Rhizopogon togasawariana sp. nov., the first report of Rhizopogon associated with an Asian species of Pseudotsuga

Mycologia, 106(1), 2014, pp. 105–112. DOI: 10.3852/13-055

Rhizopogon togasawariana sp. nov., the first report of *Rhizopogon* associated with an Asian species of *Pseudotsuga*

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Abstract: *Rhizopogon* subgenus *Villosuli* are the only members of the genus known to form an ectomycorrhizal relationship exclusively with *Pseudotsuga*. The specificity of this host relationship is unusual in that *Rhizopogon* is broadly associated with several tree genera within the Pinaceae and relationships with a host genus are typically distributed across *Rhizopogon* subgenera. Naturally occurring specimens of *R.* subg. *Villosuli* have been described only from North American collections, and the unique host relationship with *Pseudotsuga* is demonstrated only for *Rhizopogon* associated with *P. menziesii* (Douglas-fir), the dominant species of *Pseudotsuga* in North America. Species of *Pseudotsuga* are naturally distributed around the northern Pacific Rim, and *Rhizopogon* associates of other *Pseudotsuga* spp. are not yet described. Here we present the results of field sampling conducted in *P. japonica* forests throughout the Japanese archipelago and describe *Rhizopogon togasawariana* sp. nov., which occurs in ectomycorrhizal association with *P. japonica*. Placement of this new species within *R.* subg. *Villosuli* is supported by morphological and molecular phylogenetic analysis, and its implications to *Pseudotsuga-Rhizopogon* biogeography are discussed.

Key words: biogeography, ectomycorrhizae, hypogeous, Japan, *Rhizopogon* subgenus *Villosuli*, truffle

Introduction

Rhizopogon is a large genus of ectomycorrhizal (EM) fungi (Basidiomycota: Boletales) that produce hypogeous, truffle-like basidiomata in association with host trees of the Pinaceae. The symbiosis between *Rhizopogon* and Pinaceae occurs globally throughout the natural and anthropogenic range of the family and plays an important ecological role in the establishment and maintenance of forests (Tweig et al. 2007, Simard 2009). The foundational species concepts for genus *Rhizopogon* were established in the North American monograph of Smith and Zeller (1966), and a detailed monograph also has been produced for European *Rhizopogon* species (Martín 1996). However, few data on Asian species of *Rhizopogon* have been incorporated into phylogenetic and taxonomic studies and only a limited account of Asian *Rhizopogon* species has been published for EM associates of *Pinus* (Hosford and Trappe 1988).

Based on phylogenetic analyses of the internal transcribed spacer (ITS) of the nuclear rDNA, Grubisha et al. (2002) re-examined the infrageneric relationships of Smith and Zeller (1966). Those analyses, as well as morphological and ecological evidence corroborated by their findings, resulted in a new infrageneric classification composed of five subgenera: *Rhizopogon*, *Villosuli*, *Amylopogon*, *Roseoli* and *Versicolores*. The ITS phylogeny of Grubisha et al. (2002) found that EM associates of major conifer genera (e.g. *Pinus*, *Abies*) were distributed across multiple subgenera of *Rhizopogon*. As an exception to this trend, a host-specific relationship was resolved between the genus *Pseudotsuga* Carrière (Pinales: Pinaceae) and *R.* subg. *Villosuli*. Only one *Rhizopogon* species outside *R.* subg. *Villosuli*, *R. salebrosus* (= *R. subcaerulescens*) in *R.* subg. *Amylopogon*, has been demonstrated to form an EM relationship with *Pseudotsuga* without the presence of an alternate host (Massicotte et al. 1994). However this species forms more abundant mycorrhizae with *Pinus* species and also is capable of forming EM relationships with a range of Pinaceae hosts (Massicotte et al. 1994), arbutoid mycorrhizae with Ericaceae hosts and Monotropoid mycorrhizae with mycoheterotrophic *Pterospora andromedea* (Ericaceae) hosts (Molina et al. 1997). In contrast, members of *R.* subgenus *Villousli* consistently have been demonstrated to form mycorrhizae only with *P. menziesii* (Massicotte et al. 1994, Molina et al. 1997). The specificity of the *Pseudotsuga-R.* subg. *Villosuli* EM symbiosis suggests a single evolutionary origin of this relationship within genus *Rhizopogon*. However, the ecological synapomorphy of the *Rhizopogon-Pseudotsuga* symbiosis cannot be confirmed because the taxonomic sampling of Grubisha et al. (2002) was restricted to North

Submitted 12 Feb 2013; accepted for publication 11 Jun 2013.
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American *Rhizopogon* specimens associated only with coastal *P. menziesii* var *menziesii* and interior *P. menziesii* var *glauca*. The natural range of *Pseudotsuga* extends around the northern Pacific Rim from central Mexico to southern China (Gernandt and Liston 1999), and additional sampling from the range of all *Pseudotsuga* species is required to test thoroughly the phylogenetic distribution of the *Rhizopogon-Pseudotsuga* symbiosis.

