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The Host-Pathogen Association of *Exobasidium* in Japan Inferred from Molecular Phylogeny of ITS and Large Subunit rDNA Sequences

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ITS領域とrDNA D1/D2領域のシークエンスより解析した
日本産もち病菌の宿主―病原菌関連性

長尾英幸・糟谷大河

Summary――Phylogenetic relationships among *Exobasidium* species were estimated by the concatenated ITS and LSU sequences of 72 isolates obtained from fresh material. The phylogenetic tree suggested 6 host-specific groups for *Exobasidium* spp. with high support values. A *Rhododendron* clade comprises three groups: Group 1 includes two witches' broom causal species, *E. nobeyamense* and *E. pentasporium,* that were phylogenetically distinguished, while *E. nobeyamense, E. otanianum* and *E. cylindrosporum* were unresolved. Group 2 comprised *E. japonicum* and three species that are pathogenic to subgenus *Hymenanthes* plants. Group 3 comprised species pathogenic to section *Rhododendron* and *Tsutsusi* plants that cause leaf blister. Group 4 comprised five species pathogenic to Vaccinioideae. Groups 5 and 6 are characterized by host specificity to *Camellia* and *Symplocos*, respectively. The six groups are independent of morphological characteristics for basidiospores and colony appearances. The concatenated ITS and LSU sequences could be used to predict host preference of *Exobasidium* isolates.

Key Words: Basidiomycetes, *Exobasidium*, Ericaceae, Japan, molecular phylogenetic analysis

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1. Introduction

Members of the Exobasidiales are known as plant parasites and are classified in four families (Brachbasidiaceae, Exobasidiaceae, Cryptobasidiaceae and Graphiolaceae) based on mode of sporulation (Bauer et al. 2001). In the Exobasidiaceae, the parasitism of *Exobasidium* is recorded on different host plants in the families Escalloniaceae (Gómez and Kisimova-Horovitz, 1997, 1998), Ericaceae, Saxifragaceae, Symplocaceae and Theaceae (Nannfeldt, 1981 ; Savile, 1959 a; Ezuka, 1990 a, 1990 b, 1991 a, 1991 b). In Oceania and South America, Ericaceae subfamily Styphelioideae (Chlebicki and Chlebická, 2007 ; McNabb, 1962) is also described as including host plants. *Exobasidium* induces different types of symptoms, such as galls on leaves, buds, flowers, fruits, and trunks, leaf blisters and blast, shoestring leaf, and witches' bloom (Fig. 1). A causal relationship is not found between the plant taxa and symptoms. Rather, the symptom appearance is specific to the part of the plant infected on different host plants.

Exobasidiales is characterized by morphologically diverse taxa (Begerow et al., 1997 ; Bauer et al., 2001). The taxonomy of *Exobasidium* has been particularly controversial due to their simple morphology, the variable symptoms they induce, and their wide host range (Burt, 1915 ; Ezuka, 1990 b; Nannfeldt, 1981 ; McNabb, 1962 ; Savile, 1959 a; Sundström, 1964). Savile (1959 a) synonymized many *Exobasidium* species isolated from different host plants into a few species based on unicellular or multicellular basidiospores. Nannfeldt (1981), however, based species differentiation on the mode of basidiospore germination and cultural characteristics, which were previously studied by Sundström (1964). According to Nannfeldt-Sundström's morphological species concept, Japanese *Exobasidium* species were re-assessed by comparing the morphology of basidia, basidiospores and sterigmata, and the mode of basidiospore germination (Nagao et al. 2001, 2003 a, 2003 b, 2004 a, 2004 b, 2006). The successful inoculation tests by the isolates of *Exobasidium* on the cultivated plants of *Camellia, Rhododendron,* and *Vaccinium* species were reported (Ezuka, 1955 ; Graafland, 1960 ; Sundström, 1964 ;Nickerson and Vander Kloet, 1997). Sundström (1964) confirmed difference in pathogenicity of *E. vaccinii* "vit. id" to the susceptible *V. vitis-idaea* L.

Molecular analyses of the nuclear LSU rDNA have supported the monophyly of this group (Begerow et al. 1997 ; Bauer et al. 2001), whereas those from 18 S rDNA did not, even when only three species of *Exobasidium* were examined (Döring and Blanz 2000). A controversial species on *Saxifraga, Arctiomyces warmingii* (Rostr.) Savile, was placed in Exobasidiaceae using LSU molecular analysis (Begerow *et al.*, 2002). Savile (1959 b) erect-

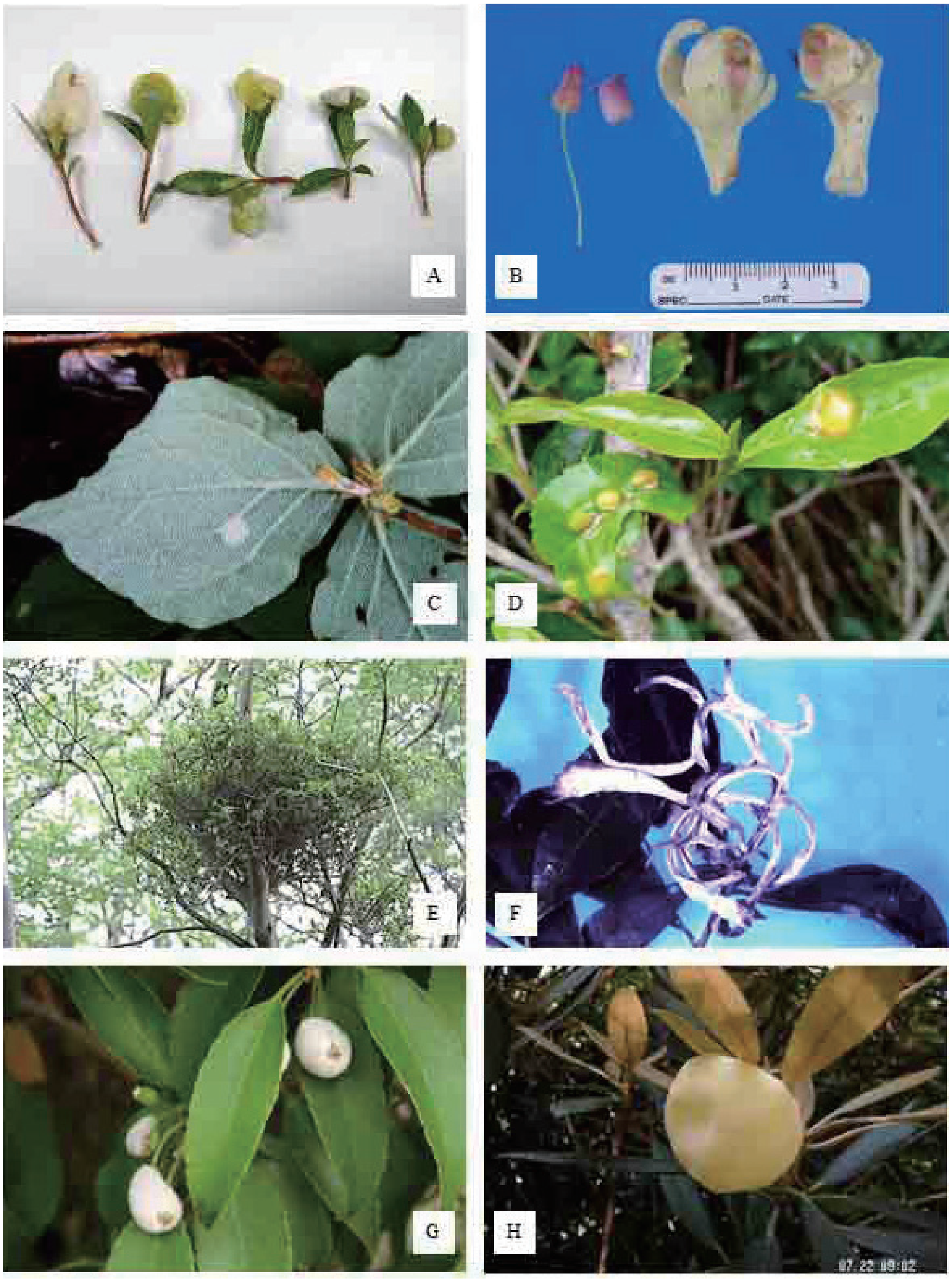

Fig. 1. Symptoms caused by *Exobasidium*. A. Gall on leaves of *Rhododendron*, B. Gall on flower of *Vaccinium*, C. Leaf blister of *Rhododendron*, D. Leaf blister blight of *Camellia*, E. Witches' broom of *Rhododendron*, F. Shoot blight of *Symplocos*, G. Fruit deformation of *Symplocos*, H. Gall on leaf of *Rhododendron*.

