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FULL PAPER

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Phylogenetic analyses of Japanese species of *Phyllosticta* sensu stricto

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Abstract Although the genus concept of *Phyllosticta* s. str. (teleomorph: *Guignardia*) as defined by van der Aa is widely accepted, the species concept is still controversial because it is often based on the morphology on host plants. In this study, the culture characteristics within *Phyllosticta* s.str. were examined, and the phylogenetic relationships among Japanese species of *Phyllosticta* s.str. and its teleomorph *Guignardia* were analyzed using 18S rDNA sequences. *Phyllosticta* s. str. formed a monophyletic clade. ITS-28S rDNA sequences extracted from fungal cultures derived from various host plants were divided into two subgroups. The first group included cultures from a wide range of host plants and were mainly derived as endophytes from a symptom-less plant. In the second group, cultures from each host plant genus formed distinct clades; these were often isolated as leaf pathogens from diverse plants. Isolates belonging to the first lineage generally grew faster on oatmeal agar. To classify species of *Phyllosticta* it is necessary to consider an integrated approach such as molecular phylogeny, host plant, colony growth, symptoms, and morphological characteristics of the conidiomata.

Key words Endophytic fungi · Phylogeny · Plant pathogenic fungi · Ribosomal DNA · Taxonomy

Introduction

The genus concept of *Phyllosticta* sensu stricto (s. str.) based on the morphological characteristics defined by van der Aa (1973) has been widely accepted. Subsequently, van der Aa and Vanev (2002) published a list of species belonging to the genus *Phyllosticta* s. str. based on the original literature and results of reexamination of herbarium specimens including numerous type specimens. Delimitation of the genus, based on morphological characteristics, and teleomorph-anamorph relationships were indicated. According to van der Aa et al. (1990) and van der Aa and Vanev (2002), only about 7% of the species of *Phyllosticta* sensu lato (s.l.) defined by Saccardo (1878, 1884) and succeeding investigators have been accepted as species of the genus *Phyllosticta* s. str. amended by van der Aa (1973). Most of the species of *Phyllosticta* s.l. were reclassified into other coelomycetous genera: 50% in *Phoma*, 20% in *Asteromella*, 5% in *Phomopsis*, and 18% in other genera of Sphaeropsidales, Melanconiales, and even some genera of Moniliales or Ascomycotina. However, species concepts within *Phyllosticta* s. str. are still controversial (Motohashi et al. 2008). Species of *Phyllosticta* s. str., including its teleomorph *Guignardia*, are regarded as host specific (monoxenic) according to results of inoculation studies (Stewart 1916; Luttrell 1946, 1948; Reusser 1964). The epithet of a new *Phyllosticta* species has been given if the host plant and fungal morphology on diseased leaves are different. Although only a rather small group of plant pathogens is really host specific, host specificity for the taxonomy of *Phyllosticta* has been strongly overestimated, and the species epithet is given by distinguishable host, morphology on diseased leaves of host plants, and culture characteristics (van der Aa 1973; van der Aa and Vanev 2002). Based on these criteria, multiple species of *Phyllosticta* s. str. have been reported to share the same host genus. For example, three species of *Phyllosticta* having distinguishable sizes and shapes of conidia and thicknesses of conidial slime layer have been reported on *Smilax* spp., namely, *Phyllosticta crypta* Bissett, *P. cumminsii* Bissett, and *P. subeffusa* (Ellis & Everh.) Tehon & G. L. Stout. (Bissett 1979). However, other examples have been shown that do not support this species concept. For example, *P. concentrica* Sacc., considered to be a plant pathogenic species, is recognized as a polyxenic (wide host range) species having indistinguishable morphology on different host plants, namely, *Cryptomeria japonica*, *Hedera*

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helix, *Heptapleurum venulosum*, *Ilex* spp., *Magnolia* spp., *Rhododendron* spp., and *Taxus* spp. (van der Aa and Vanev 2002).

Crous et al. (2006) revealed the stability of the genus *Phyllosticta* s. str. as a monophyletic clade within the Botryosphaeriaceae, except *P. flevolandica* Aa, known only as a soilborne and didymosporous species. Similarly, studies of delimitation of species and host specificity of *Phyllosticta* have been introduced using molecular phylogenetic techniques. *Guignardia endophyllicola* Okane, Nakagiri & Tad. Ito [anamorph: *P. capitalensis* Henn.], recognized as an endophytic species, has been shown to be polyxenic based on the results of comparative studies of morphology on artificial media and sequence analyses of ribosomal DNA internal transcribed spacer regions (rDNA ITS) for cultures isolated from 38 plant families (Okane et al. 2001, 2003). Similar results have been also shown by ITS-RFLP analysis with isolates of endophytic *Phyllosticta* from 17 species of tropical trees in 17 genera of 14 families in India (Pandey et al. 2003). On the other hand, acceptance of 2 species of *Guignardia* on *Citrus* spp., i.e., *Guignardia citricarpa* Kiely [anamorph: *P. citricarpa* (McAlpine) Aa] and *G. mangiferae* A. J. Roy (anamorph: *P. capitalensis*), is based on culture characteristics, growth rate, thickness of the conidial slime layer, and their nucleotide sequence data, although they have the same size of conidia (Baayen et al. 2002; Meyer et al. 2006; Peres et al. 2007).

This investigation was conducted to reveal the phylogenetic relationships among the Japanese species of *Phyllosticta* s. str. based on analyses of rDNA sequence data and cultural characteristics on artificial medium. Moreover, species concepts of both plant pathogenic and endophytic *Phyllosticta* are discussed based on the use of integrated approaches such as molecular phylogeny, host plant, colony growth, symptoms, and morphological characteristics of conidiomata.

Materials and methods

Identification and fungal isolations

Fresh leaf materials with *Phyllosticta* and/or *Guignardia* were collected in the field. Specimens for microscopic observation were made by hand-sectioning the material and mounting the section with Shear's fluid (Chupp 1940). The fungus was identified based on its host plant and morphological characteristics and size of each structure of fungus: i.e., pycnidia/ascomata, conidia and appendage/asci and ascospores, and conidiogenous cells. To obtain living cultures that originated from diseased leaves collected by the authors, the monoconidial isolation method (Nakashima and Kobayashi 1997) was used on Japanese cedar (*Cryptomeria japonica*) needle decoction agar (Ito et al. 1952) with modifications by Motohashi et al. (2008). A germinated conidium was transferred onto oatmeal agar (OMA) (Difco oatmeal agar; Becton Dickinson, Hunt Valley, MD, USA). Other cultures of *Phyllosticta* spp. and allied genera were obtained from the Microbiological Genebank, National Institute of Agrobiological Sciences (MAFF), Tsukuba, Ibaraki Prefecture, Japan, or Laboratory of Plant Protection, Department of International Agricultural Development, Tokyo University of Agriculture, Tokyo, Japan. These cultures are maintained in MAFF, Biological Resource Center, the National Institute of Technology and Evaluation (NBRC), Kisarazu, Chiba Prefecture, Japan, or Culture Collection, Laboratory of Plant Pathology, Mie University (MUCC), Tsu, Mie Prefecture, Japan (Table 1).

