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Short communication

Genetic variation among natural isolates of the ectomycorrhizal hypogenous fungus, *Rhizopogon roseolus* from Japanese pine forests inferred using AFLP markers

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ABSTRACT

Genetic variation among 45 *Rhizopogon roseolus* isolates from 21 different regions of Japan were inferred using amplified fragment length polymorphism (AFLP) markers. Using three primer pair combinations, AFLP analysis reproducibly produced a total of 223 DNA fragments, 74.4% of which were polymorphic. Pairwise dissimilarity of AFLP patterns between isolates ranged from 0.043 to 0.228. Cluster analysis and principal coordinate analysis of AFLP data generally showed four major clusters from geographically distinct areas. The findings suggested that the Japanese populations of *R. roseolus* from different geographical regions can be distinguished based on AFLP characters.

The basidiomycete *Rhizopogon roseolus* (Corda) Th. Fr. (= *Rhizopogon rubescens* (Tul. & C. Tul.) Tul. & C. Tul.), an ectomycorrhizal (ECM) hypogeous fungus that forms globose to subglobose fruiting bodies underground, is primarily associated with the members of the Pinaceae (Molina et al. 1999). In Japan, *R. roseolus*, or "Shoro" as it is known, is regarded as an excellent edible fungus that grows in Japanese black pine (*Pinus thunbergii*) forests along the coast (Imazeki and Hongo 1989). In response to a marked decrease in annual production, fruiting bodies of *R. roseolus* command a high price in local markets. Attempts to cultivate this fungus in its natural habitat have been developed with methods of improving the forest environment or planting pine seedlings that had been artificially inoculated with *R. roseolus* ectomycorrhizae (Nagasawa 2000). However, the implementation of these methods has not yet been able to increase the supply of fruiting bodies to the market. In addition to developing a method for germinating *R. roseolus* basidiospores, Kawai et al. (2008) recently revealed that this species employs a bipolar mating system. Their findings are very interesting as they open the possibility of crossbreeding *R. roseolus* cultivars. In such crossbreeding experiments, information on genetic relatedness among the natural isolates of this fungus in Japan will be very useful in the evaluation of *R. roseolus* genetic resources.

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Molecular approaches are considered to be powerful tools for investigating the inter- and intra-genetic relatedness among basidiomycete species. Indeed, such molecular data can be used in conjunction with conventional methods employing morphological, physiological and biochemical characters. The amplified fragment length polymorphism (AFLP) technique combines both classical, hybridization-based fingerprinting methods with a PCR-based fingerprinting method (Vos et al. 1995). This technique is highly sensitive and reproducible along with the ability to detect a large number of loci covering a whole genome in a single assay, so it has been used in genetic study of many organisms

Table 1 – *Rhizopogon roseolus* isolates from Japan used in this study.