ed the new genus *Arctiomyces* based on the intermediate morphological characteristics between *Exobasidium* and *Kordyana*. The legitimate name for this species is *Exobasidium warmingii* Rostr. according to its morphology (Donk, 1966 ; Müller, 1977). Even though Begerow et al. (2002) did not refer to these studies, molecular analysis supported the position of this species in Exobasidiaceae with 78 % bootstrap value by neighbor-joining analysis. On the other hand, *Exobasidium lauri* Geyl. on *Laurus novocanariensis* Rivas Martínez, Lousã, Fernández Prieto, Días, Costa & Aguiar was excluded and moved to *Laurobasidium* in Cryptobasidiaceae supported by molecular re-assessments (Begerow et al. 2002). *Laurobasidium hachijoense* (Y. Otani, Kakish. & Iijima) Kakish., Nagao & Denchev is recently legitimately described as the new combination of *E. hachijoense* Y. Otani *et al.* by the morphological evidence and molecular analysis (Kakishima et al., 2017). The phylogenetic position of this species has been placed in Cryptobasidiaceae (*Clinoconidium* spp. and *L. lauri*) by other researchers in the provisional name of '*Laurobasidium hachijoense*' (Maier et al., 2006 ; Wang et al., 2015).

Piątek et al. (2012) chose the way of Nannfeldt-Sundström's morphological analyses (Nannfeldt, 1981 ; Sundström, 1964) and applied the concatenated ITS+LSU sequence tree for the speciation of *Exobasidium darwinii* M. Piatek & M. Lutz, which systemically infects *Vaccinium reticulatum* Sm. The concatenated ITS+LSU sequence tree resolved the relationships within the cluster of six species that were unresolved using only LSU sequences. Kennedy et al. (2012) adopted the concatenated ITS+LSU sequence tree for the identification of new species *Exobasidium ferrugineae* Minnis, Kenn. & Goldberg on flowers of *Lyonia ferruginea* (Michx.) G.S. Torr.

For species identification, Nannfeldt-Sundström's morphological analyses was supported by the molecular analysis using the concatenated ITS+LSU (Kennedy et al., 2012 ; Piątek et al., 2012), whereas two different pathogenic *Exobasidium* species were recognized on one host plant either *Rhododendron* or *Vaccinium* (Nannfeldt, 1981). Japanese *Exobasidium* species on *Rhododendron* species were much better documented than those on *Vaccinium* species. We picked up the examples on *Rhododendron* species and *Symplocos lucida* Sieb. et Zucc.; i.e. *E. japonicum* Shirai, *E. pentasporium* Shirai, and *E. japonicum* var. *hypophyllum* Ezuka on *R. kampferi* Planch., *E. yoshinagae* P. Henn. and *E. nobeyamense* Nagao & Ezuka on *R. wadanum* Makino, *E. yoshinagae* and *E. otanianum* Ezuka emend. Nagao on *Rhododendron* subgen. *Tsutsusi*, *E. cylindrosporum* Ezuka and *E. kawaense* Ezuka on *R. macrosepalum* Maxim., and *E. symploci-japoniccae* Kusano & Tokubuchi and *E. symploci-japoniccae* var. *caprogenum* Nagao & S. Ogawa on *S. lucida.* The focus of the present study was to clarify the host-parasite and phylogenetic relationships with Japanese *Exobasidium* isolates using ITS and LSU sequences adopted by Piątek et al. (2012).

2. Materials and methods

Eighty-eight Japanese *Exobasidium* isolates and 7 Cryptobasidiales, i.e., four isolates of *Clinoconidium* spp. and three isolates of *L. hachijoense*, were used to extract DNA as described below. A total of 70 ITS and 82 LSU sequences were prepared (Table 1). Twenty-six sequences were retrieved from the GenBank (Table 2). ITS (Acc. No. AY854090) and LSU (Acc. No. L20287) data of *Ustilago maydis* (DC.) Corda were used as an outgroup.

The fungal DNA was extracted using the sodium dodecyl sulfate (SDS) extraction procedure by Suyama et al. (1996) and PCR amplification profiles by Virtudazo et al. (2001) and Takeuchi and Nagao (2004) were used with slight modifications. Fungal samples were scraped from the surface of 14–day-old colonies and incubated in 20 μL extraction buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % Proteinase K, and 0.01 % SDS for 1 h at 37 °C and then held for 10 min at 95 °C. The extract was then centrifuged at 6,000 rpm and the supernatant was subsequently used as the template for PCR amplification. PCR was performed using a HotstartTaq master Mix (Qiagen, Hilden, Germany). The primers ITS 1 F (Gardes and Bruns, 1993) and ITS 4 (White et al. 1990) were used for the ITS regions and NL1 and NL4 (O'Donnell, 1993) for the D1 / D2 regions of LSU rDNA (Table 3). The PCR amplification profile was set as follows: an initial denaturation step for 15 min at 95 °C; followed by 35 cycles of 30 s denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C; with a final extension period of 10 min at 72 °C. PCR products were then electrophoresed on 1 % (w/v) agarose gels containing 0.5 μg/mL ethidium bromide in TAE buffer composed of 40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH 7.4, to confirm successful amplification. PCR products were purified with MicroSpin columns S-400 HR (Amersham Biosciences Corp., Piscataway, NJ, USA) according to the manufacture's protocol. Amplified products were sequenced with a BigDye dye terminator kit (Applied Biosystems, Foster City, CA, USA). AutoSeq G-50 (Amersham Biosciences Corp., Piscataway, NJ, USA) was used to remove excess fluorescent dye-terminators from cycle sequencing reactions prior to analysis on an ABI 377 or ABI 3100 automated DNA sequencer (Perkin Elmer Co., Foster, CA, USA).

Multiple DNA sequences were initially aligned independently for both genes using Clustal W ver. 2.1 on the BBDJ web site (http://clustalw.ddbj.nig.ac.jp/index.php?lang=-ja) with default settings and the results were output to a NEXUS file. Phylogenetic analysis was conducted by PAUP* 4.3. 99.169.0 (Swofford, 1998). Gaps in sequences were treat-

Table 1. List of Japanese *Exobasidium* examined in this study.