Table 1. Sources of the materials used for molecular analysis

MUCC[a]	MAFF[b]	NBRC[c]	Fungal species	Host species	Location in Japan	GenBank accession number	
						18S	ITS-28S
0010[d]	240040	102244	*Phyllosticta aspidistricola*	*Aspidistra elatior*	Aichi	AB454176	AB454260
0011[d]	240042	102246	*Phyllosticta sphaeropsoidea*	*Aesculus turbinata*	Aichi	AB454177	AB454261
0012[d]	240041	102245	*Phyllosticta concentrica*	*Rhododendron* sp.	Tokyo	AB454178	AB454262
0014[d]	240044	102248	*Phyllosticta alliacea*	*Allium fistulosum*	Shizuoka	AB454179	AB454263
0015[d]	240045	102249	*Phyllosticta alliacea*	*Allium fistulosum*	Shizuoka	AB454180	AB454264
0016[d]	240046	102250	*Phyllosticta minima*	*Acer pycnanthum*	Aichi		AB454265
0017[d]	240047	102251	*Phyllosticta kerriae*	*Kerria japonica*	Aichi	AB454181	AB454266
0018[d]	240048	102252	*Phyllosticta* sp.	*Pieris japonica* subsp. *japonica*	Tokyo	AB454182	AB454267
0019[d]	240049	102253	*Phyllosticta ampelicida*	*Parthenocissus tricuspidata*	Aichi		AB454268
0021[d]	240050	102254	*Phomopsis* sp.	*Cercis canadensis*	Aichi	AB454182	
0024[d]	240053	102256	*Phyllosticta ligustricola*	*Ligustrum obtusifolium*	Kumamoto	AB454269	
0027[d]	240056	102257	*Phyllosticta concentrica*	*Magnolia liliiflora* var. *gracilis*	Aichi	AB454183	AB454270
0028[d]	240057	102258	*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Toyama	AB454184	AB454271
0029[d]	240058	102259	*Phyllosticta capitalensis*	*Acer* sp.	Aichi	AB454185	AB454272
0030[d]	240059	102260	*Phyllosticta capitalensis*	*Dendropanax trifidus*	Aichi	AB454186	AB454273
0031[d]	240060	102261	*Phyllosticta ardisiicola*	*Ardisia crenata*	Aichi		AB454274
0032[d]	240061	102262	*Phyllosticta concentrica*	*Hedera rhombea*	Aichi	AB454187	AB454275
0037	236403		*Phyllosticta ampelicida*	*Parthenocissus tricuspidata*	Tokyo		AB454276
0038	236703		*Phyllosticta harai*	*Aucuba japonica*	Kagoshima	AB454188	AB454277
0039	237027		*Fusicoccum aesculi*	*Chaenomeles speciosa*	Shimane		AB454278
0041	237042		*Guignardia* sp.	*Psidium guajava*	Okinawa	AB454189	AB454279
0042	237091		*Guignardia* sp.	*Farfugium japonicum*	Kanagawa	AB454190	AB454280

Table 1. *Continued*

MUCC[a]	MAFF[b]	NBRC[c]	Fungal species	Host species	Location in Japan	GenBank accession number	
						18S	ITS-28S
0043	237096		*Phyllosticta harai*	*Aucuba japonica*	Osaka	AB454191	AB454281
0044	237099		*Guignardia* sp.	*Kalmia latifolia*	Tokyo	AB454192	AB454282
0045	237100		*Guignardia ardisiae*	*Ardisia japonica*	Tochigi	AB454193	AB454283
0046	237127		*Guignardia* sp.	*Rhododendron indicum*	Tokyo		AB454284
0047	237181		*Phyllosticta* sp.	*Nerium oleander*	Okinawa	AB454194	AB454285
0049	237283		*Phyllosticta kobus*	*Magnolia kobus*	Ibaraki	AB454195	AB454286
0050	237515		*Guignardia* sp.	*Podocarpus macrophyllus*	Nagasaki	AB454196	AB454287
0051	237519		*Guignardia* sp.	*Ilex crenata*	Kanagawa	AB454197	AB454288
0052	237521		*Guignardia* sp.	*Ilex crenata*	Ibaraki		AB454289
0054	237895		*Fusicoccum aesculi*	*Eriobotrya japonica*	Chiba	AB454198	
0055	237283		*Phyllosticta kobus*	*Magnolia kobus*	Ibaraki	AB454199	
0059	238872		*Phyllosticta camelliae*	*Camellia japonica* var. *hortensis*	Kanagawa	AB454200	AB454290
0060	239155		*Fusicoccum aesculi*	*Swida controversa*	Chiba	AB454201	
0062	305187		*Phyllosticta phaseolina*	*Phaseolus vulgaris*	Japan	AB454202	
0064	305980		*Phoma destructiva*	*Lycopersicon esculentum*	Tokyo	AB454203	
0065	410091		*Phyllosticta miurae*	*Lindera praecox*	Mie	AB454204	AB454291
0066	410092		*Guignardia sawadae*	*Cryptomeria japonica*	Niigata	AB454205	AB454292
0069	410183		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454206	
0070	410184		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454207	
0071	410185		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454208	
0072	410186		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454209	
0073	410187		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454210	
0074	410188		*Botryosphaeria laricina*	*Larix kaempferi*	Hokkaido	AB454211	AB454293
0075	410303		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Miyagi		AB454294
0076	410304		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Mie	AB454212	AB454295
0077	410305		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Akita	AB454213	AB454296
0078	410306		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Akita		AB454297
0079	410307		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Japan		AB454298
0080	410308		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Ishikawa	AB454214	AB454299
0081	410309		*Phyllosticta cryptomeriae*	*Cryptomeria japonica*	Tokyo		AB454300
0083	410349		*Phyllosticta populorum*	*Populus* sp.	Tokyo	AB454215	
0084	410350		*Phyllosticta alcides*	*Populus tremula* var. *sieboldii*	Yamagata	AB454216	
0085	410351		*Phyllosticta alcides*	*Populus* sp.	Tokyo	AB454217	
0087	410353		*Phyllosticta harai*	*Aucuba japonica*	Tokyo	AB454218	AB454301
0088	410354		*Phyllosticta azevinhi*	*Ilex pedunculosa*	Hiroshima	AB454219	AB454302
0089	410355		*Phyllosticta gardeniicola*	*Gardenia jasminoides*	Tokyo	AB454220	AB454303
0091	410609		*Guignardia* sp.	*Podocarpus macrophyllus*	Tokyo	AB454221	AB454304
0092	410706		*Guignardia cryptomeriae*	*Cryptomeria japonica*	Kyoto	AB454222	AB454305
0096	410710		*Guignardia cryptomeriae*	*Thujopsis dolabrata*	Ishikawa	AB454223	
0097	410712		*Guignardia cryptomeriae*	*Cryptomeria japonica*	Kyoto	AB454224	
0098	410717		*Guignardia cryptomeriae*	*Cryptomeria japonica*	Kyoto	AB454225	
0099	625040		*Phomopsis amygdali*	*Amygdalus persica*	Niigata	AB454226	
0100	625042		*Phomopsis amygdali*	*Amygdalus persica*	Niigata	AB454227	
0101	625044		*Phomopsis amygdali*	*Amygdalus persica*	Shizuoka	AB454228	
0103	712088		*Phyllosticta petasitidis*	*Farfugium japonicum*	Mie	AB454229	
0105	726550		*Phoma lycopersici*	*Lycopersicon esculentum*	Miyazaki	AB454230	
0106	726583		*Phoma macrostoma* var. *incolorata*	*Cynara scolymus*	Mie	AB454231	
0107	726585		*Phoma exigua* var. *exigua*	*Cynara scolymus*	Mie	AB454232	
0112[d]		102265	*Phyllosticta sphaeropsoidea*	*Aesculus carnea*	Toyama	AB454233	AB454306
0113[d]	240051	102266	*Phyllosticta fallopiae*	*Fallopia japonica*	Aichi	AB454234	AB454307
0114[d]	240054	102267	*Phyllosticta capitalensis*	*Cercis canadensis*	Aichi		AB454308
0116[d]		102268	*Guignardia* sp.	*Sarcandra glabra*	Hyogo	AB454235	AB454309
0117[d]		102269	*Phyllosticta gardeniicola*	*Gardenia jasminoides*	Aichi	AB454236	AB454310
0118[d]	240055	102270	*Guignardia* sp.	*Ardisia crenata*	Aichi	AB454237	AB454311
0119[d]		102271	*Guignardia* sp.	*Triteleia bridgesii*	Aichi	AB454238	AB454312
0120[d]		102272	*Phyllosticta ampelicida*	*Parthenocissus tricuspidata*	Aichi		AB454313
0121[d]		102273	*Phyllosticta aspidistricola*	*Aspidistra elatior*	Shizuoka		AB454314
0122[d]		102274	*Phyllosticta capitalensis*	*Aspidistra elatior*	Aichi	AB454239	AB454315
0123[d]		102275	*Phyllosticta minima*	*Acer pycnanthum*	Aichi		AB454316
0124[d]		102276	*Phyllosticta* sp.	*Pachysandra terminalis*	Hokkaido	AB454240	AB454317
0125[d]			*Phyllosticta* sp.	*Pyrola asarifolia* subsp. *incarnata*	Nagano	AB454241	AB454318
0147[d]			*Phyllosticta concentrica*	*Rhododendron keiskei*	Nagano	AB454242	AB454319
0148[d]		102284	*Phyllosticta minima*	*Acer crataegifolium*	Nagano		AB454320
0149[d]			*Phyllosticta hamamelidis*	*Hamamelis japonica*	Gifu	AB454243	AB454321
0150[d]			*Phyllosticta hamamelidis*	*Hamamelis japonica* var. *discolor* f. *obtusata*	Aomori	AB454244	AB454322

