerellaceous clade (*Mycosphaerella* with *Ramichloridium* anamorph), and *Lachnum* spp. Among these, some unidentified endophytes with strong support were inserted. Phylogenetic relationships of these unidentified endophytes and the aforementioned groups were not clarified.

The Basidiomycota clade was subdivided into two strongly supported clades. One included *Bjerkandera, Fomes*, and *Polyporus*. All these are known as white rot fungi, decomposing lignin in the tree. However, none of them are known as a a pathogen from *A. japonica*. *Fomes fomentarius* is a stem heart rot pathogen of *Acer, Betula, Carpinus, Larix, Populus, Prunus, Quercus, Sorbus, Tilia,* and *Ulmus* (Kobayashi, 2007) . It is particularly well known to occur on *Fagus crenata* (Kishi, 1998). Although *F. crenata* was associated with *A. japonica* in high altitude, the site where *F. fomentarius* was isolated was about 50 m altitude where *A. japonica* was not associated with *F. crenata*. It is therefore suggested that some pathogenic fungi, having a selectivity to their host may survive as endophytes in other plant when they are away from their primary habitat.

Basidiomycetes are infrequently isolated as endophytes (Bills and Polishook, 1991), and claimed to be isolation bias (Stone *et al*., 2004). However, Oses *et al*. (2006) and Arnold *et al*. (2007) detected basidiomycetes from environmental DNA, suggesting that more numbers of basidiomycetous endophytes exist than expected. Oses *et al*. (2006) elucidated the possible contribution of basidiomycetous endophytes to white rot decay of trees.

Xylariales is also known as white rot fungi. More than several groups of lignin decomposing fungi were found to contribute to decompose *A. japonica* leaves, together with brown rot fungi. Xyalrialean isolates shear about 14% of the obtained isolates in the present study. Okane *et al*. (1997) reported that xylariaceous fungi and *Phyl-*



losticta spp. comprised of about 50% of obtained isolates in evergreen plants in subtropical area. Xylariales seems to be cosmopolitan in the evergreen broad-leaved trees in subtropical area.

The other clade in Basidiomycota is composed of brown rot fungi *Fomitopsis* and *Laetiporus*, and *Cyphellopsis* and *Malassezia*. Substrate selectivity of the latter two is not known.

Biodiversity of fungal endophytes in *Aucuba japonicae* in Sagami Sea and its adjacent areas

In the inventory of Japanese fungi (Katumoto, 2010), 26 species were listed from *A. japonica*. Of these, *Glomerella cingulata* (anamorph: *Colletotrichum gloeosporioides*) is known as a pathogen of the anthracnose of *A. japonica* (Kishi, 1998). It is also known as a pathogen of various plants. *Diaporthe aucubae* and *Phomopsis aucubae* are also known as canker disease pathogens. The sequences of these fungi were not registered in GenBank, and further study is required to identify the isolates obtained in the present study with certainty. *Phyllosticta harai* is known for brown rot fungus, but the sequences of the isolates obtained in the present study was not identical with it.

Elucidation of more comprehensive endophytic mycobiota requires multiple media and large scale collecting because isolation of singletons may be ruled by chance. Due to the distribution of the host, fungi specific to *A. japonica* may contain east Asian elements, and extensive studies in other areas to compare the results with those in the present study will contribute to reveal the fungal diversity hot spot in Asia.

Some endophytic fungi are known to stay on the leaves after the leaves were fallen from the tree, and contribute in decomposition as saprophytes (Promputtha *et al*, 2007). Field mycologi-

Fig. 3. Overall topology of a neighbor-joining (NJ) tree of endophytic fungi from *Aucuba japonica* with reference sequences from GenBank inferred from ITS-5.8S rRNA sequences. A1 and A2 represent Ascomycota clade, B represents Basidiomycota clade. Details of each clade are shown in Figs 4, 5 and 6, respectively.