We conducted field collections throughout the range of *P. japonica* (Shiras.) Beissn. (togasawara) on the Japanese islands of Honshu and Shikoku with the goal of collecting specimens of *Rhizopogon* associated with *P. japonica* to further test the evolutionary hypotheses of the *Rhizopogon-Pseudotsuga* symbiosis. Presented here are the results of that sampling and the description of a new species of *Rhizopogon* found fruiting in association with *P. japonica*. Interspecific relationships of this new species within *R.* subg. *Villosuli* and their evolutionary implications are discussed.

MATERIALS AND METHODS

Field collection.—The range of *P. japonica* is restricted to isolated, mountainous sites in western Japan 500–1000 m on Shikoku Island (Kochi Prefecture) and the Kii Peninsula (Nara, Wakayama and Mie prefectures) on Honshu Island (Farjon 2010). Two field sites where *P. japonica* forests could be accessed were located in Kochi and Nara prefectures, and field collections were conducted 29 Jun–9 Jul 2010. Field collecting was conducted by gently raking the top 10–20 cm of the organic horizon and mineral soil layers under *P. japonica* trees. Fresh basidiomata were photographed and clean samples of gleba from each basidioma were stored in 20% DMSO buffer (Hosaka 2009) for DNA extraction. Twenty percent DMSO buffer was prepared by first mixing 250 mL 0.5 M EDTA, 50 mL 1 M Tris pH 8.0, 100 g NaCl, then applying heat until nearly all NaCL was dissolved. Distilled H_2O was added to a total volume of 375 mL, the solution was autoclaved and 2 M Na_2 SO_3 (sterilized) was added to a total volume of 400 mL. Finally DMSO was added to a total solution volume of 500 mL. Basidiomata were dried at 45 C with a food dehydrator (Snackmaster Express FD-60, Nesco/American Harvest, Two Rivers, Wisconsin) within 24 h of collection.

To confirm the host relationship of basidiomata, it was desirable to identify *P. japonica* EM root tips colonized by *Rhizopogon* species. Whole root samples were collected from under the drip line of *P. japonica* trees by raking through surface soil and coarse woody debris. Only roots with visible EM tips were collected and, because of the diverse community structure of *P. japonica* forests, these roots were potentially from one of three Pinaceae hosts, *P. japonica*, *Abies firma* and *Tsuga sieboldii*. To increase the number of EM tips retrieved from *P. japonica*, whole live seedlings of *P. japonica* were collected along with native soil. Soil balls and seedling roots were moistened, wrapped in plastic bags and processed within 96 h of collection. Loose whole root samples were moistened and stored in plastic bags for up to 5 wk at 4 C until processing was possible. Both seedling and loose roots were washed in filtered water, and EM root tips were observed under a dissection stereoscope. Individual EM root tips were excised from the root system, photographed and stored in 20% DMSO buffer for DNA extraction. All seedlings were potted and deposited as specimens at the Botanic Garden of the National Museum of Nature and Science (TNS) in Tsukuba, Japan.

DNA extraction and taxonomic analysis.—DNA extraction from *Rhizopogon* basidiomata was performed by grinding preserved gleba tissue in a mortar and pestle with 2 × CTAB buffer followed by processing with the GeneClean III DNA extraction kit (MP Biomedicals) following manufacturer recommendations. DNA extraction from EM root tips was performed with a CTAB buffer extraction protocol. Preserved EM root tips were rinsed in distilled water under a dissection stereoscope and ground in 2 × CTAB buffer with a FastPrep cell lysis device (MP Biomedicals, Solon, Ohio). Samples were extracted with phenol:chloroform:isoamyl alcohol, and DNA was precipitated with 3 M sodium acetate (pH 5.2) and 95% ethanol.