Isolate	Species	Voucher	Host plant	Locality	GenBank accession No.	
					d1 /d2	ITS
EOS44 (K44)	*Exobasidium otanianum*		*Rhododendron dilatatum* var. *satsumense*	Kagoshima Pref.	AB178257	AB180373
EOS59 (K59)	*Exobasidium otanianum*		*Rhododendron dilatatum* var. *satsumense*	Kagoshima Pref.	AB178256	AB180374
IFO 30151	*Exobasidium shiraianum*		*Rhododendron degronianum*	Japan	AB177595	
IFO 30152	*Exobasidium bisporum*		*Eubotryoides grayana* var. *oblongifolia*	Japan	AB177596	AB180368
IFO 30393	*Exobasidium reticulatum*		*Camellia sinensis*	Japan	AB177569	AB180369
IFO 30394	*Exobasidium reticulatum*		*Camellia sinensis*	Japan	AB177570	
IFO 30756	*Exobasidium japonicum*		*Rhododendron lateriticum*	Japan		AB180370
IFO 7790	*Exobasidium symploci-japonicae* var. *symploci-japonicae*		*Symplocos lucida*	Japan	AB177597	AB180363
IFO 9942	*Exobasidium bisporum*		*Eubotryoides grayana* var. *glabra*	Japan	AB177598	AB180364
IFO 9959	*Exobasidium yoshinagae*		*Rhododendron reticulatum*	Japan	AB177599	AB180365
IFO 9960	*Exobasidium otanianum*		*Rhododendron retuculatum* f. *retuculatum*	Japan	AB177600	AB180366
IFO 9961	*Exobasidium pieridis-ovalifoliae*		*Lyonia ovalifolia* ssp. *neziki*	Japan	AB177601	AB180367
IFO 9962	*Exobasidium kishianum*		*Vaccinium hirtum* var. *pubescens*	Japan	AB177602	
MAFF 238175	*Exobasidium japonicum*	TSH-B0009	*Rhododendron obtusum* var. *kaempferi*	Shizuoka Pref.		AB178941
MAFF 238176	*Exobasidium japonicum*	TSH-B0011	*Rhododendron indicum*	Ibaraki Pref.	AB177548	AB180315
MAFF 238177	*Exobasidium cylindrosporum*	TSH-B0127	*Rhododendron macrosepalum*	Shizuoka Pref.	AB176713	
MAFF 238178	*Exobasidium cylindrosporum*	TSH-B0015	*Rhododendron* × *pulchrum*	Gunma Pref.	AB178243	
MAFF 238179	*Exobasidium pentasporium*		*Rhododendron obtusum* var. *kaempferi*	Nagano Pref.		AB180316
MAFF 238330	*Laurobasidium hachijoense*		*Cinnamomum japonicum*	Tokyo Metropol.	AB177566	
MAFF 238578	*Exobasidium camelliae*		*Camellia japonica*	Chiba Pref.	AB176712	AB180317
MAFF 238579	*Exobasidium cylindrosporum*	TSH-B0107	*Rhododendron* × *pulchrum*	Ibaraki Pref.		AB180318
MAFF 238580	*Exobasidium dubium*	TSH-B0074	*Rhododendron yedoense* var. *yedoense* f. *yedoense*	Hokkaido Pref.	AB178242	
MAFF 238581	*Exobasidium dubium*	TSH-B0076	*Rhododendron yedoense* var. *yedoense* f. *yedoense*	Hokkaido Pref.	AB178250	
MAFF 238582	*Exobasidium dubium*	TSH-B0077	*Rhododendron yedoense* var. *yedoense* f. *yedoense*	Hokkaido Pref.	AB177563	AB180319
MAFF 238583	*Exobasidium miyabei*	TSH-B0017	*Rhododendron dauricum*	Tokyo Metropol.	AB177550	AB180320
MAFF 238585	*Exobasidium gracile*	TSH-B0108	*Camellia sasanqua*	Shizuoka Pref.		AB180321
MAFF 238586	*Exobasidium gracile*	TSH-B0069	*Camellia sasanqua*	Tokyo Metropol.		AB180322
MAFF 238587	*Exobasidium japonicum*	TSH-B0046	*Rhododendron obtusum* var. *kaempferi*	Nagano Pref	AB177586	AB180323
MAFF 238588	*Exobasidium japonicum*	TSH-B0063	*Rhododendron obtusum* var. *kaempferi*	Kyoto Pref.		AB180324
MAFF 238589	*Exobasidium japonicum*	TSH-B0072	*Rhododendron obtusum* var. *kaempferi*	Hokkaido Pref.	AB177571	AB180325
MAFF 238590	*Exobasidium japonicum*		*Rhododendron obtusum* var. *kaempferi*	Ibaraki Pref.	AB178246	
MAFF 238591	*Exobasidium japonicum*		*Rhododendron obtusum* var. *kaempferi*	Ibaraki Pref.	AB177568	AB180326
MAFF 238592	*Exobasidium japonicum*	TSH-B0125	*Rhododendron kiusianum*	Kagoshima Pref.		AB180327
MAFF 238594	*Exobasidium miyabei*	TSH-B0075	*Rhododendron dauricum*	Hokkaido Pref.	AB177561	AB180328
MAFF 238595	*Exobasidium miyabei*	TSH-B0087	*Rhododendron dauricum*	Hokkaido Pref.	AB177579	AB180330
MAFF 238596	*Exobasidium nobeyamense*	TSH-B0001	*Rhododendron wadanum*	Nagano Pref.	AB178247	AB180329
MAFF 238597	*Exobasidium nobeyamense*	TSH-B0002	*Rhododendron wadanum*	Nagano Pref.	AB177582	AB180331
MAFF 238598	*Exobasidium nobeyamense*	TSH-B0004	*Rhododendron wadanum*	Nagano Pref.	AB177583	AB180332
MAFF 238599	*Exobasidium nobeyamense*	TSH-B0003	*Rhododendron wadanum*	Ibaraki Pref.	AB177585	AB180333
MAFF 238600	*Exobasidium pentasporium*		*Rhododendron obtusum* var. *kaempferi*	Nagano Pref.	AB177581	AB180334
MAFF 238601	*Exobasidium pentasporium*		*Rhododendron obtusum* var. *kaempferi*	Nagano Pref.	AB177567	AB180335
MAFF 238602	*Exobasidium shiraianum*	TSH-B0023	*Rhododendron degronianum*	Nagano Pref.	AB177549	AB180336
MAFF 238603	*Exobasidium shiraianum*	TSH-B0024	*Rhododendron degronianum*	Nagano Pref.	AB177584	AB180337
MAFF 238604	*Exobasidium shiraianum*	TSH-B0025	*Rhododendron degronianum*	Nagano Pref.	AB178248	AB180338
MAFF 238605	*Exobasidium symploci-japonicae* var. *symploci-japonicae*	TSH-B0040	*Symplocos lucida*	Shimane Pref.	AB176711	AB180339
MAFF 238606	*Exobasidium yoshinagae*		*Rhododendron wadanum*	Nagano Pref.	AB177551	AB180340
MAFF 238607	*Exobasidium yoshinagae*		*Rhododendron wadanum*	Nagano Pref.	AB177590	AB180341
MAFF 238608	*Exobasidium cylindrosporum*	TSH-B0012	*Rhododendron* sp.	Aomori Pref.	AB178245	