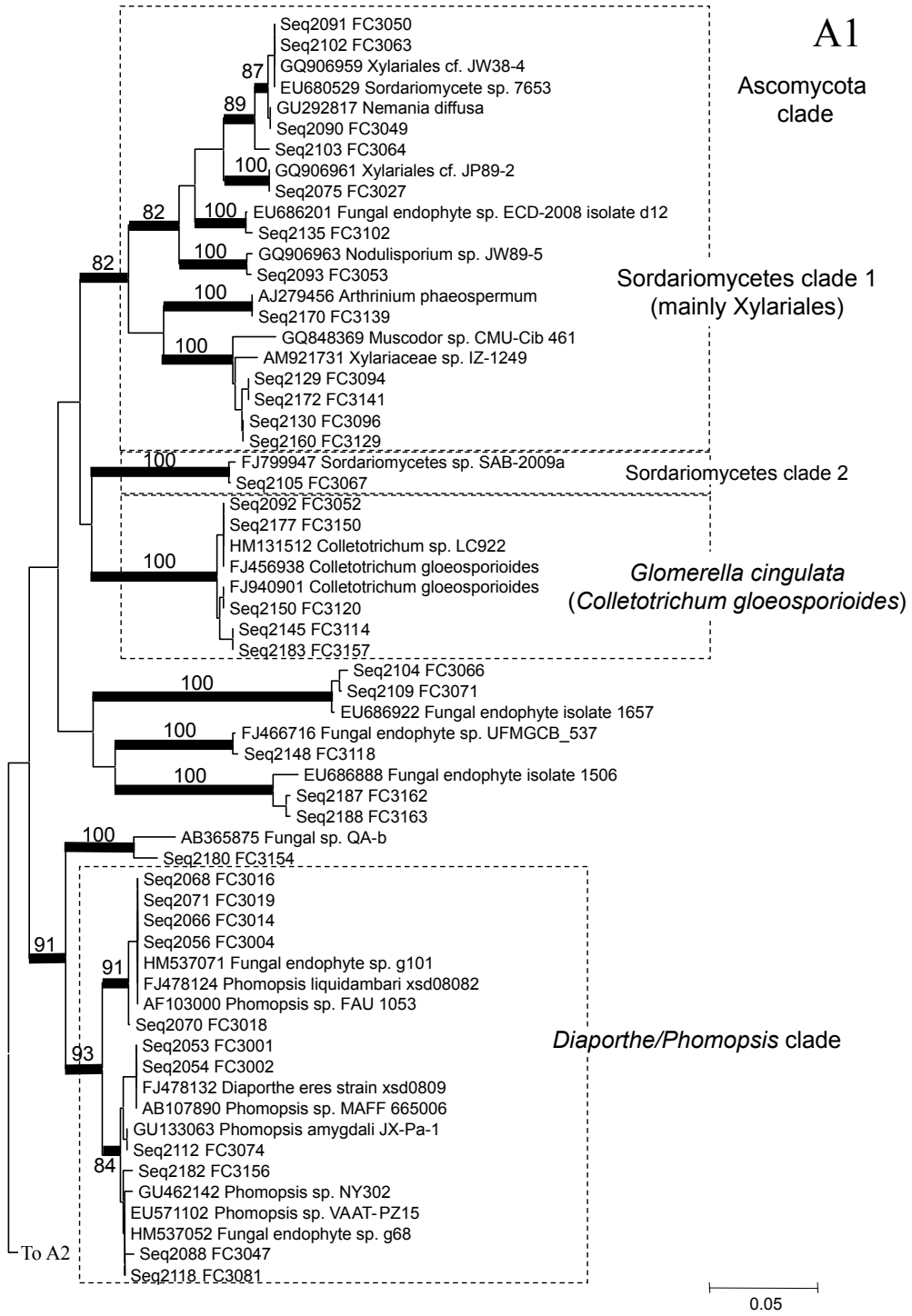


Fig. 4. Part of neighbor-joining (NJ) tree (clade A1 in Fig. 3) of endophytic fungi from *Aucuba japonica* with reference sequences from GenBank inferred from ITS-5.8S rRNA sequences. Remarkable fungal groups are indicated. Bootstrap values (BP) of 1000 replications are shown where BP exceeded over 80%. Caldes with BP>80% are shown with thickened branches.