Taxonomic placement of Japanese *Rhizopogon* was determined with both morphological and molecular analysis. Sections of basidiomata were prepared for microscopic analysis by mounting in 5% KOH, Melzer's reagent or distilled water as indicated. KOH mounts were prepared by rehydrating the basidioma section in distilled water, squashing the tissue with a cover slip and flooding the slide with 5% KOH. Host tree identity of EM root tips was determined by direct association of root samples with individual *P. japonica* seedlings. Basidiomata and fungal symbionts present in EM root tips were identified by PCR amplification of the ITS rDNA with the fungal-specific primer pair ITS1-F and ITS4 (White et al. 1990, Gardes and Bruns 1993) with several PCR protocols. Sequencing reactions were performed with a BigDye® Terminator kit on an ABI 3730 capillary electrophoresis device (Applied Biosystems, Foster City, California). Before sequencing of fungal ITS rDNA from EM root tips, EM root tip DNA was screened by PCR amplification with the microsatellite primers Rve1.34F and Rve1.34R (Kretzer et al. 2003b) following the PCR protocol of Kretzer et al. (2000). These microsatellite PCR primers were developed for *R. vinicolor* and *R. vesiculosus* but also were effective in amplifying microsatellite products from DNA isolated from our Japanese *Rhizopogon* basidiomata. Microsatellite PCR products were visualized on 2% agarose gels with ethidium bromide staining, and those EM root tip DNAs that were found to produce strong, clear bands were subjected to PCR amplification and sequencing of the ITS rDNA. The ITS rDNA locus was selected for taxonomic identification and phylogenetic analysis because it was used successfully to infer *Rhizopogon* phylogenies in Grubisha et al. (2002) and Kretzer et al. (2003a); it has a clearly defined ''barcode gap'' between intra- and interspecies variation, and it has been formalized as the universal DNA barcode marker for Fungi (Schoch et al. 2012). Putative identities were assigned to

fungal ITS sequence data with GenBank BLAST webserver available through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/).

Subgeneric affiliation of Japanese *Rhizopogon* collections was investigated by morphological examination and by molecular phylogenetic analysis. Quantification of taxonomic characters was determined by measuring microscopic features from each basidioma as follows: 12 basidiospores, 3–10 basidioles and four peridial/rhizomorph hyphae. Intact, recognizable basidia were scarce, and a total of three basidia were observed from all the dried specimens examined. The ITS sequence data from basidiomata and EM root tips were incorporated into a sequence dataset compiled from the datasets of Grubisha et al. (2002) and Kretzer et al. (2003a). Duplicate sequences from the dataset of Kretzer et al. (2003a) that were shared with the dataset of Grubisha et al. (2002) were removed. Also included in this dataset are three ITS sequences each of *R. vesiculosus* (GenBank HQ385849.1, HQ385850.1 HQ385854.1) and *R. vinicolor* (GenBank HQ385847.1, HQ385848.1, HQ3 85853.1) from Luoma et al. (2011) and one ITS sequence of the *R. rudus* holotype collection (GenBank AF377107.1) from Bidartondo and Bruns (2002). Alignment of this combined dataset was performed by Clustal W 1.4 (Thompson et al. 1994) as implemented in BioEdit 7.0.9.0 (Hall 1999) with the default settings and then edited with BioEdit. The most appropriate model of nucleotide substitution for this dataset was determined with jModelTest (Guindon and Gascuel 2003, Posada 2008). Maximum likelihood (ML) phylogenetic analysis was performed with RAxML 7.2.6 (Stamatakis 2006) with the GTRGAMMA model of nucleotide substitution and 1000 bootstrap replicates. The alignment file in this analysis is available at TreeBASE (http://treebase.org) under study ID S14328.

RESULTS

Field collections and morphological analyses.—Seven *Rhizopogon* basidiomata were retrieved from three collections near Kawakami village, Nara Prefecture, Japan (34°15′50.3″N, 136°06′26.3″E, 887 m). Dissection of the root collections from both Kochi and Nara prefecture sites yielded a total of 105 *P. japonica* EM root tips from seedlings and another 407 EM root tips from loose, whole root samples. Two of the *P. japonica* tips collected from seedlings at the Nara prefecture site possessed ITS sequences with nearly 100% identity to *Rhizopogon* basidioma ITS sequences from the same site. These two EM tips both were collected from a single *P. japonica* seedling; this seedling possessed only one other EM tip with identical morphology to the sequenced *Rhizopogon* EM tips and had two other EM tips with a different morphology.