Table 1. *Continued*

MUCC[a]	MAFF[b]	NBRC[c]	Fungal species	Host species	Location in Japan	GenBank accession number	
						18S	ITS-28S
0151[d]			*Phyllosticta hamamelidis*	*Hamamelis japonica* subsp. *megalophylla*	Gunma	AB454245	AB454323
0152[d]			*Phyllosticta hamamelidis*	*Hamamelis japonica*	Aichi	AB454246	AB454324
0153[d]			*Phyllosticta hamamelidis*	*Hamamelis japonica*	Ibaraki	AB454247	AB454325
0154[d]	240062		*Guignardia alliacea*	*Allium fistulosum*	Shizuoka	AB454248	AB454326
0155[d]	240063		*Guignardia alliacea*	*Allium fistulosum*	Shizuoka	AB454249	AB454327
0156[d]			*Phyllosticta minima*	*Acer pycnanthum*	Nagano		AB454328
0158[d]			*Phyllosticta* sp.	*Gaultheria shallon*	Japan	AB454250	AB454329
0159[d]			*Phyllosticta capitalensis*	*Hydrangea quercifolia*	Aichi	AB454251	AB454330
0206[d]			*Phyllosticta cruenta*	*Polygonatum odoratum* var. *pluriflorum*	Mie		AB454331
0207			*Phyllosticta capitalensis*	*Ginkgo biloba*	Japan	AB454252	AB454332
0208			*Phyllosticta capitalensis*	*Pittosporum tobira*	Japan	AB454253	AB454333
0209			*Phyllosticta capitalensis*	*Eriobotrya japonica*	Japan	AB454254	AB454334
0210			*Phyllosticta capitalensis*	*Ligustrum lucidum*	Japan	AB454255	AB454335
0211			*Phyllosticta capitalensis*	*Lithocarpus edulis*	Japan	AB454256	AB454336
0212			*Phyllosticta capitalensis*	*Nerium oleander* var. *indicum*	Japan		AB454337
0213			*Phyllosticta capitalensis*	*Osmanthus fragrans* var. *aurantiacus* f. *aurantiacus*	Japan	AB454257	AB454338
0214			*Phyllosticta capitalensis*	*Robinia pseudoacacia*	Japan	AB454258	AB454339
0215			*Phyllosticta capitalensis*	*Cercidiphyllum japonicum*	Japan	AB454259	AB454340
0409[d]			*Phyllosticta* sp.	*Fraxinus angustifolia*	Tokyo		AB454341
0410[d]			Phyllosticta conjac	Amorphophallus rivieri	Aichi		AB454342
0411[d]			*Guignardia* sp.	*Disanthus cercidifolius*	Kumamoto		AB454343
0412[d]			*Phyllosticta* sp.	*Podocarpus macrophyllus*	Toyama		AB454344
0413[d]			*Phyllosticta* sp.	*Paeonia lactiflora* var. *trichocarpa*	Tokyo		AB454345
0425[d]			*Phyllosticta* sp.	*Nandina domestica* cv. *otafukunanten*	Tokyo		AB454346
0426[d]			*Phyllosticta* sp.	*Cercis chinensis*	Aichi		AB454347
0428			*Phyllosticta* sp.	*Oxypetalum* sp.	Fukuoka		AB454348
0432			*Phyllosticta* sp.	*Rhaphiolepis indica* var. *umbellata*	Kagoshima		AB454349
0433			*Phyllosticta* sp.	*Schefflera heptaphylla*	Okinawa		AB454350
0435			*Guignardia* sp.	*Smilax china*	Okinawa		AB454351
0436			*Phyllosticta* sp.	*Aucuba japonica*	Ibaraki		AB454352
0437			*Phyllosticta* sp.	*Cinnamomum insularimontanum*	Tokyo		AB454353
0440			*Phyllosticta* sp.	*Pittosporum tobira*	Kagoshima		AB454354
0441			*Guignardia* sp.	*Podocarpus macrophyllus*	Fukuoka		AB454355
0443[d]			*Phyllosticta capitalensis*	*Davidia involucrata*	Tokyo		AB454356
0521			*Phyllosticta cordylinophila*	*Cordyline fruticosa*	Kagoshima		AB454357
0522			*Guignardia* sp.	*Leucothoe keiskei*	Japan		AB454358
0523			*Guignardia* sp.	*Alnus sieboldiana*	Tokyo		AB454359
0524			*Guignardia* sp.	*Ilex chinensis*	Fukuoka		AB454360
0542	240199		*Phyllosticta capitalensis*	*Spathiphyllum* sp.	Okinawa		AB454361
0543	240200		*Phyllosticta* sp.	*Rohdea japonica*	Ibaraki		AB454362
0544[d]			*Phyllosticta* sp.	*Aucuba japonica*	Tochigi		AB454363
0547			*Phyllosticta* sp.	*Gelsemium sempervirens*	Tokyo		AB454364
0548			*Phyllosticta* sp.	*Amacrinum* sp.	Tokyo		AB454365
0549			*Phyllosticta concentrica*	*Hedera rhombea*	Tokyo		AB454366
0550			*Phyllosticta* sp.	*Cyclamen persicum*	Tokyo		AB454367
0551			*Phyllosticta* sp.	*Liriodendron tulipifera*	Tokyo		AB454368
0552			*Phyllosticta* sp.	*Chlorophytum comosum*	Tokyo		AB454369
0553			*Phyllosticta* sp.	*Leucothoe catesbaei*	Tokyo		AB454370
0554			*Phyllosticta* sp.	*Gardenia jasminoides*	Tokyo		AB454371
0555			*Phyllosticta* sp.	*Vaccinium* sp.	Tokyo		AB454372
0556			*Phyllosticta* sp.	*Pachysandra terminalis*	Tokyo		AB454373
0562			*Phyllosticta cruenta*	*Polygonatum odoratum* var. *pluriflorum*	Tokyo		AB454374