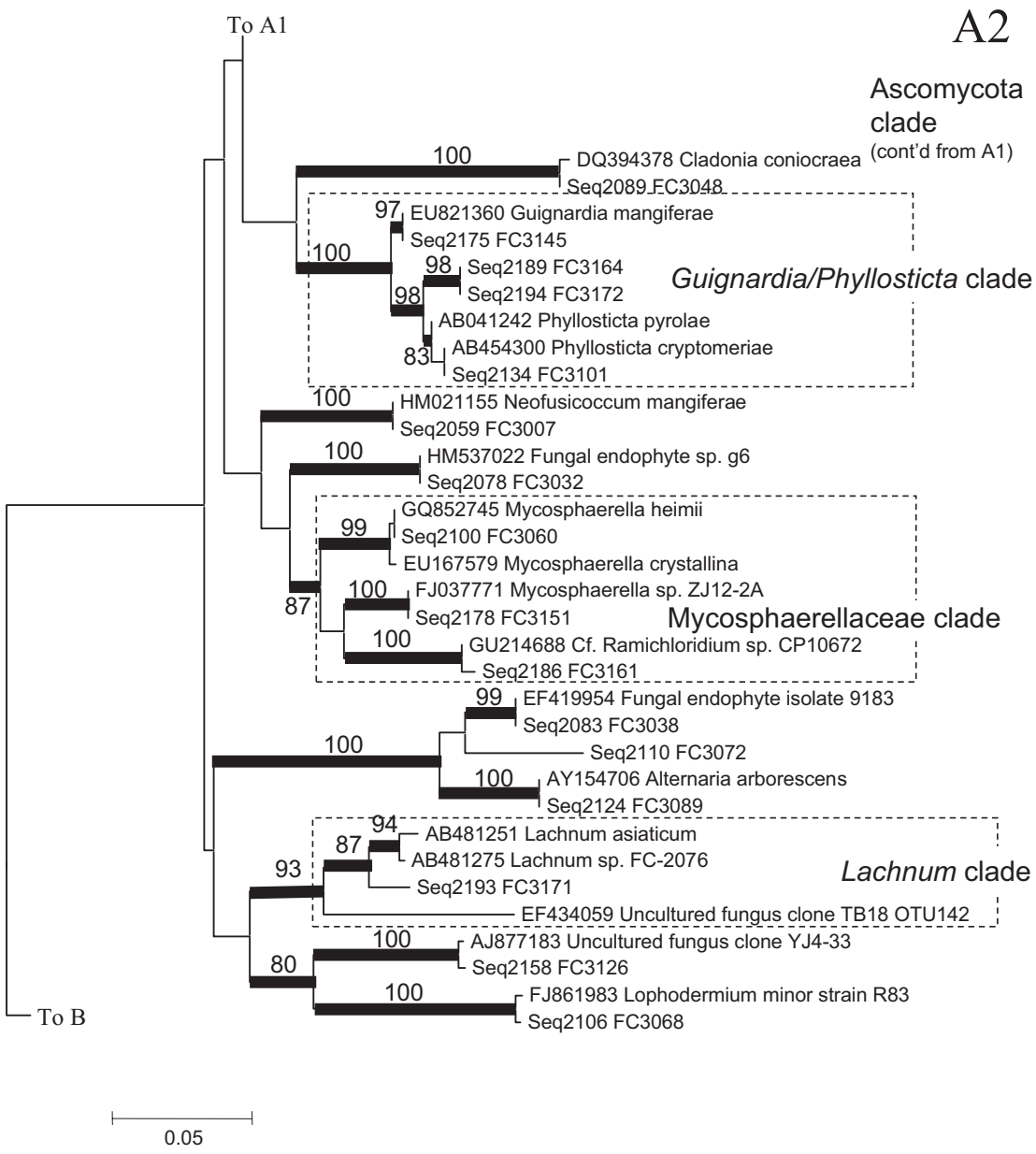


Fig. 5. Part of neighbor-joining (NJ) tree (clade A2 in Fig. 3) of endophytic fungi from *Aucuba japonica* with reference sequences from GenBank inferred from ITS-5.8S rRNA sequences. Remarkable fungal groups are indicated. Bootstrap values (BP) of 1000 replications are shown where BP exceeded over 80%. Caldes with BP>80% are shown with thickened branches.