Morphological analysis.—The morphological characteristics of Japanese *Rhizopogon* basidiomata closely resemble those typical of *R.* subg. *Villosuli*, which is characterized by a peridial epicutis and rhizomorphs of loosely woven, brown-walled hyphae as viewed in KOH and dark granules in the peridial subcutis when viewed in H_2O (FIG. 1G, H) (Grubisha et al. 2002). Taxonomic descriptions were compiled from examination of the seven basidiomata and two EM root tips retrieved in Nara Prefecture. All basidiomata and EM root tips possess near identical ITS rDNA sequences.

Molecular and phylogenetic analyses.—All DNA from EM root tips sampled from mature trees tested negative with microsatellite PCR screening. Furthermore, ITS sequences amplified from these DNA were not found to match data from Rhizopogonaceae available in the NCBI GenBank database. As such, sequence data from these EM root tips are not presented here. ML phylogenetic analysis clearly places Japanese *Rhizopogon* basidiomata and root tips collected from *P. japonica* seedlings as members of a single, well supported clade within *R.* subg. *Villosuli* (FIG. 2). However, the relationship between the distinct species clades within *R.* subg. *Villosuli* remains unclear. Sequence data for *R. rudus* also is included in this ITS phylogeny. Although previously classified as *R.* section *Amylopogon* by Smith and Zeller (1966), sequence data from this species and *R. zelleri* are highly similar. Phylogenetic affinity of *R. rudus* with other members of *R.* subg. *Villosuli* was suggested by Bidartondo and Bruns (2002), as well as Smith and Zeller (1966), and the results presented here provide grounds for formal placement of *R. rudus* within *R.* subg. *Villosuli.*

TAXONOMY

Rhizopogon togasawariana A.B. Mujic, K. Hosaka & J.W. Spatafora, sp. nov. MycoBank MB804649

FIG. 1A–H

Macrocharacters: Basidiomata. 13–23 × 9–13 mm. Hypogeous, oblong to subglobose or lobed. Base often pinched or flattened with a basal tuft of brown rhizomorphs. Peridium. White, quickly staining brown to dull vinaceous red upon disturbance, stains fading to greenish brown with patches of red persisting. Drying to a vinaceous red. Rhizomorphs. Pale brown when immature, maturing or bruising to dark brown. Interwoven into the peridium as palmate hyphal fans that diverge from rhizomorphs in the surrounding substrate. Typically arising from a single hemisphere of the basidioma and coalescing at a central tuft that emanates and branches into the surrounding substrate. Rhizomorphs are easily destroyed during excavation of basidiomata. Gleba. Composed of homogenous lacunose folds of tissue forming irregular locules. White at immaturity,