[a] MUCC; Lab. of Plant Pathology, Mie University
[b] MAFF; the Microbiological Genebank, National Institute of Agrobiological Sciences
[c] NBRC; Biological Resource Center, the National Institute of Technology and Evaluation
[d] Isolations collected by authors in this study

rDNA extraction and polymerase chain reaction (PCR) amplification

All cultures were grown on OMA plates at 22°C. Agar blocks with mycelium were cut from the plates with a sterilized plastic straw. Extraction of whole-cell culture DNA was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of the two internal transcribed spacers (ITS1 and ITS2) including the 5.8S rDNA gene region and 28S rDNA region was performed by PCR using primer sets ITS5 and NL4 according to O'Donnell (1993) and White et al. (1990). Similarly, the 18S rDNA region was amplified using primers no. 1 (5′-CTGGTTGATCCTGCCAGT-3′) (Hendriks et al. 1989) and NS8 (White et al. 1990). PCR on a T Gradient Thermocycler (Biometra, Goettingen, Germany) was performed in a 25-µl reaction mixture containing 5 µl template DNA, 0.5 µl KOD-plus DNA polymerase (Toyobo, Osaka, Japan), 0.375 µl each primer, 2.5 µl 10× PCR buffer, 2.5 µl dNTPs (2 mM), 2 µl $MgSO_4$ (25 mM), and 11.75 µl distilled water. The thermal cycler program was as follows: 2 min at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 60°C (for 18S region) or 56°C (for ITS and 28S region), 110 s (for 18S region) or 70 s (for ITS and 28S region) at 68°C, with a final extension period of 10 min at 68°C. Following amplification, the PCR products were purified with the Agencourt AMPure PCR Purification System (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions.

DNA sequencing

Amplified products were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730xl DNA Analyzer DNA Sequencing System (Applied Biosystems). For amplification for sequencing of the 18S region, the primers no. 1, NS2, NS3, NS4, NS5, NS6, NS7, and NS8 (White et al. 1990) were used. Similarly, for amplification of the ITS and 28S rDNA region sequencing, the primers ITS5, ITS2, ITS3, ITS4, NL1, and NL4 (White et al. 1990; O'Donnell 1993) were used. For removal of the unincorporated dye terminators, dye-labeled products were purified with the Agencourt CleanSEQ (Beckman Coulter) according to the manufacturer's instructions.

Phylogenetic analyses

The obtained sequences in this study were aligned with MAFFT version 6.240 with E-INS-i option (Katoh et al. 2002, 2005). Sequences were truncated at the 5′- and 3′-ends and manually aligned when necessary using BioEdit version 7.09 (Hall 1999). At this point, ambiguously aligned sites were removed. The alignment files of 18S and ITS-28S rDNA were deposited in TreeBASE (http://www.treebase.org/treebase/) under the accession number of PIN 1102. Phylogenetic trees were obtained from the data using neighbor-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP), and Bayesian phylogenetic analyses. The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via the Akaike information criterion (AIC) (Akaike 1974) using PAUP* version 4.0b10 (Swofford 2002) and MrModeltest version 2.2 (Nylander 2004). As the result of calculations, 18S, ITS1, and 28S regions were fitted to a general time-reversible model with a proportion of invariable sites and a gamma-shaped distributed rate (GTR + I + G) (Tavaré 1986). ITS2 and 5.8S regions were fitted to an evolutionary model of GTR + G and K80 + I (Kimura 1980), respectively. The combined dataset of the ITS and the 28S regions was fitted to the GTR + I + G model.

NJ and MP analyses with the selected evolutionary model were performed in PAUP*. For NJ analyses, distance was measured by the maximum-likelihood model as the selected evolutionary model. Ties were broken randomly when encountered. MP analysis was performed for 100 replications with different random starting points using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. Alignment gaps were treated as missing data, and all characters were unordered and had equal weight. The branch-swapping algorithm was tree-bisection-reconstruction (TBR). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The best tree topology of MP trees was conducted using the Kishino–Hasegawa likelihood test (Kishino and Hasegawa 1989) on PAUP*. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated.

The strength of the internal branches from the resulting tree was tested by bootstrap (BS) analysis (Felsenstein 1985) using 1000 replications in both distance and parsimony analysis. Moreover, decay indexes (DI) (Bremer 1988, 1994; Donoghue et al. 1992) were calculated from MP trees and the dataset using AutoDecay version 5.04 for Perl (http://www.bergianska.se/), with almost the same options as the parsimonious method used in this study and PAUP*.

Bayesian phylogenetic analyses with the selected evolutionary model were done using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). It was launched with random starting trees for 5×10^6 (in ITS and 28S regions) and 10×10^6 (in 18S region) generations, and the Markov chains were sampled every 100 generations. To ensure that the Markov chain did not become trapped in local optima, we used the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm, performing the estimation with four incrementally heated Markov chains. In each of these analyses, the first 7 682 000 in 18S and 3 705 000 in ITS and 28S generations were discarded as burn-in. The remaining trees were summarized in a 50% majority-rule consensus tree, yielding the probabilities of each clade being monophyletic.

In 18S rDNA analysis, *Guignardia mangiferae* (AB041247–AB041249), *Phyllosticta pyrola* (Ehrenb.) Allesch. (AB041250), *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* (DQ109961), and *Phoma herbarum* Westend. (AY337712) were added to the data set, and

Table 2. Growth of isolates after 7 days in darkness at 22° and 28°C on oatmeal agar (OMA)

Group 1a				Group 1b			
MUCC	Host species	22°C (mm)	28°C (mm)	MUCC	Host species	22°C (mm)	28°C (mm)
0012	*Rhododendron* sp.	31	45	0010	*Aspidistra elatior*	6	0
0027	*Magnolia liliiflora* var. *gracilis*	42	50	0011	*Aesculus turbinata*	19	22
0029	*Acer* sp.	30	46	0016	*Acer pycnanthum*	17	18
0043	*Aucuba japonica*	31	38	0017	*Kerria japonica*	28	42
0065	*Lindera praecox*	28	41	0018	*Pieris japonica* subsp. *japonica*	15[a]	18[a]
0066	*Cryptomeria japonica*	34	47	0024	*Ligustrum obtusifolium*	17[a]	6[a]
0112	*Aesculus carnea*	31	48	0028	*Cryptomeria japonica*	29	18
0113	*Fallopia japonica*	34	52	0031	*Ardisia crenata*	33	42
0122	*Aspidistra elatior*	34	50	0038	*Aucuba japonica*	20	20
0208	*Pittosporum tobira*	26	37	0045	*Ardisia japonica*	16[a]	20[a]
0212	*Nerium oleander* var. *indicum*	29	46	0049	*Magnolia kobus*	0	0
0213	*Osmanthus fragrans* var. *aurantiacus* f. *aurantiacus*	27	42	0050	*Podocarpus macrophyllus*	24	26
				0052	*Ilex crenata*	0	0
0215	*Cercidiphyllum japonicum*	32	50	0116	*Sarcandra glabra*	26	20
0410	*Amorphophallus rivieri*	33	47	0117	*Gardenia jasminoides*	17	19
0411	*Disanthus cercidifolius*	33	52	0118	*Ardisia crenata*	33	40
0413	*Paeonia lactiflora* var. *trichocarpa*	34	55	0124	*Pachysandra terminalis*	40	27
0426	*Cercis chinensis*	27	41	0125	*Pyrola asarifolia* subsp. *incarnata*	49	61
0435	*Smilax china*	33	50				
0443	*Davidia involucrata*	34	46[a]	0148	*Acer crataegifolium*	15	17
0542	*Spathiphyllum* sp.	33	47	0149	*Hamamelis japonica*	16	22
0543	*Rohdea japonica*	39	55	0156	*Acer pycnanthum*	13	19
				0206	*Polygonatum odoratum* var. *pluriflorum*	0	4
				0409	*Fraxinus angustifolia*	27	39
				0412	*Podocarpus macrophyllus*	9	11
				0425	*Nandina domestica* cv. *otafukunanten*	24	40
				0432	*Rhaphiolepis indica* var. *umbellata*	26	28
				0433	*Schefflera heptaphylla*	39	44
				0440	*Pittosporum tobira*	5	6[a]
				0521	*Cordyline fruticosa*	26	32
				0524	*Ilex chinensis*	29	21
				0544	*Aucuba japonica*	18	17

[a] Isolations that produced yellow pigmentation on oatmeal agar (OMA)

Peziza echinospora (AF006309) and *Phillipsia domingensis* (AF006315) were used as outgroup taxa, based on Lutzoni et al. (2004).