Isolate	Species	Voucher	Host plant	Locality	GenBank accession No.	
					d1 /d2	ITS
MAFF 238609	*Exobasidium cylindrosporum*	TSH-B0014	*Rhododendron* × *pulchrum*	Gunma Pref.	AB178244	
MAFF 238610	*Exobasidium woronichinii*	TSH-B0022	*Rhododendron brachycarpum*	Nagano Pref.	AB177588	AB180342
MAFF 238611	*Exobasidium otanianum*	TSH-B0058	*Rhododendron hyugaense*	Miyazaki Pref.	AB177576	AB180343
MAFF 238612	*Exobasidium otanianum*	TSH-B0059	*Rhododendron hyugaense*	Miyazaki Pref.	AB177554	AB180344
MAFF 238613	*Exobasidium otanianum*	TSH-B0061	*Rhododendron retuculatum* f. *glabrescens*	Miyazaki Pref.	AB177593	AB180345
MAFF 238614	*Exobasidium dubium*	TSH-B0078	*Rhododendron yedoense* var. *yedoense* f. *yedoense*	Hokkaido Pref.	AB177564	AB180346
MAFF 238616	*Exobasidium inconspicuum*	TSH-B0080	*Vaccinium hirtum* var. *pubescens*	Hokkaido Pref.	AB177556	AB180347
MAFF 238617	*Exobasidium woronichinii*	TSH-B0081	*Rhododendron brachycarpum*	Hokkaido Pref.	AB177557	AB180348
MAFF 238618	*Exobasidium woronichinii*	TSH-B0085	*Rhododendron brachycarpum*	Hokkaido Pref.		AB180349
MAFF 238619	*Exobasidium inconspicuum*	TSH-B0086	*Vaccinium hirtum* var. *pubescens*	Hokkaido Pref.		AB180350
MAFF 238620	*Exobasidium symploci-japonicae* var. *caprogenum*	TSH-B0090	*Symplocos lucida*	Fukuoka Pref.	AB177559	AB180351
MAFF 238621	*Exobasidium pachysporum*	TSH-B0121	*Vaccinium uliginosum*	Tochigi Pref.	AB177573	AB180352
MAFF 238622	*Exobasidium woronichinii*	TSH-B0018	*Rhododendron brachycarpum*	Nagano Pref.	AB178240	
MAFF 238623	*Exobasidium kishianum*	TSH-B0070	*Vaccinium hirtum* var. *pubescens*	Aomori Pref.	AB177577	AB180353
MAFF 238624	*Exobasidium kishianum*	TSH-B0071	*Vaccinium smallii*	Aomori Pref.	AB177555	AB180354
MAFF 238625	*Exobasidium woronichinii*	TSH-B0116	*Rhododendron brachycarpum*	Tochigi Pref.	AB177572	AB180355
MAFF 238662	*Exobasidium cylindrosporum*	TSH-B0128	*Rhododendron macrosepalum*	Shizuoka Pref.	AB177589	AB180356
MAFF 238663	*Exobasidium cylindrosporum*	TSH-B0129	*Rhododendron* × *mucronatum*	Shizuoka Pref.	AB177580	AB180357
MAFF 238664	*Exobasidium japonicum*	TSH-B0049	*Rhododendron obtusum* var. *kaempferi*	Nagano Pref.	AB177587	AB180358
MAFF 238665	*Laurobasidium hachijoense*	TSH-B0068	*Cinnamomum japonicum*	Tokyo Metropol.	AB177562	AB180359
MAFF 238666	*Exobasidium woronichinii*	TSH-B0083	*Rhododendron brachycarpum*	Hokkaido Pref.	AB177578	AB180360
MAFF 238667	*Exobasidium woronichinii*	TSH-B0114	*Rhododendron brachycarpum*	Tochigi Pref.	AB177565	AB180361
MAFF 238668	*Exobasidium vaccinii*	TSH-B0120	*Vaccinium vitis-idaea*	Tochigi Pref.	AB177560	AB180362
MAFF 238674	*Exobasidium gracile*	NIAES1471008	*Camellia sasanqua*	Ibarak Pref.		AB180684
MAFF 238677	*Exobasidium otanianum*	NIAES20561	*Rhododendron retuculatum* f. *retuculatum*	Hiroshima Pref.		AB180683
MAFF 238810	*Exobasidium symploci-japonicae* var. *symploci-japonicae*	NIAES20520	*Symplocos lucida*	Fukuoka Pref.		AB180677
MAFF 238811	*Exobasidium symploci-japonicae* var. *symploci-japonicae*	NIAES20521	*Symplocos lucida*	Fukuoka Pref.	AB178255	AB180678
MAFF 238824	*Exobasidium japonicum*	NIAES20571	*Rhododendron obtusum* var. *kaempferi*	Aomori Pref.	AB178251	AB180679
MAFF 238826	*Exobasidium japonicum*	NIAES20572	*Rhododendron obtusum* var. *kaempferi*	Aomori Pref.	AB178253	AB180681
MAFF 238830	*Exobasidium caucasicum*	NIAES20542	*Rhododendron aureum*	Nagano Pref.	AB178254	AB180682
MAFF 239439	*Exobasidium nobeyamense*	INM2–15278–052254	*Rhododendron wadanum*	Tochigi Pref.	AB180378	AB180375
MAFF 239440	*Exobasidium nobeyamense*	INM2–15278–052256	*Rhododendron wadanum*	Tochigi Pref.	AB180379	AB180376
MAFF 239441	*Exobasidium vexans*	INM2–15278–052298	*Camellia sinensis*	Shizuoka Pref.	AB180380	
MAFF 239442	*Exobasidium reticulatum*	INM2–15278–052327	*Camellia sinensis*	Shizuoka Pref.	AB180381	AB180377
MAFF 306193	*Exobasidium pieridis*		*Lyonia ovalifolia* ssp. *neziki*	Shizuoka Pref.	AB177575	
MAFF 306194	*Exobasidium pieridis-ovalifoliae*		*Lyonia ovalifolia* ssp. *neziki*	Shizuoka Pref.	AB177552	
MAFF 306198	*Exobasidium cylindrosporum*		*Rhododendron oomurasaki*	Shizuoka Pref.	AB177574	
TUK-E02	*Laurobasidium hachijoense*		*Cinnamomum japonicum*	Tokyo Metropol.	AB176715	
TUK-E11	*Exobasidium hemisphaericum*		*Rhododendron brachycarpum*	Yamanashi Pref.	AB177591	
TUK-E13	*Exobasidium hemisphaericum*		*Rhododendron brachycarpum*	Yamanashi Pref.	AB176716	
TUK-E21	*Exobasidium gracile*		*Camellia sasanqua*	Ibarak Pref.	AB176714	
TUK-E26	*Exobasidium gracile*		*Camellia sasanqua*	Ibarak Pref.		AB180371
TUK-E30	*Exobasidium gracile*		*Camellia sasanqua*	Ibarak Pref.	AB177592	
TUK-E44	*Exobasidium pieridis-ovalifoliae*		*Lyonia ovalifolia* ssp. *neziki*	Niigata Pref.	AB176710	
TUK-MA01	*Clinoconidium globosum*		*Cinnamomum daphnoides*	Kagoshima Pref.	AB178259	
TUK-MA02	*Clinoconidium globosum*		*Cinnamomum daphnoides*	Kagoshima Pref.	AB178260	
TUK-S703	*Clinoconidium onumae*		*Cinnamomum japonicum*	Kagoshima Pref.	AB178258	
TUK-S720	*Clinoconidium onumae*		*Cinnamomum japonicum*	Kagoshima Pref.	AB177594	

EOS: Laboratory of Plant Pathology, Faculty of Agriculture, Kagoshima University; IFO: Institute of Fermantation, Osaka; MAFF: Genebank in National Institute of Agrobiological Sciences; TUK: Laboratory of Plant Parasitic Mycology, Institute of Agriculture and Forestry, University of Tsukuba

Table 2. List of the referance species and its accession number of Large subunit rDNA.

Species	Host	DDBJ Acc. No.
Exobasidium arescens	*Vaccinium myrtillus*	AF352057
E. bisporum	*Eubtryoides grayana*	AF487386
E. vaccinii	*Vaccinium vitis-idaea*	AF487398
E. japonicum	*Rhododendron indicum = Rhododendron lateritium*	AF487388
E. karstenii	*Andromeda polifolia*	AF487389
E. myrtilli	*Vaccinium myrtillus*	AF487390
E. oxycocci	*Vaccinium oxycoccos*	AF487391
E. pachysporum	*Vaccinium ulginosum*	AF487392
E. pieridis-ovalifoliae	*Lyonia ovalifolia= Lyonia neziki*	AF487393
E. rhododendri	*Rhododendron ferrugineum*	AF009856
E. rostrupii	*Vaccinium oxycoccos*	AF009857
E. shiraianum	*Rhododendron degronianum*	AF487395
E. sundstroemii	*Andromeda polifolia*	AF487396
E. symploci-japonicae	*Symplocos sp.*	AF487397
E. vaccinii	*Vaccinium vitis-idaea*	AF009858
E. vaccinii	*Vaccinium vitis-idaea*	AJ406400
E. warmingii	*Saxifraga bryoides*	AF487380
E. yoshinagae	*Rhododendron reticulatum*	AF487399

Table 3. The primers used in this study.