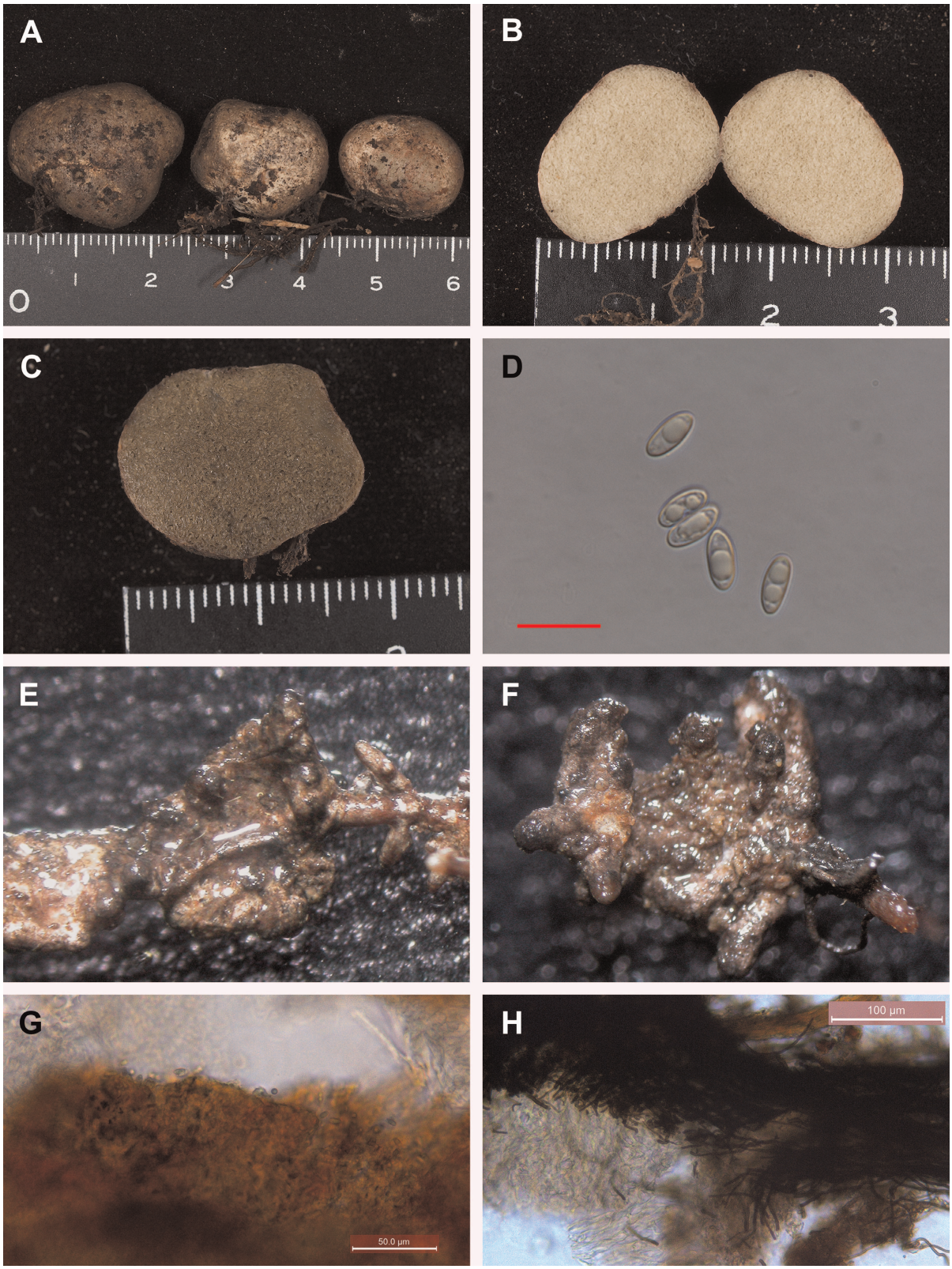

FIG. 1. Macroscopic and microscopic features of *Rhizopogon togasawariana* sp. nov. basidiomata and EM root tips associated with *Pseudotsuga japonica*. A–C. Basidiomata with the staining reaction of fresh peridium (A), immature gleba (B) and mature gleba (C) with basal rhizomorphs. D. Basidiospores, bar = 10 µm. E–F. Ectomycorrhizal root tips formed by *P. japonica* and *R. togasawariana* showing black fungal mantle with small amounts of underlying white hyphae visible (E). G. H_2O mount of