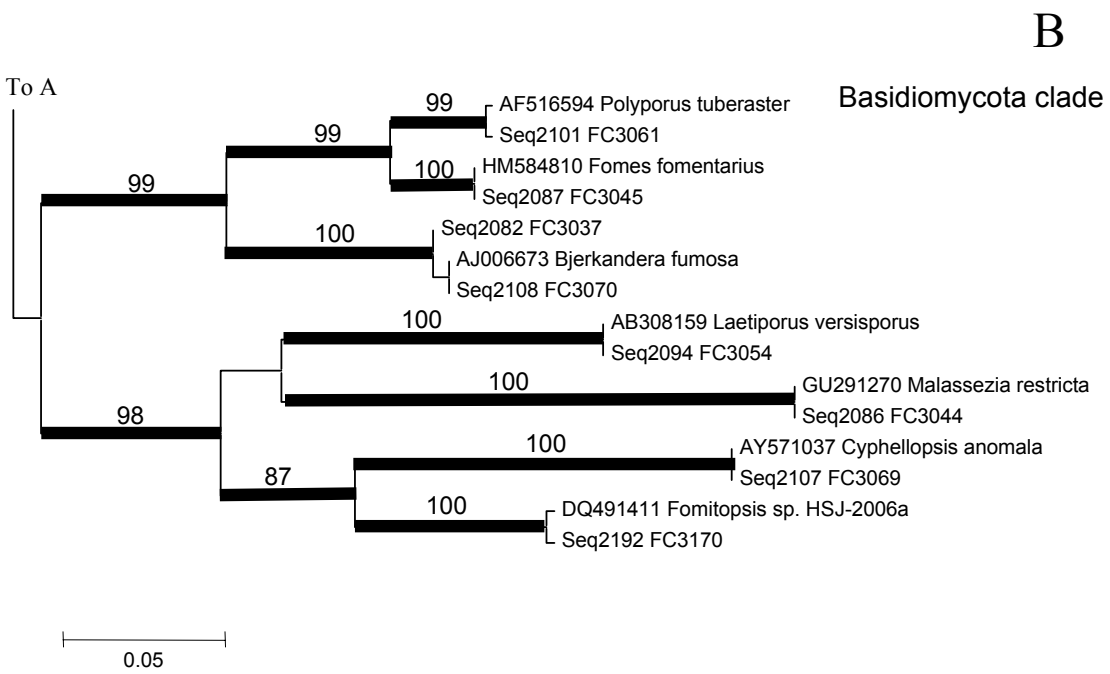


Fig. 6. Part of neighbor-joining (NJ) tree (clade B in Fig. 3) of endophytic fungi from *Aucuba japonica* with reference sequences from GenBank inferred from ITS-5.8S rRNA sequences. Remarkable fungal groups are indicated. Bootstrap values (BP) of 1000 replications are shown where BP exceeded over 80%. Caldes with BP>80% are shown with thickened branches.

cal studies should also be carried out by collecting the fruiting body of the fungi on *A. japonica in situ* to give a reference to the identification of the isolates with only sterile hyphae.

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分子同定に基づいた相模灘とその近傍地域におけるアオキの内生菌相

出川洋介・西村幹雄・平山由美子・細矢　剛

相模灘地域における真菌類相に与えている因子を検討するため，アオキの内生菌を調査した．アオキの葉を相模灘地域の 7 地点で採集し，表面殺菌によって 2 種類の培地で分離された内生菌 141 株のバーコード領域である ITS-5.8S リボソーム RNA 領域をシークエンスし，既存のデータベースのデータをもとに，36 分類群に分子同定した．もっとも多く分離されたのは *Glomerella cinguilata*（アナモルフ：*Colletotrichum gloeosporoioides*），*Diaporthe*（アナモルフ：*Phyllosticta*）フンタマカビ類，クロサイワイタケ類で，大部分は子嚢菌であり，わずかな担子菌類も含まれていた．分離培地や葉の部位によっては明確な菌類相の違いはなかったが，菌類相は採集地の高度とそれによるホストへの環境因子に影響を受けていることが推察された．