ITS1 (White et al., 1990)	TCCGTAGGTGTAACCTGCGG
ITS1F (Gardes and Bruns, 1993)	CTTGGTCATTTAGAGGAAGTAA
ITS4 (White et al., 1990)	TCCTCCGCTTATTGATATGC
D1 /D2	
NL-1 (O'Donnelle, 1993)	GCATATCAATAAGCGGAGGAAAAG
NL-4 (O'Donnelle, 1993)	GGTCCGTGTTTCAAGACGG

ed as “missing”. All molecular characters were unordered and given equal weight. Phylogenetic trees were examined in the default setting by neighbor-joining (NJ) method and maximum parsimony (MP) analysis under a heuristic search. Bootstrap values for branch support were assessed with 1000 bootstrap pseudo-replicates with 10 random taxon additions per bootstrap replicate (Felsenstein, 1985). Maximum likelihood was also applied and evaluated by quartet puzzling analysis with 1000 puzzling steps (Schmidt and von Haeseler, 2003 ; Reaz et al., 2014). The quality of the data is evaluated by a support value. A higher support value corresponds to a greater confidence of the bipartition (Schmidt and von Haeseler, 2003).

3. Results

3-1 Phylogenetic analysis for LSU (D1 /D2)

The alignment included 635 total characters of which 406 are constant (proportion = 0.63937), 76 variable characters are parsimony uninformative, while 153 characters are parsimony informative. Neighbor-joining analysis for the datasets of D1 /D2 regions indicated that *Exobasidium* is distinguished from Cryptobasidiaceae (*Clinoconidium* and *Laurobasidium*) with high bootstrap value but the resolution among *Exobasidium* species is poor (Fig. 2). A group composed of *Exobasidium symploci-japonicae* pathogenic to *S. lucida* was supported by high bootstrap value with 99 %, whereas *Exobasidium* spp. pathogenic to *Camellia, Rhododendron, Saxifraga*, and Vaccinioideae were phylogenetically paraphyletic supported by moderate bootstrap value in the neighbor-joining analysis. These groupings were recognized in both the maximum parsimony and the maximum likelihood analyses (Figure is not presented). Some *Exobasidium* spp. pathogenic to *Rhododendron* appeared to be polyphyletic.

3-2 Phylogenetic analysis for ITS regions

The alignment included 822 total characters of which 369 are constant (proportion = 0.448905), 139 variable characters are parsimony uninformative, while 314 characters are parsimony informative. Twice as many parsimony–informative characters were found in the ITS regions as in the D1 /D2 regions of LSU. Topology of neighbor-joining, maximum parsimony and maximum likelihood analyses were similar to those of the D1 /D2 region of LSU but showed higher bootstrap values (Fig. 3). *Exobasidium otanianum* pathogenic to *Rhododendron* spp. appeared to be polyphyletic. One group was pathogenic to *R. dilatatum* Miq. var. *satsumense* T.Yamaz. and *R. hyugaense* (T. Yamaz.) T. Yamaz., and another was pathogenic to *R. reciculatum* f. *reciculatum* D. Don ex G. Don in the maximum likelihood analysis. However, the quartet-puzzling value didn't support two groups.

3-3 Phylogenetic analysis for concatenated ITS+LSU sequences

The alignment included 1479 total characters of which 847 are constant (proportion = 0.572684), 229 variable characters are parsimony uninformative, while 403 characters are parsimony informative. As shown in the phylogenetic tree of ITS regions, the concatenated sequences also produced a similar topology of neighbor-joining, maximum parsimony, and maximum likelihood analyses with higher bootstrap and quartet-puzzling values (Fig. 4). From the phylogenetic tree, the following host-specific groups are recognized; Group 1 composes *E. nobeyamense, E. otanianum, E. cylindrosporum* and *E. pentasporium*, Group 2 *E. japonicum, E. shiraianum, E. caucasicum,* and *E. woronichinii*. Group 3 *E. dubium, E. miyabei*, and *E. yoshinagae*, Group 4 *E. bisporum, E. pieridis-ovalifoliae*, *E. pachysporum, E. inconspicuum, E. kishianum*, and *E. vaccinii*, Group 5 *E. camelliae* and *E. reticulatum* and Group 6 *E. symploci-japonicae*. Groups 1 to 4 are pathogenic to Ericaceae and supported by high bootstrap and quartet-puzzling values.

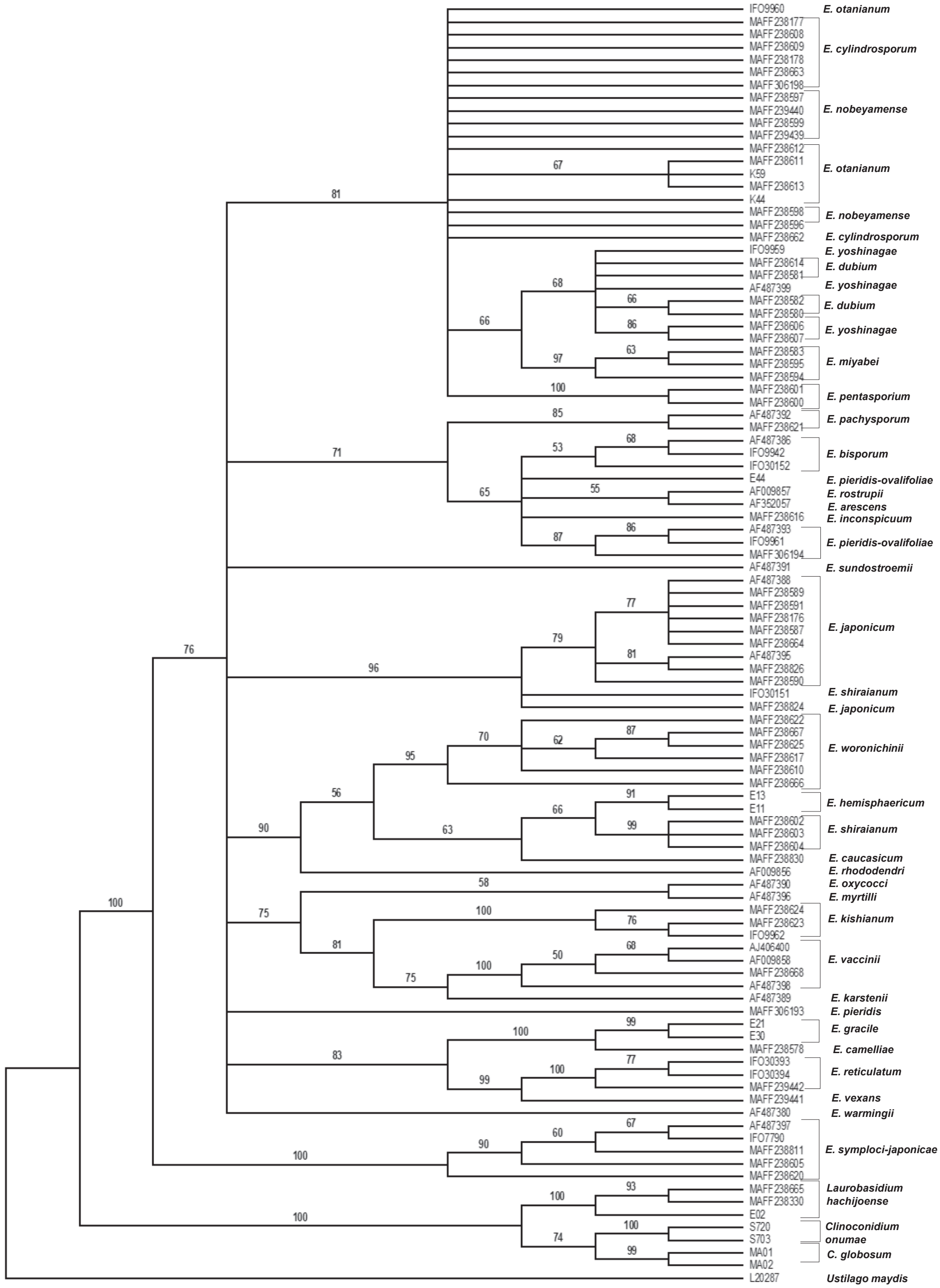

Fig. 2. Topology obtained by neighbor-joining analysis of D1 /D2 domain of LSU rDNA sequences of Japanese isolates of *Exobasidium* and relatives rooted with *Ustilago maydis*. NJ bootstrap values of 1000 replicates are indicated. Values smaller than 50 % are not shown.