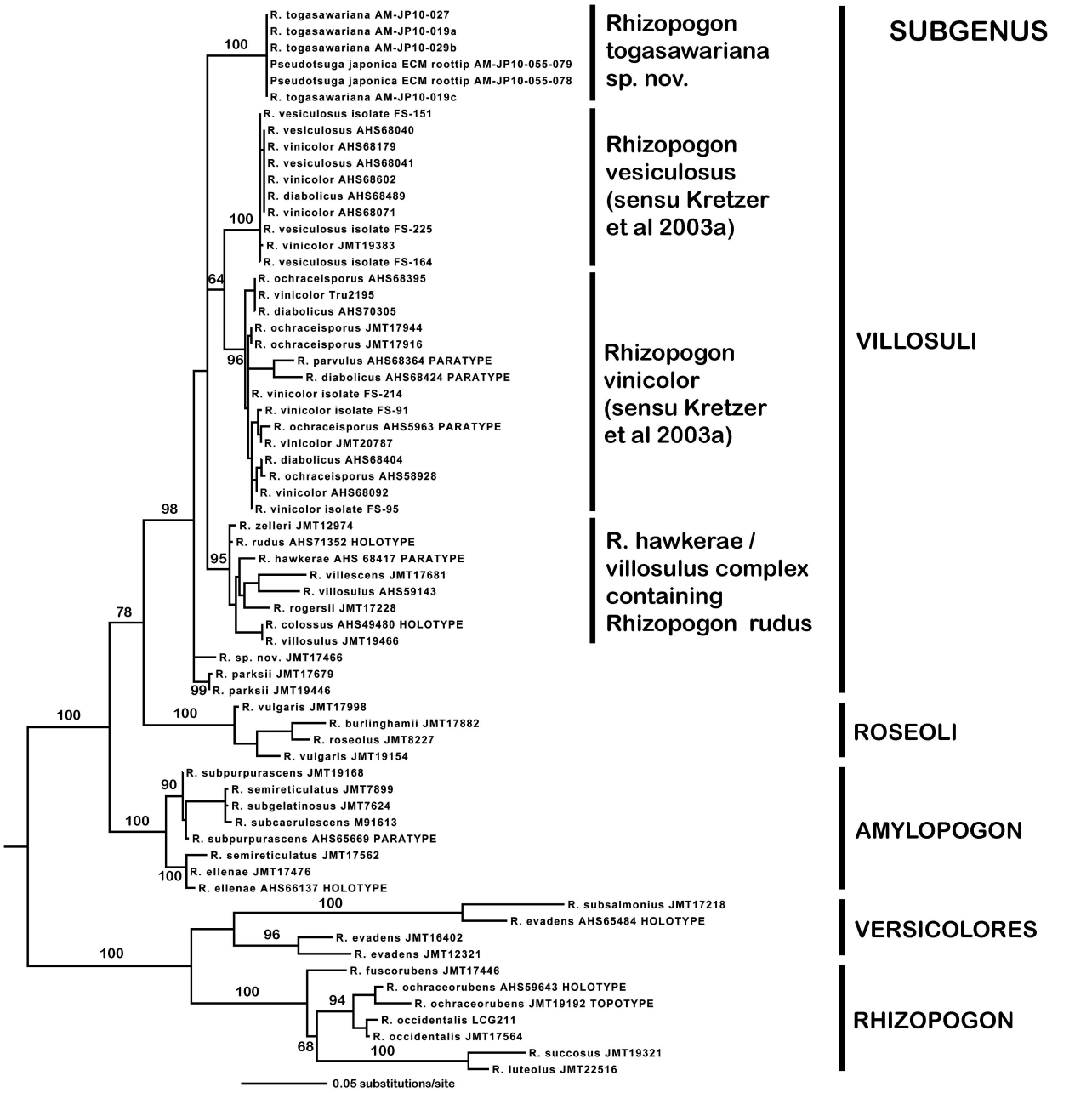

FIG. 2. RAxML analysis of the ITS rDNA of 65 *Rhizopogon* taxa using 1000 rapid bootstrap replicates. Bootstrap support values of major branches exceeding 60% are noted along branches. This analysis included the ITS datasets of Kretzer et al. (2003a), Grubisha et al. (2002), and information on sequence data can be found within these studies. Taxon names from the Kretzer et al. (2003a) and Grubisha et al. (2002) datasets are presented here as they appeared in the original publications for the purpose of reference. Taxon names redetermined or synonymized to *R. vinicolor* or *R. vesiculosus* by Kretzer et al. (2003a) are noted in clade labels. ITS sequences of *R. vinicolor* and *R. vesiculosus* sporocarps from Luoma et al. (2011) are listed under the collection numbers FS-91, FS -95, FS-214, FS-151, FS-164 and FS-225. New sequences generated in this study and included in this analysis include four sequences from *R. togasawariana* sp. nov. basidiomata and two sequences from *P. japonica* EM root tips. One representative sequence from the *R. togasawariana* sp. nov. holotype is deposited in GenBank KC542888.

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peridium subcutis with deposits of red pigment and dark black granules. H. Five percent KOH mount of peridium with the epicutis of dark brown interwoven hyphae and the underlying subcutis of hyaline yellowish hyphae.

darkening through maturation from yellow-brown to light rusty cinnamon-brown. Pliant and soft during immaturity, developing a cartilaginous-rubbery texture at maturity. Columella absent. *P. japonica* ectomycorrhiza. Subtuberculate. *P. japonica* rootlets surrounded by a mantle of dark black *Rhizopogon* hyphae. Inner hyphae of the mantle white under black exterior. Mantle of adjacent rootlets fused and partially obscuring detail of underlying plant superstructure.

Microcharacters. Basidiospores. 6.8–8.6 × 2.5–3.5 μm. Ellipsoid. Hyaline individually and light rusty cinnamon-brown in mass as viewed in deionized water. Often with a basal scar appearing as a slight longitudinal indentation. Basidia. 8.3–17.1 × 5.3–10.1 μm and clavate to cylindrical. Ephemeral and collapsing upon maturity. Basidioles. 8.3–17.1 × 5.3–10.1 μm. Clavate to subcylindrical. Peridium. Up to 160 μm thick with a subcutis of hyaline yellowish hyphae 2.2–5.7 μm diam and oriented parallel to the surface of the peridium. Patches of vinaceous pigment are abundant between subcutis hyphae in dried specimens, often obscuring detail. Subcutis with sparse, embedded black granules that are visible in H2O and dissolve into a green pigment in KOH. Epicutis scanty, occasionally distributed in irregular patches over subcutis at the base of the basidioma and more regularly in regions of rhizomorph attachment. Epicutis composed of irregularly septate, loosely woven brown-walled hyphae, 2.8–5.6 μm diam, as viewed in 5% KOH. Rhizomorphs. Interwoven directly into the epicutis and formed from hyphae identical in appearance to those of the epicutis. Individual hyphae tightly packed into a longitudinally corrugate bundle. Gleba trama. Divergent to interwoven. Composed of refractive-gelatinous hyaline hyphae, 3.6–5.4 μm diam. Subhymenium. Ramose. Subhymenium cells often swollen near septation with hymenial elements. Individual cells highly variable in size and shape, 7.9–36.1 × 4.1–13.8 μm. Clamp connections absent from all observed hyphae.