Colony growth

For a comparative study of the growth of mycelial colonies, 52 cultures isolated from 44 plant species in 38 genera of 29 families were examined (Table 2). Mycelial colonies grown on OMA plates at 22°C in darkness for 2–4 weeks were transferred to OMA plates and incubated at 22° and 28°C under dark conditions. Growth of the colonies was measured 7 days after incubation.

Results

Molecular phylogenetic analyses

The 18S rDNA sequences of the 91 strains of coelomycetous fungi were aligned with 7 sequences obtained from the DNA database (see Table 1). The aligned data matrix of 93 sequences consisted of 1723 characters, of which 241 characters were variable and 171 characters were phylogenetically informative for parsimony analysis. The MP analysis using PAUP* generated 424 equally parsimonious trees with 341 steps (CI = 0.7988, RI = 0.9514, RC = 0.7600). Although slight differences in small branching orders of the terminal branches and branch length were observed, tree topologies were generally consistent among all 424 trees (data not shown). Moreover, topology of the tree generated by the Bayesian phylogenetic analysis (Fig. 1) was similar to the NJ and MP trees. As shown in Fig. 1, the 84 sequences obtained were divided into four groups (groups 1 to 4). All groups were strongly supported with 100% in BS values in the NJ analysis, 97% or higher in the MP analysis, 2 or higher in the DI, and 0.98 and higher in the Bayesian PP. Group 1, without exception, consisted of sequences of *Phyllosticta* s. str. and its teleomorph, *Guignardia*, containing 59 newly obtained sequences in this study and 4 sequences of *Guignardia* obtained from the DNA database. Group 2 mainly consisted of sequences of *Fusicoccum/Botryosphaeria*, including the type species of the genus, *Fusicoccum aesculi* Corda. As the exceptions, the sequences of *Guignardia cryptomeriae* Sawada on *Cryptomeria japonica*

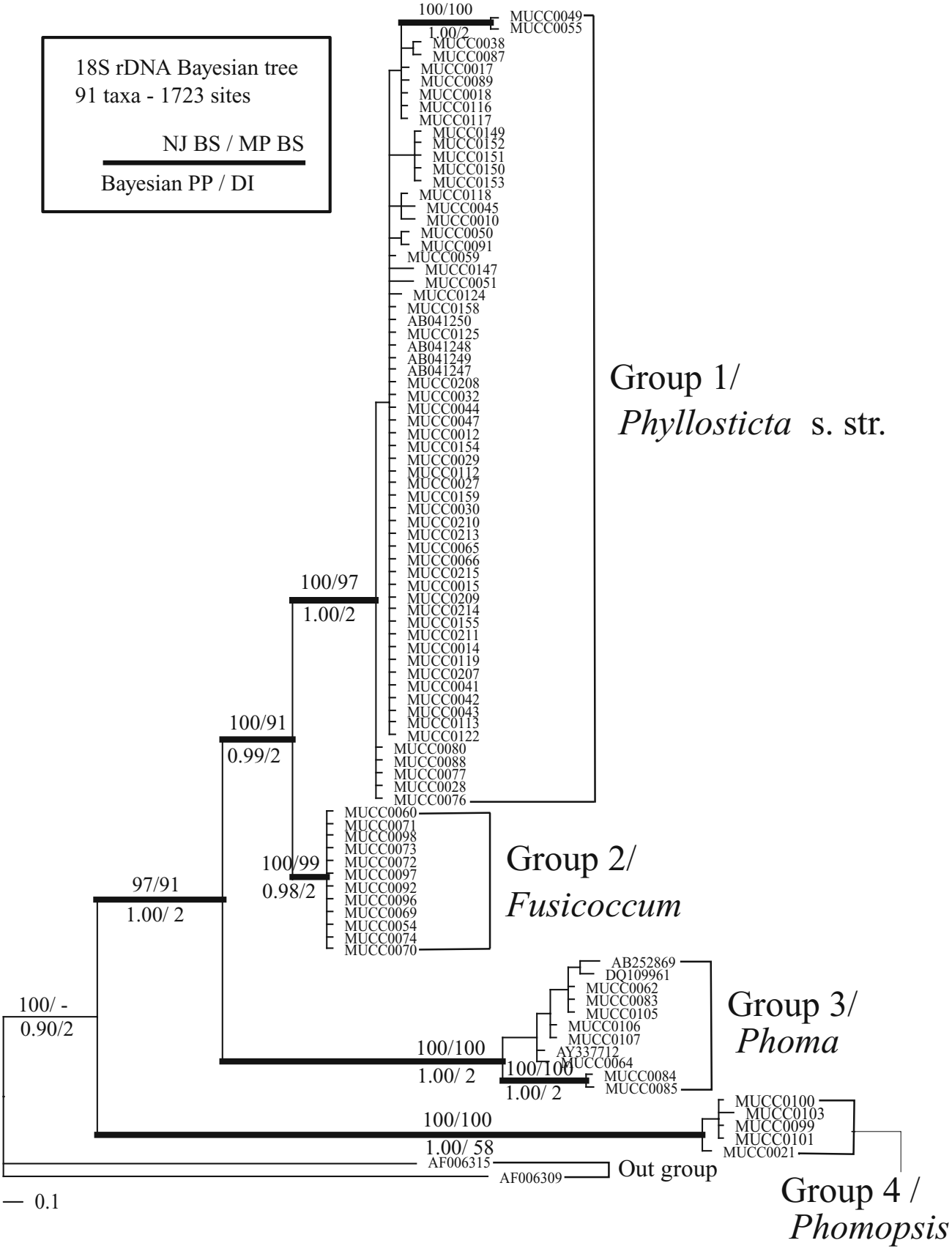

Fig. 1. Phylogenetic tree based on nuclear 18S rDNA sequence for genera *Fusicoccum* (teleomorph: *Botryosphaeria*), *Phoma*, *Phmopsis*, and *Phyllosticta* (teleomorph: *Guignardia*) inferred by Bayesian analysis. Nodes of support values are shown above and below. *NJ BS*, neighbor-joining bootstrap values; *MP BS*, maximum-parsimony bootstrap values; *Bayesian PP*, estimates of posterior probabilities; *DI*, decay index. Nodes supported by >90 NJ and MP BS, >0.95 Bayesian PP, and >2 decay index are indicated by *thick lines*

(MUCC0092, -0097, and -0098) and on *Thujopsis dolabrata* (MUCC0096) were located in group 2. Group 3 consisted of sequences of another coelomycetous genus, *Phoma*. *Phyllosticta populorum* Sacc. & Roum. (MUCC0083) and *P. alcides* Sacc. (MUCC0084 and -0085) were included as exceptions. Similarly, group 4 consisted of sequences of *Phomopsis*, but included an exception sequence, *Phyllosticta petasitidis* Ellis & Everh. (MUCC103).

