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Full paper

A new *Rhizopogon* species associated with *Pinus amamiana* in Japan

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ABSTRACT

Pinus amamiana is an endangered *Pinus* species found only on Yakushima Island and Tanegashima Island, Japan. We surveyed remaining *P. amamiana* forests and found some sporocarps of *Rhizopogon* (Boletales), many species of which exhibit strict host specificity to a narrow range of Pinaceae trees and play critical roles in host establishment. Based on morphological characteristics and molecular phylogeny, here we describe *Rhizopogon yakushimensis* sp. nov. This new species belongs to a new clade, phylogenetically related to the subgenera *Versicolores* and *Rhizopogon*. We also confirmed its ectomycorrhizal association with *P. amamiana* by comparing rDNA ITS sequences between the sporocarps and ectomycorrhizal root tips.

1. Introduction

Rhizopogon Fr. is a genus belonging to Boletales, Basidiomycota, and it forms ectomycorrhizal (ECM) associations with Pinaceae, specifically *Pinus* and *Pseudotsuga* (Molina, Trappe, Grubisha, & Spatafora, 1999). While most ECM fungi are compatible with a broad range of host trees, *Rhizopogon* species usually show strict specificity to a narrow range of hosts (e.g., Koizumi & Nara, 2016; Rusca, Kennedy, & Bruns, 2006). Spores of some *Rhizopogon* species survive for many years in soil (Bruns et al., 2009) and predominate soil spore-bank communities of ECM fungi (Huang, Nara, Zong, & Lian, 2015; Taylor & Bruns, 1999). Thus, the soil spore banks of *Rhizopogon* serve as the primary ECM inocula for regenerating host seedlings after disturbance, playing a critical role in the establishment and regeneration of specific pines (Baar, Horton, Kretzer, & Bruns, 1999; Castellano & Trappe, 1985; Peay, Garbelotto, & Bruns, 2009; Simard, 2009).

Despite this ecological importance, few surveys on *Rhizopogon* species have been conducted in Japan. Yet, limited surveys have clearly indicated that different Pinaceae host species are associated with unique *Rhizopogon* species. For example, *R. roseolus* Tul. & C. Tul. and *R. succosus* A. H. Sm. are associated with two-needle pines; *Pinus densiflora* Siebold &Zucc. and *Pinus thunbergii* Parl. (Hosford & Trappe, 1988), *R. togasawariana* Mujic, K. Hosaka & Spatafora is specific to *Pseudotsuga japonica* (Shiras.) Beissn (Mujic, Hosaka, & Spatafora, 2014). and *R. alpinus* T. Koizumi & K. Nara to *Pinus pumila* (Pall.) Regel (Koizumi & Nara, 2016). However, these previously surveyed Pinaceae hosts account for four of nine potential host species (Ono, Oba, & Nishida, 1989); thus, there may still be undescribed *Rhizopogon* species in association with unsurveyed host species.

Pinus amamiana Koidz. is a five-needle *Pinus* tree species endemic to Japan, which is distributed only on Yakushima Island and Tanegashima Island (Ono et al., 1989). This species is designated as Endangered by the IUCN Red List (Katsuki & Farjon, 2013). The number of remaining *P. amamiana* trees was estimated to be approximately 2000 (Katsuki & Farjon, 2013), while the recent outbreak of pine wilt nematode disease has reduced the size of the remaining population (Nakamura, Akiba, & Kanetani, 2001). While ECM fungi have not been surveyed in *P. amamiana* forests, there may be some *Rhizopogon* that are species specific to *P. amamiana* and play critical roles in its seedling establishment as demonstrated in other Pinaceae species (Castellano & Trappe, 1985; Hosford & Trappe, 1988; Baar et al., 1999; Peay et al., 2009; Simard, 2009; Mujic et al., 2014; Koizumi & Nara, 2016; Murata, Nagata, & Nara, 2017). Discovering such *Rhizopogon* species may contribute to the conservation of this endangered pine.

In this study, we collected sporocarps of *Rhizopogon* under *P. amamiana* in Yakushima Island and examined their morphological characteristics and phylogenetic placement. Based on unique phylogenetic position and morphological characters, we propose *Rhizopogon yakushimensis* sp. nov. as the first *Rhizopogon* species associated with the endangered *P. amamiana*.

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2. Materials and methods

2.1. Field collection

In June 2016, a field survey was conducted on Yakushima Island, located in southwestern Japan, and three sporocarps of *R. yakushimensis* were collected. The ECM host trees growing on the site were *P. amamiana* and *Castanopsis sieboldii* (Makino) Hatusima ex Yamazaki et Masiba; however, all sporocarps were collected near *P. amamiana* trees. After recording the macroscopic features (described below), the collected sporocarps were placed in a plastic bag with silica gel and transported to the laboratory. ECM roots of *P. amamiana* were also collected in the same forests.

2.2. DNA extraction

Total DNA was extracted from inner tissues of the three fresh sporocarps or ECM tips using a modified cetyltrimethyl ammonium bromide method (Nara, Nakaya, Wu, Zhou, & Hogetsu, 2003). The nuclear ribosomal internal transcribed spacer (ITS) regions, including ITS1, 5.8S and ITS2, were amplified using the primers ITS1F and ITS4 (Gardes & Bruns, 1993). We used a Multiplex PCR Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Amplified PCR products were purified using the PCR and Sequence Reaction Clean-up Kit (with exonuclease I and alkaline phosphatase, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sequenced directly on the ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The ITS sequence was deposited at the DNA Data Bank of Japan (LC216339, LC216340).