Etymology. Togasawara (Japanese): Traditional name given to *Pseudotsuga japonica*.

Habitat and distribution. Found in mixed forests in ectomycorrhizal association with *P. japonica*. Known only from the type collections below. Presumably occurring throughout the range of *P. japonica*.

Specimens examined. JAPAN: NARA PREFECTURE: near Kawakami village, 34°15′50.3″N, 136°06′26.3″E, 887 m. Fruiting approximately 10 cm below surface of a thick organic layer near the surface of the mineral horizon. Basidiomata appressed to the primary root of a large *P. japonica*, 6 Jul 2010. *Alija Bajro Mujic AM-JP10-019* (HOLOTYPE: TNS TNS-F-47724, ISOTYPE: OSC 147941); Fruiting approximately 15 m from holotype collection at the interface of mineral soil and organic layer. 7 Jul 2010. *Alija Bajro Mujic AM-JP10-027* (PARATYPE: OSC 147942); Fruiting approximately 2 m from holotype collection at the interface of mineral soil and organic layer. 7 Jul 2010. *Alija Bajro Mujic AM-JP10-029* (PARATYPE: TNS TNS-F-47725)

DISCUSSION

The ecological, phylogenetic and morphological evidence presented here indicates strong support for inclusion of *R. togasawariana* in *R.* subg. *Villosuli*. This is the first account of any *Rhizopogon* species forming an EM symbiosis with *P. japonica*, and the placement of *R. togasawariana* in *R.* subg. *Villosuli* is consistent with the suggested hypothesis of a single evolutionary origin for the *Rhizopogon-Psuedotsuga* symbiosis (Grubisha et al. 2002). In North America, species of *R.* subg. *Villosuli* are prevalent members of the fungal community, forming EM symbioses with *P. menziesii* throughout all stand age classes and are especially abundant in younger stands (Luoma et al. 2006, Twieg et al. 2007). It also has been noted that *R. vinicolor* and other members of *R.* subg. *Villosuli* are especially abundant members of the fungal communities forming EM root tips with seedlings after disturbance in managed populations of *P. menziesii* (Colinas et al. 1994). Similar trends also have been observed within other *Rhizopogon* subgenera associated with species of *Pinus* where *Rhizopogon* are common members of the spore bank community colonizing seedlings (Kjøller and Bruns 2003, Izzo et al. 2006b) and show increased colonization success after fire disturbance (Izzo et al. 2006a, Peay et al. 2009). At its type locality, *R. togasawariana* does not appear to be a common component of fungal communities forming EM root tips with mature *P. japonica* (root-tip data presented here, Dr Kazuhide Nara pers comm). However, this is not sufficient evidence to determine the relative abundance or ecological role of *R. togasawariana* in *P. japonica* forests. *Rhizopogon* species associated with *Pinus muricata* also are sampled infrequently as EM root tips from mature forests (Taylor and Bruns 1999). Yet, *Rhizopogon* species persist as resistant propagules in the soil of these same forests and are among the most abundant colonizers of *P. muricata* seedlings after disturbance by wildfire (Baar et al. 1999) as well as in bioassays (Baar et al. 1999, Taylor and Bruns 1999). While it is not possible to speculate on the ecology or frequency of *R. togasawariana*, given the limited sampling presented here, the capability of *R. togasawariana* to form EM symbioses with *P. japonica* is consistent with the ecology of *R.* subg. *Villosuli*.

Both microscopic and macroscopic characters of *R. togasawariana* are typical of *R.* subg. *Villosuli*.