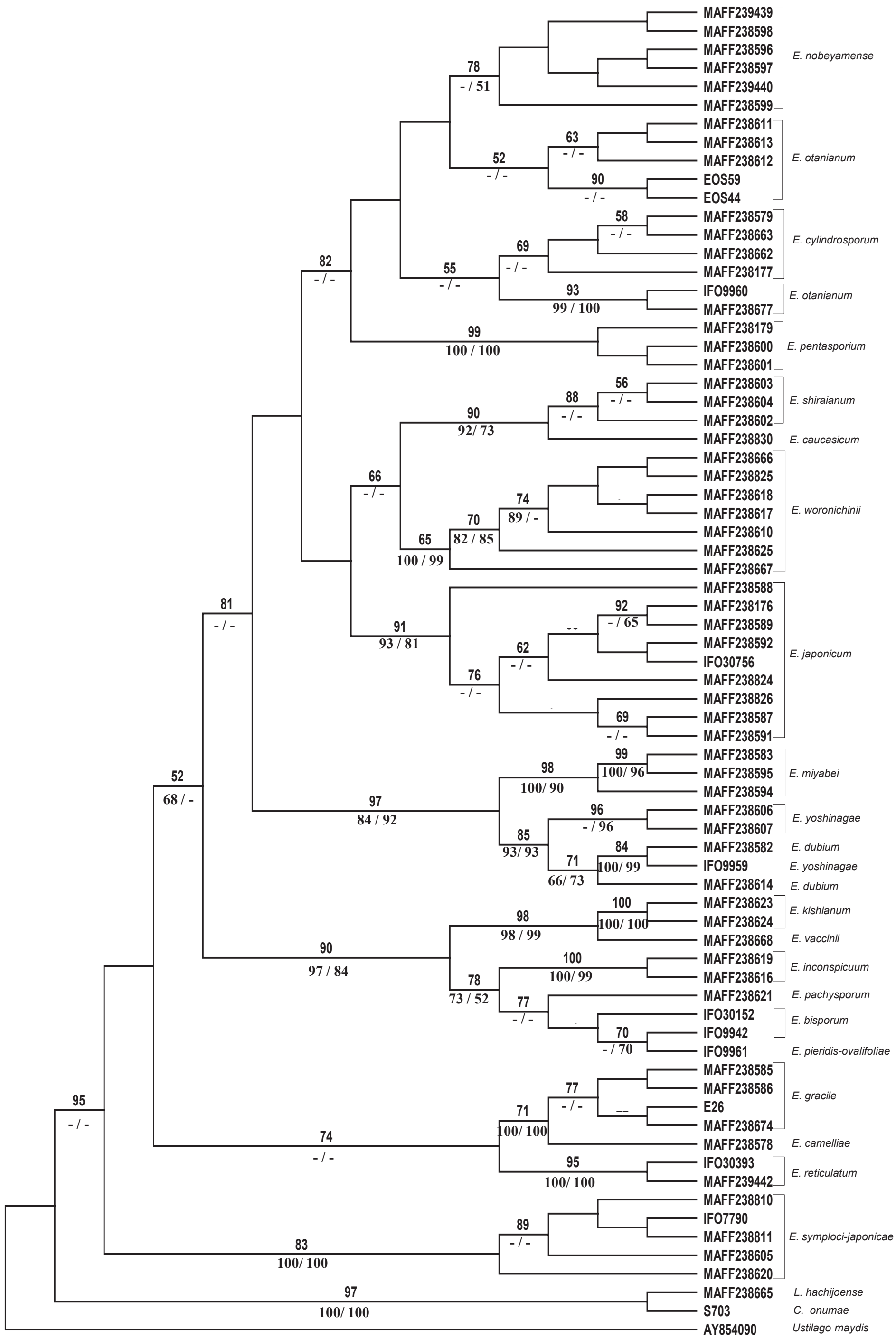

Fig. 3. Topology obtained by maximum likelihood analysis of ITS sequences of Japanese isolates of *Exobasidium* and relatives rooted with *Ustilago maydis*. Quartet puzzling support value of 1000 puzzling steps is indicated above the branch and NJ and MP bootstrap values of 1000 replicates are indicated below the branch from left to right. Values smaller than 50 % are not shown.

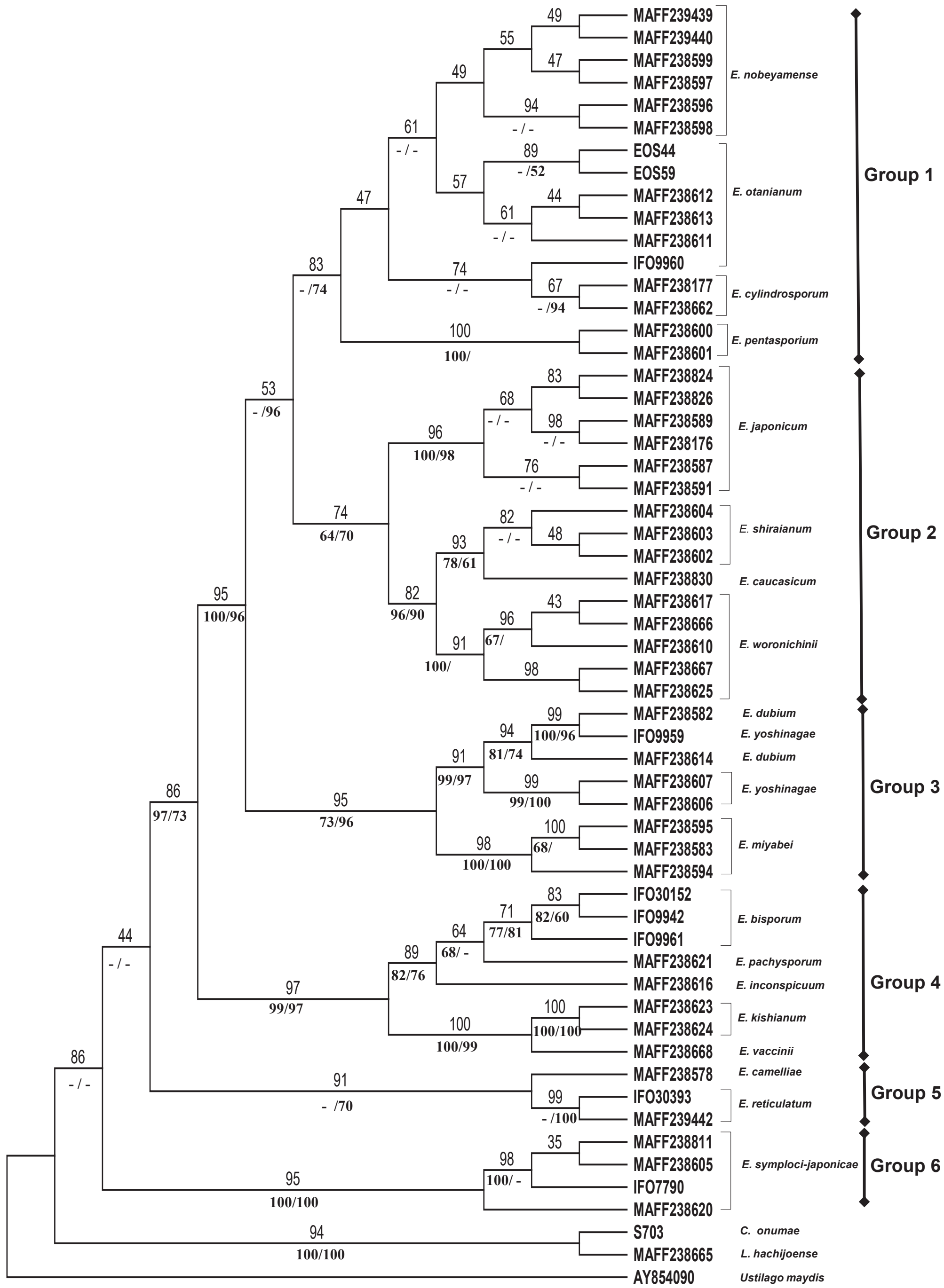

Fig. 4. Topology obtained by maximum likelihood analysis of the concatenated ITS+LSU sequences of Japanese isolates of *Exobasidium* and relatives rooted with *Ustilago maydis*. Quartet puzzling support value of 1000 puzzling steps is indicated above the branch and NJ and MP bootstrap values of 1000 replicates are indicated below the branch from left to right. Values smaller than 50 % are not shown.