2.3. Phylogenetic analysis

To infer the phylogenetic placement of *R. yakushimensis* within the genus *Rhizopogon* and to confirm the ECM association with *P. amamiana*, ITS sequences from the sporocarp specimens and ECM tips were incorporated into the *Rhizopogon* sequence dataset of Grubisha, Trappe, Molina, and Spatafora (2002). Additional sequences in GenBank (AB839390, AB636449 and JQ991778) that showed high similarities with our sequences according to BLAST results were also included. Sequences of *Chroogomphus vinicolor* (Peck) O.K. Mill. (L54095), *Gomphidius oregonensis* Peck (L54114), *Alpova trappei* Fogel (AF074920) and *Truncocolumella citrina* Zeller (L54097) were added to the dataset as the outgroup. Fifteen sequences from Grubisha et al. (2002) were short or contained long indels (AF062927, AF062928, AF071437 and AF071441–AF071-5452; all belongs to subgenus *Villosuli* that is specific to *Pseudotsuga* trees) and thus were eliminated from our final dataset. After confirming the phylogenetic position of *R. yakushimensis* within the entire genus *Rhizopogon* using this dataset, we performed a detailed phylogenetic analysis that excluded distantly related species to investigate the relationship between *R. yakushimensis* and the two closely related subgenera *Rhizopogon* and *Versicolores*. In this detailed analysis, *R. roseolus* (AF058315), *R. burlinghamii* A. H. Sm (AF058303). and two *R. vulgaris* (Vittad.) M. Lange sequences (AF062931, AF062934) were used as the outgroup.

Alignments of these datasets were performed using MUSCLE (Edgar, 2004) implemented in MEGA 7 (Kumar, Stecher, & Tamura, 2016) with the default settings. Poorly aligned positions were eliminated using Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), allowing gap positions within the final blocks. The most appropriate nucleotide substitution model was determined using the MEGA7 model selection option (Kumar et al., 2016) for each dataset. Maximum likelihood (ML) phylogenetic analyses were performed using MEGA 7 with the determined substitution model, i.e., the GTR + G + I model for both the entire and detailed analyses and 1000 bootstrap replicates. Neighbor-joining (NJ) phylogenetic analyses were also performed using MEGA 7, with the Maximum Composition Likelihood model and 1000 bootstrap replicates.

2.4. Morphological characterization

The colors and textures of the sporocarp surface and inner parts (glebae, longitudinal section) were examined in fresh samples before transportation to the laboratory. Color reactions were examined in the peridium and gleba of dried specimens with 5% (w/v) KOH solution, $FeSO_4$ and Melzer's reagents in the laboratory.

In the laboratory, the microscopic features of the specimens were observed under a light microscope (ECLIPSE E600; Nikon, Tokyo, Japan). The length and width of 50 mature spores randomly selected from each type specimen were measured, and their standard deviations were calculated. While the paratype specimen was immature, only mature ones were measured after excluding small immature spores. The morphologies of basidia, brachybasidiole, peridium and trama were also observed. The means and standard deviations of these structures were calculated from at least 20 replicates. Color reactions of these tissues were examined by mounting a section in 5% KOH and $FeSO_4$. The amyloid reaction was examined with Meltzer's reagent. One of the three sporocarps was overmatured when brought back to the laboratory, so we could not use this sporocarp for morphological examination of the sporocarps.

Rhizopogon brunneicolor A. H. Sm. and *Rhizopogon molligleba* A. H. Sm. described in Smith and Zeller (1966) share some common morphological traits with the sporocarps collected in this study. Thus, the above morphological observation was applied to their holotype specimens (MICH 66092 and MICH 69154 for *R. brunneicolor* and *R. molligleba*, respectively). These type specimens were not good enough to obtain ITS sequences.

3. Results

3.1. Molecular phylogenetic analyses

ITS sequences from three *Rhizopogon* sporocarp specimens and ECM roots of *P. amamiana* collected in the same forest were identical (nucleotide similarity = 100%, only results of type specimens are shown in Fig. 1). These sequences formed a monophyletic group with an uncultured *Rhizopogon* sequence from a subtropical pine forest in China (JQ991778) and environmental sequences from a *Pinus massoniana* forest in China (AB636449 and AB839390). This clade was placed in a sister position to the combined subgenera *Rhizopogon* and *Versicolores* (Fig. 1), but the branching support was weak in the entire dataset (BS of ML analysis = 37; Supplementary Fig. S1). The alignment file is available at TreeBase (accession no. S20628).

3.2. Taxonomy

Rhizopogon yakushimensis Y. Sugiyama, M. Murata & K. Nara, sp. nov. Fig. 2.

MycoBank no: MB 820196.

Sporocarps 8–12 × 6–11 mm, oblong to subglobose, sessil, covered with orange to dark-brown branching rhizomorphs. Branching rhizomorphs densely covering the entire surface. The texture of the surface dry to a little moist but not lubricous or viscid, appressed fibrillose. Peridium white when young, becoming

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