Microscopic examination of *R. togasawariana* basidiomata revealed dark brown interwoven epicutis hyphae in KOH mounts and dark granules in H_2O mounts of the subcutis (FIG. 1G, H). *R. togasawariana* macroscopically is most similar to *R. vinicolor* and *R. vesiculosus* (sensu Kretzer et al. 2003a), displaying similar, yet differentiable, gross morphology and color-staining reactions upon exposure and drying. The collections of *R. togasawariana* examined in this study ranged from immature (gleba entirely white; FIG. 1B) to early maturity (gleba brown but basidioma still firm; FIG. 1C). The peridium of all specimens examined was white upon exposure and quickly stained brown with areas of pronounced red (FIG. 1A). After several minutes the staining reaction progressed to dark greenish brown with areas of red persisting. This is similar to staining reaction of the peridium in basidiomata of both *R. vinicolor* and *R. vesiculosus* (Smith and Zeller 1966, Kretzer et al. 2003a), but *R. vinicolor* differs in that the peridium of immature basidiomata stains purely pink or rose-red and yellow patches often are present on the peridium at early maturity. Both of these characters are lacking in *R. togasawariana*. *R togasawariana* is differentiated microscopically from *R. vesiculosus* by the lack of vesicles near rhizomorph attachments and from both *R. vinicolor* and *R. vesiculosus* by the lack of pronounced truncation at the base of its basidiospores (FIG. 1D). The molecular sequence data as well as the ecological, microscopic and macroscopic characteristics presented here all differentiate *R. togasawariana* as a unique species in *R.* subg. *Villosuli*.

It is unclear whether *R. togasawariana* should be classified in either of the two established sections of *R.* subg. *Villosuli*, *R.* sect. *Villosuli* or *R.* sect. *Vinicolores* (Grubisha et al. 2002). The molecular phylogeny (FIG. 2) supports *R. togasawariana* as a distinct clade separate from the type species of either *R.* sect. *Villosuli* (*R. villosulus*) or *R.* sect. *Vinicolores* (*R. vinicolor*). The dark granules in the subcutis and presence of an epicutis of dark brown hyphae are similar to those of *R.* sect. *Villosuli*. Whereas the brown to red staining reaction of the fresh subcutis, tendency of the subcutis to dry red and the restricted distribution of epicutis tissue to regions of the peridium near rhizomorph attachment are similar to those of *R.* sect. *Vinicolores*. Given that *R. togasawariana* basidiomata possess a suite of morphological traits that intergrades between those typical of both *R.* sect. *Villosuli* and *R.* sect. *Vinicolores*, and the lack of phylogenetic support, *R. togasawariana* is not classified here into an existing section of *R.* subg. *Villosuli*. Due to the limited sampling of *R.* subg. *Villousli* in Asia, it also might be possible that *R. togasawariana* represents an undescribed section to the subgenus.

The oldest fossil records for *Pseudotsuga* are known from North America (Hermann 1985) and are suggestive of a geographic origin of the genus in North America. Strauss et al. (1990) proposed a stepwise model of migration for *Pseudotsuga* from North America into eastern Asia, and molecular phylogenetic analyses (Strauss et al. 1990, Gernandt et al. 1999, Wei et al. 2010) are consistent with a North American origin for the genus, followed by species diversification in Asia and North America. The support for a single origin of the *Rhizopogon-Pseudotsuga* symbiosis within *Rhizopogon* and the phylogeography of *Pseudotsuga* results in the development of the hypothesis that the evolutionary origin of the symbiosis likely predates divergence of extant *Pseudotsuga* species from a common ancestor. The ML phylogeny presented here provides strong support for the single-origin theory of the *Rhizopogon-Pseudotsuga* EM symbiosis. However, interspecific relationships are not well resolved and *Rhizopogon* EM associates of other Asian *Pseudotsuga* spp. are unknown. Additional sampling in the *Pseudotsuga* forests of Taiwan and China as well as more robust molecular datasets are needed to test whether *R.* subg. *Villosuli* also originated in North America and co-migrated with *Pseudotsuga*.

ACKNOWLEDGMENTS

Field collections in Japan were conducted with the valuable assistance and guidance of Dr Nobuyuki Tanaka at the Kochi Prefectural Makino Botanical Garden Herbarium and Dr Masakuni Kimura at the Museum of Forests and Headwaters, Kawakami. K. Nishibori, M. Otsuka, K. Uno, Y. Hirayama and S. Vandruff provided laboratory assistance. This work was supported by the National Science Foundation and the Japan Society for the Promotion of Science through an East Asia and Pacific Summer Institutes Fellowship (NSF award No. 1015355) to Alija Bajro Mujic.

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