4. Discussion

By concatenating the sequences from the LSU and ITS regions, the most parsimony-informative characters are available among these sequence alignments in this study. Therefore, the phylogenetic analysis of maximum likelihood showed clades depended on the species with high quartet-puzzling support values except *E. nobeyamense, E. otanianum*, and *E. cylindrosporum.* In general, six proposed phylogenetic groups were resolved by neighbor-joining, maximum parsimony and maximum likelihood analyses with high bootstrap and quartet-puzzling support values. Previously, concatenated ITS+LSU data were reported to resolve relationships within clades with high bootstrap values (Piątek et al., 2012 ; Fig. 2, Wang et.al., 2015 ; Fig. 2 D). Even with a concatenated ITS+LSU data tree, missing ITS data may lead to different results or a topology like an LSU tree (Kennedy et al., 2012). In our concatenated ITS +LSU trees, *E. hemisphaericum* in Group 2 and *E. vexans* in Group 5 were excluded because ITS data for these isolates were not obtained.

Rapid separation of the Exobasidiomycetidae into several groups was suggested by the short internal distances and the small bootstrap values obtained from LSU analysis (Begerow et al., 1997). Begerow et al. (2002) proposed a plausible evolutionary scenario for *Exobasidium*. In the interpretation of a topology obtained by LSU with 18 *Exobasidium* spp., *Exobasidium* is presumed to arise as a pathogen on the Ericaceae ancestor (Begerow et al., 2002). In their interpretation of an LSU phylogenetic tree, the dichotomy occurred between the *Exobasidium* species on Theales and on the Ericaceae, and further between the *Exobasidium* species on *Rhododendron* and those on Vaccinioideae. Even though the two groupings for the species pathogenic to Vaccinioideae were demonstrated by different studies (Begerow et al., 2002 ; Piątek et al., 2012 ; Brewer et al., 2014 ; and Wang et al., 2015), we wonder whether the results from less informative sequences are reliable. We agree with the statement of Brewer et al. (2014) that molecular phylogenetic studies have supported Nannfeldt's species concept (Begerow et al. 2002 ; Kennedy et al. 2012 ; Piątek et al. 2012) . However, the phylogenetic tree of LSU alone did not resolve the relationships of *Vaccinium* parasites (Fig. 2).

As more distinctive grouping can be proposed by the concatenated ITS+LSU, we focused on the concatenated ITS+LSU tree. Our results also suggested a possible dichotomy between *Camellia* and the Ericaceae but support values were not significant, while a dichotomy between *Symplocos* and the Ericaceae had high support value.

Multiple gene analysis and protein-coding genes including *RPB1*, *RPB2*, and *TEF1*

(Wang et.al., 2015 ;Figs. 1 and 3) didn't suggest a remarkable difference compared with a concatenated ITS+LSU. Further examination should be applied with more informatic and protein-coding genes to identify crucial branching points.

Nannfeldt (1981) compiled the available information for *Exobasidium* in Europe and sifted through the host-specific and omnivorous infections in the Ericales. Referring to Sundström (1964), Nannfeldt (1981) proposed nine new species and a new combination from the examinations of *E. vaccinii* sensu auctt. p.p. and others. *Exobasidium* pathogenic to Vaccinioideae often attack a few species and different part(s) of their hosts. As ITS data of *Exobasidium* pathogenic to Ericaceae was not sufficient to resolve the clades, we also added LSU data of those *Exobasidium* species for the further analysis. The concatenated ITS+LSU of those *Exobasidium* may resolve the phylogenetic distinction. Ezuka (1991 b) disputed the host range of *Exobasidium bisporum* Sawada ex Ezuka since Sawada (1950) reported two different genera as host plants; i. e. *Vaccinium axillare* Nakai, *Eubotryoides grayana* (Maxim.) H. Hara var. *oblongifolia* (Miq.) Ohwi, and *E. grayana* var. *glabra* (Komatsu ex Nakai) H. Hara. Ezuka (1991 b) added *E. grayana* var. *hypoleuca* (Nakai) H. Hara and *V. oldhamii* Miq. as new host plants. Morphology and cultural characteristics of isolates from both *Vaccinium* spp. and *Eubotryoides* spp. were indistinguishable (Sawada, 1950 ; Ezuka, 1991 b). In our study *E. bisporum* from *Vaccinium* spp. was not available.

In the Ericaceae host plants, *Exobasidium* pathogenic to the Vaccinioideae forms Group 4, which involves at least three genera and causes leaf blister and leaf blight. Basidiospores are 0–1 to multi-septate and germinate or bud. Consequently, colonies are composed of pseudo-hyphae or yeast-like cells. Despite such diverse characteristics, *Exobasidium* pathogenic to the Vaccinioideae forms a group with high support value. On the contrary, *Exobasidium* pathogenic to *Rhododendron* is divided into three groups. Groups 1 and 2 may have a common ancestor and show similar morphology in terms of 0–1 to multi-septate basidiospores, germ-tube type, and pseudo-hyphal growth, while the pathogenicity is diverted in Group 1. Four *Exobasidium* infect four *Rhododendron* with three different symptoms. Among them, *Exobasidium pentasporium* (Group 1) and *E. japonicum* (Group 2) infect *Rhododendron kaempferi* var. *kaempferi* and cause witches' broom and leaf gall, respectively. The same result by phylogenetic analysis using the concatenated ITS+LSU was recently published (Shibata and Hirooka, 2022). The phylogenetic tree supported independent causal agents. In Group 2, *Exobasidium* pathogenic to the subgen. *Hymenathes* cause leaf blight. Group 3 shows a common pathogenicity as leaf blister with small round, flat symptom, and similar morphology. Host plants belong to subgen. *Rhododendron* and *Tsutsusi*. *Exobasidium* pathogenic to *Camellia* is placed in

Group 5 with higher bootstrap and support values in NJ, MP, and ML analyses. *Exobasidium* pathogenic to *Symplocos* is placed in Group 6 with higher values. Both groups involve a variety of modes of infection, modes of basidiospore germination, and colony growth. Host specificity and multi-septate basidiospores are common features in these two groups.

The outstanding issue of two different virulence types of *Exobasidium* species on *Rhododendron* host plants were explained by placing *Exobasidium* species in different clades, i. e. *E. japonicum* (Group 2) and *E. pentasporium* (Group 1) on *R. kampferi*, *E. yoshinagae* (Group 3) and *E. nobeyamense* (Group 1) on *R. wadanum*, *E. yoshinage* (Group 3) and *E. otanianum* (Group 1) on *Rhododendron* subgen. *Tsutsusi*. The supporting values on the point of dichotomy for Group 1 and 2 from Group 3 were 95 / 100 / 96 by ML, NJ, and MP, respectively. But those for separating Group 1 from Group 2 were relatively moderate, 53 /—/ 96 by ML, NJ, and MP, respectively. Hence, two different virulence types of *Exobasidium* species remain on the same genus of host plant but belong to different concatenated ITS+LSU clades. The case of *E. symploci-japoniccae* and *E. symploci-japoniccae* var. *caprogenum* on *S. lucida* will be investigated whenever *Symplocos*-specific *Exobasidium* species are found. The rarely recognized species *E. japonicum* var. *hypophyllum* and *E. kawaense* will be examined when available.