A total of 112 ITS-28S sequences of *Phyllosticta* s. str. were aligned with three outgroup sequences from the results of Bayesian phylogenetic analysis for the 18S regions. The aligned data matrix of 115 sequences consisted of 1252 characters, of which 76 sites were excluded from the analysis because of ambiguity of alignment. Of the remaining 1176 characters, 302 characters were variable and 238 characters were phylogenetically informative for parsimony analysis. MP analysis using PAUP* resulted in the construction of 292 equal MP trees with 739 steps (CI = 0.5467, RI = 0.9052, RC = 0.4949). Tree topologies, which exhibited only slight differences in tiny branching orders of the terminal branches and in branch length, were generally consistent among the 292 trees. One of the trees with the highest log-likelihood value was selected as the best tree (tree not shown). The tree topology in the Bayesian tree was similar to the NJ and MP trees (Fig. 2): a total of 112 sequences of *Phyllosticta* s. str. and its teleomorph *Guignardia* split into small lineages of two subgroups, 1a and 1b. These lineages were well supported with BS values in the NJ and MP analyses. Similarly, these lineages were supported by the DI and Bayesian PP analysis (BS in NJ and MP = 100%, DI = 15, PP = 1.00). Group 1a consisted of 52 sequences of *Phyllosticta* s. str. and its teleomorph, *Guignardia*. This group mainly consisted of cultures isolated from intact leaves without leaf spot. For example, the border of the leaf spot was unclear in the specimen from which MUCC0029 was isolated (see Fig. 4). A similar topology within group 1a was found for the result of MP trees using a dataset of only ITS sequences, including the data for *G. mangiferae* (from AB041233 to AB041241) (tree not shown). Group 1b consisted of several distinct clades represented by isolates from the host genera *Acer*, *Aradisa*, *Aspidistra*, *Cryptomeria*, *Gardenia*, *Hamamelis*, *Ilex*, *Leucothoe*, *Pachysandra*, *Parathenocissus*, *Podocarpus*, and *Polygonatum*. Typical leaf spots were observed on these plants, i.e., surrounded by a dark brown border in the specimens from which MUCC0016, -0148, -0123, and -0156 were isolated (see Fig. 4).

The isolates of *P. concentrica* [teleomorph: *G. philoprina* (Berk. & M.A. Curtis) Aa], which had been isolated from various plants, were scattered throughout groups 1a (MUCC0012, -0027, -0032, -0066 as synonym *G. sawadae* Tak. Kobay. and 0549) and 1b (MUCC0028 and -0075–0081 as synonym *P. cryptomeriae* Kawam., -0049 as synonym *P. kobus* Henn., -0088 as synonym *P. azevinhi* Torrend, and -0147).

Colony growth

Colonies were black, greenish toward margin, turning black with a lobed greenish margin. Mycelium was dark brown, very dense, but not forming aerial mycelium on OMA. The growth of isolates used in this study is shown in Table 2 and Fig. 3. The isolates belonging to group 1a on the phylogenetic tree of ITS-28S (Fig. 2) grew rapidly at both 22° and 28°C. MUCC0029, belonging to group 1a, grew faster than MUCC0016, -0148, and -0156, which belong to group 1b; however, these four isolates were derived from *Acer*. Similar differences were observed between group 1a and 6 isolates from *Ardisia* spp., *Aseculus* spp., *Aspidistra*, *Aucuba japonica*, *Cryptomeria japonica*, *Ilex* spp., *Magnolia* spp., and *Pittosporum tobira*. Yellow pigmentation was observed at the colony margin in MUCC0018, -0024, and -0045 at 22° and 28°C, and for MUCC0443 and -440 at 28°C.

Discussion

Phylogenetic relationships among the Japanese isolates of *Phyllosticta* s. str., its teleomorph, *Guignardia*, and related coelomycetous genera, namely *Phoma*, *Fusicoccum* (teleomorph: *Botryosphaeria*), and *Phomopsis*, were investigated in this study using NJ, MP, and Bayesian phylogenetic analyses.

In the trees constructed based on the 18S rDNA sequences, four monophyletic clades represented by the respective genera were formed with few exceptions (see Fig. 1). These monophyletic clades were well supported with 98%–100% in BS, 2–58 in DI, and 0.98–1.00 in the Bayesian PP, respectively. For the exceptions, isolates MUCC0092, -0096, -0097, and -0098 were obtained from a culture collection and were supposed to be of *Guignardia cryptomeriae* Sawada. However, they were placed in group 2, which consisted of *Botryosphaeria/Fusicoccum* species. Culture characteristics of these isolates, having well-developed aerial mycelium and a gray to dark-colored colony on OMA, with ovoid, fusiform, or ellipsoid conidia, differed from the typical characteristics of *Guignardia*/*Phyllosticta* isolates having a colony colored black, greenish toward margin, and conidia globose, ellipsoid, or obovoide in shape, surrounded by a slime layer, with a apical appendage. In Japan, Guignardia canker disease of *Cryptomeria japonica* and *Thujopsis dolabrata*, caused by *G. cryptomeriae*, has been reported by Kobayashi (1957a,b, 1962) and Sawada (1950). Miyashita (2001) suggested that the causal pathogen, *G. cryptomeriae*, should be transferred to the genus *Botryosphaeria* on the basis of the results of molecular analysis based on ITS regional sequences. This observation is supported in the present study using the sequences of the 18S rDNA region.

Similarly, *Phyllosticta alcides* Sacc. (MUCC0084 and 0085) and *P. populorum* Sacc. & Roum. (MUCC0083) belonged to group 3, which consists of *Phoma* spp. These two species were treated previously as identical to *Phoma macrostoma* Mont. var. *macrostoma* (Gruyter et al. 2002) and *Phoma macrostoma* Mont. (van der Aa and Vanev 2002), respectively. Similarly, an isolate of *Phyllosticta petasitidis* Ellis & Everh. (MUCC0103) belonging to group 4 was identified as a species of *Phomopsis* based on the shape