As Begerow et al. (2002) showed the position of *E. warmingii* pathogenic to *Saxifraga* in Exobasidiaceae, our LSU tree also supported placing *E. warmingii* in the Ericaceae clade, but all clades in Ericaceae were paraphyletic. A homology search retrieved on 13 Nov. 2022 indicated the top five choices as *A. warmingii* (Acc. No. MT223875), *Exobasidium* sp. (Acc. No. OP374143), *Exobasidium* sp. (Acc. No. ON557301), *E. rhododendri* (Acc. No. OP763657), and *E. cylindrosporum* (Acc. No. CP096880). Wang et al. (2015 ; Fig. 4) and Crous et al. (2020 ; Fig. 1) also presented the position of *E. warmingii* in Exobasidiaceae in an LSU tree. Crous et al. (2020) picked up the closest hits of ITS, LSU, and *tef*1 from a megablast search of NCBI's GenBank nucleotide database and considered that closely related to species are *Exobasidium* and *Muribasidiospora.*

Exobasidium causes overgrowth symptoms such as gall formation on buds, fruits, and leaves, blistering, blight, and malformation on shoots, including witches' broom, shoestring, and red shoot in the different host families (Fig. 1). Li and Guo (2010) concluded that phylogenetic relationships among 22 *Exobasidium* species corresponded to the host plants and symptoms. Our studies showed that fruit malformation is caused only by *Exobasidium* pathogenic to *Vaccinium* (Brewer et al., 2014) in Group 4, *Camellia* in Group 5, and *Symplocos* in Group 6. Witches' broom is also caused on *R. wadanum* and *R. kaempferi* var. *kaempferi* as mentioned above. Leaf gall is caused on *R. kaempferi* var. *kaempferi*, *Rhododendron* subsp. *Hymenathes*, and *Vaccinium* spp. These symptoms are neither host specific nor related with the examined sequence groups except Group 3 (leaf blister on *Rhododendron* spp.).

Although basidiospore morphology and mode of basidiospore germination were thought to be an important taxonomic character within *Exobasidium*, results of the present study show that they are poor guidelines to support phylogenetic relationships (Fig. 5). For example, basidiospores with the same number of septa were placed into several different clades. *Exobasidium japonicum* and *E. pentasporium* have 0–1–septated basidiospores, while these two species grouped in different clades (Fig. 4). *Exobasidium japonicum* was erroneously synonymized to *E. vaccinii* due to basidiospore morphology as stated previously (Savile, 1959 a). Mode of basidiospore germination also distinguished these two species (Sundström, 1964 ; Nannfeldt, 1981). Phylogenetic trees showed different positions of these species in Groups 2 and 4, respectively. Therefore, basidiospore size and the number of septa poorly reflect phylogenetic relationships. In addition, the mode of basidiospore germination does not reflect phylogenetic relationships. *Exobasidium symploci-japonicae* var. *symploci-japonicae* germinates via a germ-tube, whereas var. *caprogenum* does by budding (Nagao et al. 2003 b). These two varieties formed a monophyletic group within the phylogenetic trees (Figs. 3 and 4). In Group 2, *E. woronichinii* germinated by a germ-tube, whereas *E. caucasicum* and *E. shiraianum* by budding (Nagao et al. 2004 a).

Nannfeldt (1981) discussed the life cycles and symptoms of *Exobasidium* spp. and considered how to infect the host plant referring to the interior persisting mycelia. Monocarpic and polycarpic infections were explained according to a manner of symptom. Mode of basidiospore germination, either germ-tube or budding conidia, may be favorable to infection on certain host plants. For symptom development, there is no specific characteristics related to infection by budding conidia. *Taphrina* species germinate from ascospores by budding but grow in the form of pseudo-mycelium in the hypertrophied tissue (Nagao and Katumoto, 1998). Yeast-like growth of *Exobasidium* on the surface of media and leaves may be transformed to pseudo-hyphal growth in the host plant tissue. Nature of budding yeast has been known by the microtubule regulation and β-tubulin genes are related (Bode et al., 2003). Phylogenetic analysis with β-tubulin gene sequences may give insight to the common ancestor of species of *Exobasidium* with yeast-like growth.

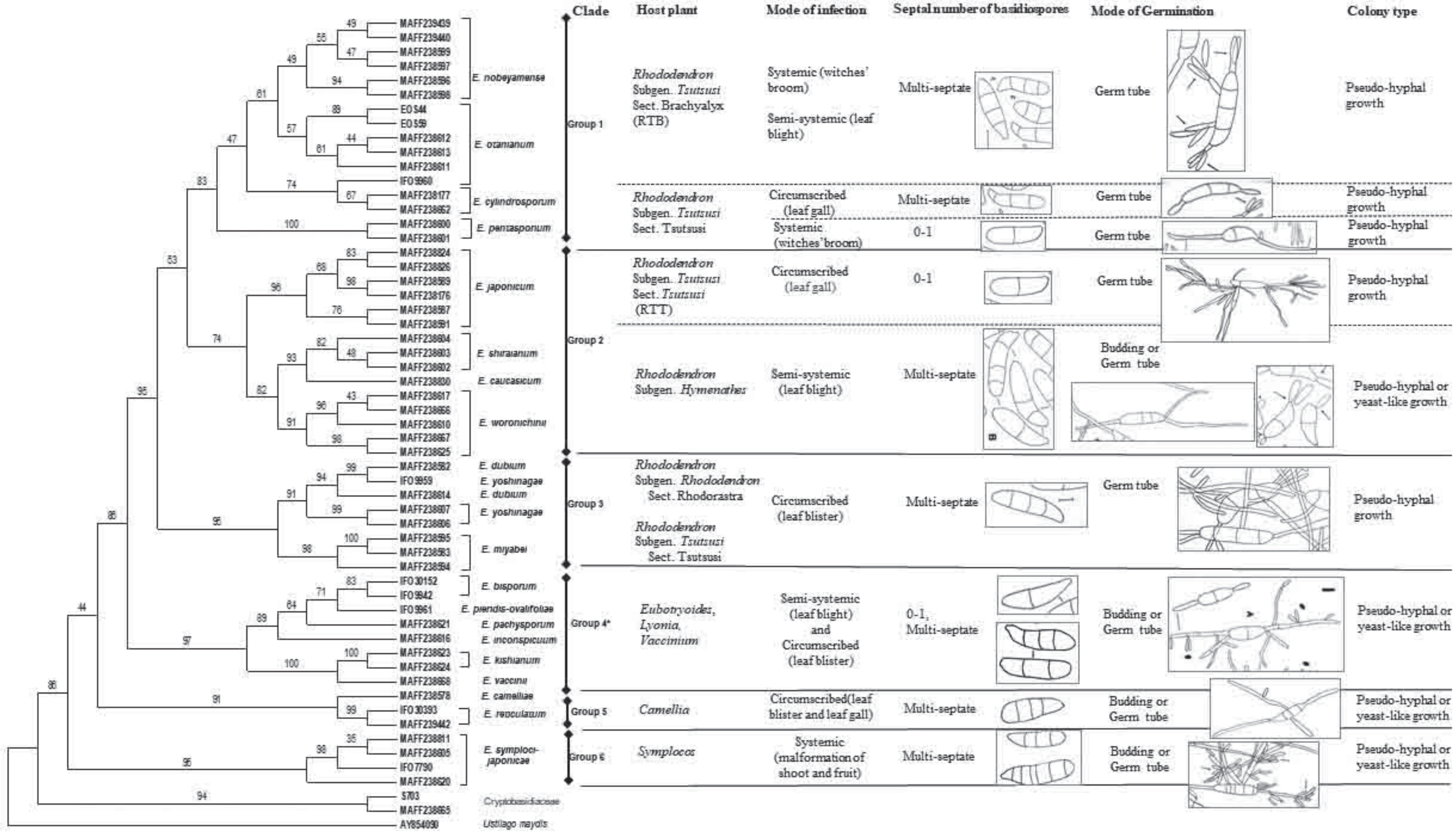

Fig. 5. Schematic comparison based on maximum likelihood analysis of the concatenated ITS+LSU sequences groupings with host plant, mode of infection, septal number of basidiospores, mode of basidiospore germination, and colony type.

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