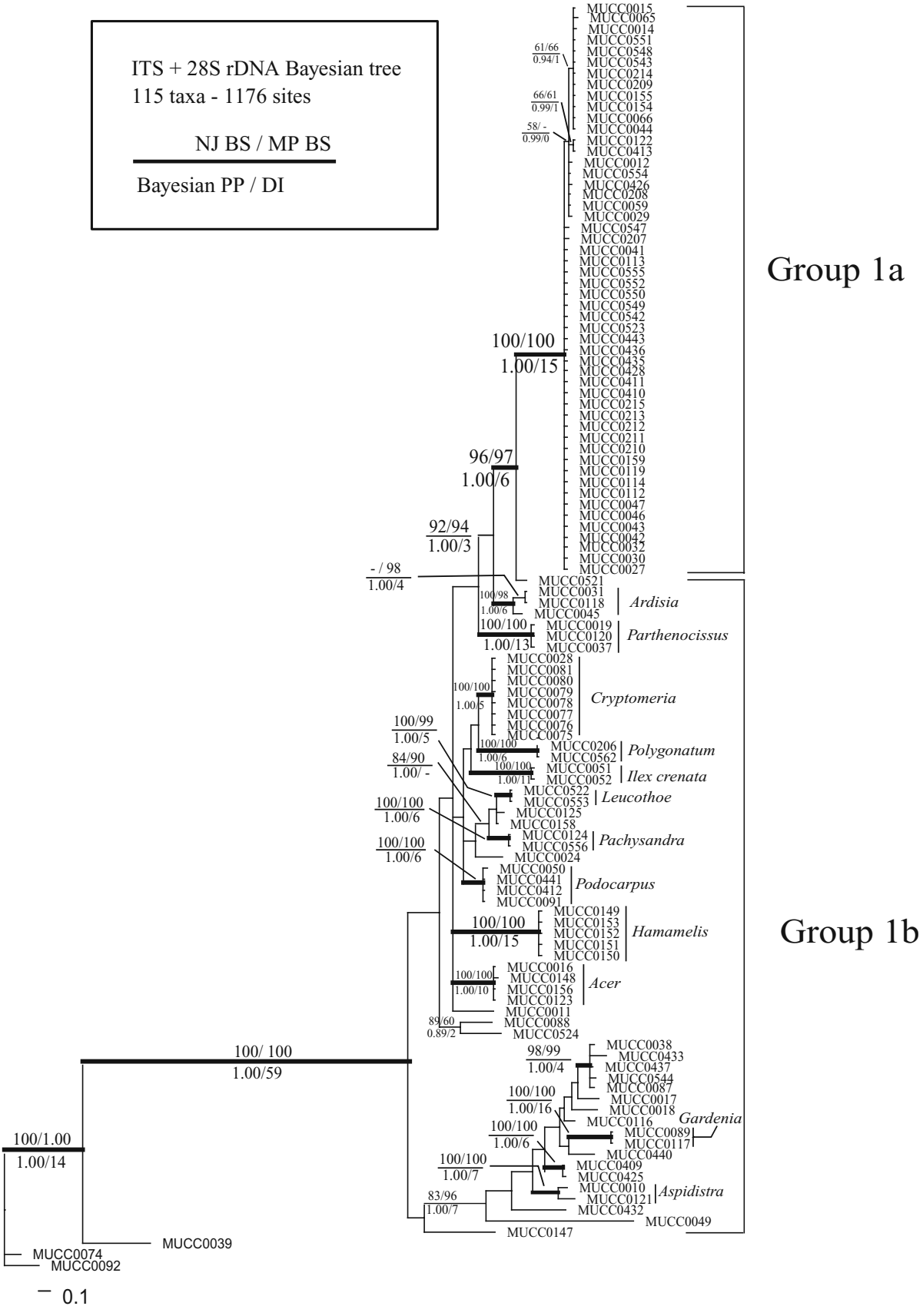

Fig. 2. Phylogenetic tree based on nuclear internal transcribed spacer ITS and 28S rDNA sequence for *Phyllosticta* s. str. inferred by Bayesian analysis. Nodes of support values are shown above and below. *NJ BS*, neighbor-joining bootstrap values; *MP BS*, maximum-parsimony bootstrap values; *Bayesian PP*, estimates of posterior probabilities; *DI*, decay index. Nodes supported by >90 NJ and MP BS, >0.95 Bayesian PP, and >2 decay index are indicated by *thick lines*

of fusiform conidia on OMA. Thus, the monophyletic clade (group 1) obtained from the phylogenetic analyses of the 18S rDNA region showed that *Phyllosticta* s. str. and its teleomorph *Guignardia* diverged from a single ancestral taxon.

The phylogenetics tree of ITS-28S rDNA of *Phyllosticta* s. str. and its teleomorph *Guignardia* was divided into two subgroups, 1a and 1b, using NJ, MP, and Bayesian analyses (see Fig. 2). Group 1a consisted of isolates from many different host species. Okane et al. (2003) concluded that *G. endophyllicola* was a polyxenic species inhabiting intact leaves of 67 plant species in 54 genera of 38 families. Baayen et al. (2002) reported that *G. mangiferae* (including *G. endophyllicola* as a synonymous species) is an endophytic polyxenic species isolated from woody plants belonging to numerous families. Okane et al. (2003) speculated that *G. endophyllicola* is a primitive species that has not established strict host specificity. However, the present phylogenetic analyses of ITS-28S regions suggest that the host range of *G. mangiferae* differentiated from monoxenic into polyxenic. In other words, the fungus might have established a niche as an endophytic fungus in various host plants without killing the host cells. On the other hand, it might have been enclosed or limited in a cell by the protective reaction of host plants after the invasion. For those who adopt the latter hypothesis, it is thought that *G. mangiferae* had sought a suitable host plant by host-jumping. Within plant parasitic fungi, a powdery mildew genus, *Golovinomyces*, has been used as a typical example (Matsuda and Takamatsu 2003) of host expansion by host-jumping. If so, the question remains: Is there any species that already specialized in a specific host in group 1a? In the phylogenetic tree of ITS-28S (see Fig. 2), group 1a included three subgroups having one or two site changes in the ITS2 and 28S regions. Among the isolates belonging to group 1a, *Guignardia alliacea* Motohashi, Jun. Nishikawa & C. Nakash. (Motohashi et al. 2008) (MUCC0014, -0015) has strong pathogenicity to *Allium fistulosum*. This species may provide supporting evidence of host-jumping. Hereafter, these strains of subgroups within group 1a might differentiate into a new species within suitable and specific host plants after many years. To confirm the hypothesis of host-jumping in the isolates of group 1a, it is necessary to observe the movement of mycelium and response of plant cells in the living tissues after inoculation and also to examine additional samples.

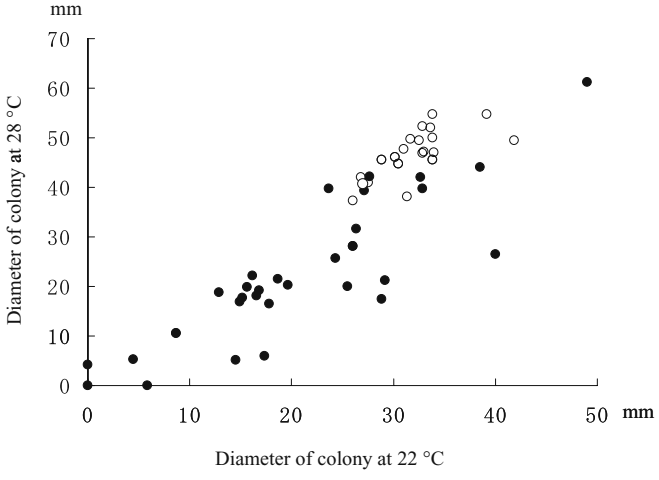

Fig. 3. Colony diameter when grown on oatmeal agar medium for 7 days. ○, Isolates belonging to group 1a; ●, isolates belonging to group 1b

Analyses showed the presence of several clades in group 1b, each of which was parasitic on a single host genus: *Phyllosticta ampelicida* (Engelm.) Aa on *Parthenocissus tricuspidata*, *P. cryptomeriae* Kawam. (synonym: *P. concentrica* Sacc.) on *Cryptomeria japonica*, *P. cruenta* (Fr.) J. Kickx f. on *Polygonatum odoratum* var. *pluriflorum*, *Phyllosticta* sp. and *Guignardia* sp. on *Podocarpus macrophyllus*, *P. hamamelidis* Peck on *Hamamelis japonica*, and *P. minima* (Berk. & M.A. Curtis) Underw. & Earle on *Acer* spp. These monophyletic clades were supported by 100% in BS in NJ and MP analyses, 4 or higher in DI, and 0.98 in Bayesian

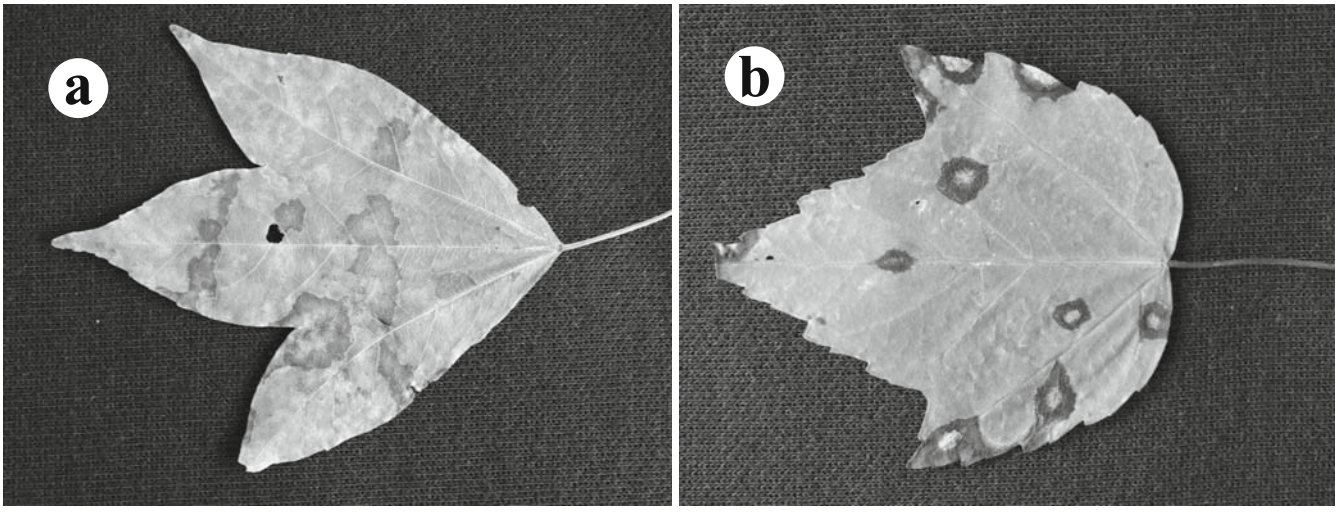

Fig. 4. Comparison of symptoms among *Phyllosticta* groups 1a and 1b on *Acer*. **a** Symptoms caused by MUCC0029 (group 1a). **b** Symptoms caused by MUCC0016 (group 1b), same as symptoms caused by MUCC0148, -0123, and -0156

PP. From this result, the respective isolates placed in each monophyletic clade in group 1b should be recognized as separate species. In group 1b, this phenomenon is exemplified by the species concept of *Phyllosticta* s. str. in which species epithets have been acceptable for isolates from diseased leaves of host plants with distinguishable morphology and culture characteristics (van der Aa 1973; van der Aa and Vanev 2002). The phylogeny of *Phyllosticta* within group 1b is not in accord with the phylogeny of higher plant taxa because both monocotyledonous and dicotyledonous host plants are scattered throughout the group.

Isolates of the polyxenic species *P. concentrica* (teleomorph: *G. philoprina*), known as a exceptional species having various host plants regardless of van der Aa's species concept (van der Aa 1973), were scattered throughout groups 1a and 1b. It thus appears appropriate to treat *P. concentrica* as an unsubstantiated "complex species" composed of several lineages. *Phyllosticta cryptomeriae* has been treated as a synonymous species of *P. concentrica*, based on the description of morphological characteristics without the observation of type specimens (van der Aa and Vanev 2002). The isolates of *P. cryptomeriae* that formed an individual monophyletic clade differed from *P. concentrica* (Fig. 2; BS = 100 in NJ and MP, DI = 5, PP = 1.00). *Guignardia sawadae* Tak. Kobay. (Kobayashi and Sasaki 1975) has been described as the teleomorph *P. cryptomeriae* based on the fruiting body formed on *Cryptomeria japonica*. Although eight isolates of *P. cryptomeriae* formed a monophyletic clade in group 1b, the isolate of *G. sawadae* (MUCC0066) located to group 1a with the polyxenic (endophytic) species, *G. mangiferae*. A difference in colony growth rate was observed between the isolates (see Table 2). As a result, we suggest that *P. cryptomeriae* should be redescribed as a independent species. Similarly, *Phyllosticta kobus* from *Magnolia kobus* (MUCC0049) and *Phyllosticta azevinhi* Torrend from *Ilex crenata* (MUCC0051 and 0052) should also each be treated as separate species.

Although some isolates were obtained from the same host plant species, these were placed separately into the monoxenic clade (group 1b) and the polyxenic clade (group 1a). Isolates belonging to the separate clades should be treated as separate species. Bissett (1979) reported differences among three species of *Phyllosticta* on *Smilax* spp. based on size and shape of conidia and thickness of the slime layer. Similarly, Baayen et al. (2002) reported that the slime layer of conidia of *P. citricarpa* is thinner than that of *P. capitalensis*. Moreover, the colony growth rate of *P. citricarpa* was slower than that of *P. capitalensis*, and the former produced yellow pigmentation on OMA. The isolates from *Aspidistra elatior* are distinguishable from each other in size of conidia (9.5–12.5 × 8.5–10 μm on MUCC0010 and 0121, 7.4–11 × 6.1–7.5 μm on MUCC0122). However, differences in size and shape of conidia and thickness of slime layer among the isolates from *Acer* spp. and *Aesculus* spp. were not observed. As described above, isolates of group 1a tended to grow fast at both temperatures (Table 2, Fig. 3). However, this does not necessarily differentiate between the isolates of group 1a and 1b. Similarly, the ability to produce yellow pigmentation at the colony margin on OMA was a poor marker for discrimination between these groups. Either character, conidial size or colony growth rate, may be a distinguishable point for grouping discrete isolates originating from the same host plant species. In addition, the leaf symptoms on *Acer* spp. were easily and clearly distinguishable between group 1a and group 1b (Fig. 4). The different symptoms may exemplify the different behavior of the fungi, i.e., plant pathogens or endophytes (Fig. 4).

The present study has clarified phylogenetic relationships among Japanese species of *Phyllosticta* s.l., especially between plant pathogenic and endophytic species. Phylogenetic analyses of plant pathogenic species support the validity of the species concept of van der Aa and Vanev (2002). Molecular analyses of rDNA were revealed to be useful for reconstruction of the classification of *Phyllosticta*, although group 1a consisted of phenotypically definable plant pathogenic species, namely, *P. alliaceae*, *P. cameriae*, *P. concentrica*, *P. conjac*, *P. cryptomeriae*, *P. fallopiae*, *P. miurae*, *P. sphaeropsoidea*, and isolates of unidentified species, some of which are similar or identical with *P. capitalensis* known as endophytic fungi in ITS-28S rDNA sequence. This result shows classification within group 1a using single-locus analysis should not be considered. Moreover, *Phyllosticta alliaceae*, having a small difference in its ITS-28S rDNA sequence, has strong pathogenicity to *Allium* plants with severe symptoms (Motohashi et al. 2008). The acquisition of a specific host plant must be recognized as the point of speciation. Speciation and host specificity within group 1a must be revealed by the more detailed phylogenetic analyses using multiregional sequences, as Taylor et al. (2000) proposed. Similarly, morphology (size and thickness of slime layer of conidia) or colony growth on medium were unsuitable for recognition of species having different nucleotides from the same host genus. In the classification of the genus *Phyllosticta*, it is necessary to consider an integrated approach, such as molecular phylogeny, host, colony growth rate, symptoms, and shape of conidia.

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