Isolate	Geographic location	Host	Year/month	Origin (Original description)	Accession no.
Iwate-1	Rikuzentakata-shi, Iwate	P. t	2003/07	Miyagi Pref. (30–41)	AB685718
Miyagi-1	Naruse-cho, Miyagi	P. t	1998/05	Miyagi Pref. (30–15)	AB685719
Miyagi-2	Sendai-shi, Miyagi	P. t	2003/07	Miyagi Pref. (30–42)	AB685386
Ibaraki-1	Asahi-son, Ibaraki	P. t	1999/04	Ibaraki Pref. (AT678)	AB685720
Ibaraki-2	Naka-shi, Ibaraki	P. d	1998/04	Ibaraki Pref. (AT632)	GQ179956
Ibaraki-3	Naka-shi, Ibaraki	P. t	–	NITE (33151)	NITE[a]
Ibaraki-4	Kashima-shi, Ibaraki,	P. t	1998/03	Ibaraki Pref. (AT630)	GQ17955
Niigata-1	Nakajo-cho, Niigata	P. t	2002/-	FFPRI (RR02Ni)	AB685721
Niigata-2	Ryozu-shi, Niigata	P. t	2005/11	Niigata Pref. (Nsado2)	AB685387
Niigata-3	Ryozu-shi, Niigata	P. t	2005/11	Niigata Pref. (Nsado3)	AB685388
Niigata-4	Ryozu-shi, Niigata	P. t	2005/11	Niigata Pref. (Nsado4)	AB685389
Niigata-5	Ryozu-shi, Niigata	P. t	2005/11	Niigata Pref. (Nsado5)	AB685390
Ishikawa-1	Nomi-shi, Ishikawa	P. t	2005/11	Ishikawa Pref. (C)	AB685722
Shizuoka-1	Kakegawa-shi, Shizuoka	P. t	2002/11	Shizuoka Pref. (1)	AB685723
Kyoto-1	Amino-cho, Kyoto	P. t	–	NITE (32812)	NITE[a]
Tottori-1	Tottori-shi, Tottori	P. t	1991/11	TMIC (31952)	AB685391
Tottori-2	Tottori-shi, Tottori	P. t	1991/11	TMIC (31956)	AB685392
Tottori-3	Tottori-shi, Tottori	P. t	1992/04	TMIC (32018)	AB685393
Tottori-4	Tottori-shi, Tottori	P. t	1992/04	TMIC (31983)	AB685394
Tottori-5	Tottori-shi, Tottori	P. t	1992/04	TMIC (31984)	AB685395
Tottori-6	Tottori-shi, Tottori	P. t	1992/04	TMIC (31985)	AB685396
Tottori-7	Tottori-shi, Tottori	P. t	1992/11	TMIC (32770)	AB685397
Tottori-8	Tottori-shi, Tottori	P. t	2005/04	This study	AB685410
Tottori-9	Tottori-shi, Tottori	P. t	2005/04	This study	AB685411
Tottori-10	Tottori-shi, Tottori	P. t	2005/04	This study	AB274244
Tottori-11	Tottori-shi, Tottori	P. t	2005/04	This study	AB685412
Tottori-12	Tottori-shi, Tottori	P. t	2005/04	This study	AB685413
Shimane-1	Izumo-shi, Shimane	P. t	2002/04	Shimane Pref. (106)	AB685724
Shimane-2	Shinji-cho, Shimane	P. t	2001/04	Shimane Pref. (107)	AB685725
Yamaguchi-1	Hikari-shi, Yamaguchi	P. t	1988/04	Yamaguchi Pref. (1)	AB685726
Tokushima-1	Kainan-cho, Tokushima	P. t	1996/11	Tokushima Pref. (TSYO1)	AB685727
Tokushima-2	Kainan-cho, Tokushima	P. t	1997/04	Tokushima Pref. (OSATO1)	AB685398
Kochi-1	Oogata-cho, Kochi	P. t	–	Kochi Pref. (20710)	AB685399
Kochi-2	Oogata-cho, Kochi	P. t	–	Kochi Pref. (20711)	AB685400
Kochi-3	Oogata-cho, Kochi	P. t	–	Kochi Pref. (20712)	AB685401
Kochi-4	Oogata-cho, Kochi	P. t	1998/03	Kochi Pref. (20714)	AB685402
Saga-1	Karatsu-shi, Saga	P. t	–	Saga Pref. (1)	AB685403
Saga-2	Karatsu-shi, Saga	P. t	–	Saga Pref. (2)	AB685404
Saga-3	Karatsu-shi, Saga	P. t	–	Saga Pref. (4)	AB685405
Saga-4	Karatsu-shi, Saga	P. t	–	Saga Pref. (B)	AB685406
Saga-7	Karatsu-shi, Saga	P. t	2001/-	FFPRI (RR01N)	AB685728
Miyazaki-1	Miyazaki-shi, Miyazaki	P. t	2003/-	FFPRI (RR03H)	AB685729
Kagoshima-1	Hioki-shi, Kagoshima	P. t	2002/03	Ibaraki Pref. (KM8)	AB685407
Kagoshima-2	Hioki-shi, Kagoshima	P. t	1999/-	FFPRI (RR99E)	AB685408
Kagoshima-3	Kagoshima-shi, Kagoshima	P. t	2003/-	FFPRI (RR03S)	AB685409

P. d, *Pinus densiflora*; P. t, *Pinus thunbergii*. –, unknown. FFPRI, Forestry and Forest Products Research Institute Kyushu Research Center; Ibaraki Pref., Ibaraki Prefectural Forestry Research Institute; Ishikawa Pref., Ishikawa-Ken Forest Experiment Station; Kochi Pref., Kochi Information, Research and Training Center; Miyagi Pref., Miyagi Prefectural Forestry Research Institute; Niigata Pref., Niigata Forestry and Forest Products Research Institute; NITE, National Institute of Technology and Evaluation (Biological Resource Center); Saga Pref., Saga Prefectural Forest Experiment Station; Shimane Pref., Shimane Prefecture Mountainous Region Research Center; Shizuoka Pref., Shizuoka Prefecture Forestry and Forest Products Research Institute; TMIC, Tottori Mycological Society Culture Collection; Tokushima Pref., Tokushima Agriculture, Forestry and Fisheries Technology Support Center Forest and Forestry Research Institute; Yamaguchi Pref., Yamaguchi Prefectural Forestry Guidance Institute.

[a] http://www.nbrc.nite.go.jp/alphabet/03_R.html.

Table 2 – Number of DNA fragments detected by AFLP analysis.

Primer combination	Total number of DNA fragments	Number of DNA fragments in each isolate	Total number of polymorphic DNA fragments	Percentage of polymorphic DNA fragments
*Eco*RI + AC/*Mse*I + CA	65	27–40 (31.8)	51	78.4
*Eco*RI + AC/*Mse*I + CC	61	14–27 (21.4)	51	83.6
*Eco*RI + CA/*Mse*I + CA	97	40–52 (45.5)	66	68.0
Total	223	82–114 (98.6)	168	75.3

Numbers in parentheses indicate the average value.

(Blears et al. 1998). Recently, also in basidiomycetes, AFLP techniques are applied to construct genetic map (Forche et al. 2000; Lind et al. 2005; Terashima et al. 2002b) and to study phylogenetic relationships and genetic diversity among cultivars (Terashima et al. 2002a, 2004, Matsumoto et al. 2003).

In this study, we estimated the level of the genetic variation among 45 *R. roseolus* isolates (Table 1) from different geographic regions in Japan using AFLP markers.

Forty-five isolates have been isolated from sporocarps aseptically and stored by different research institutes (Table 1). Taxonomic identification of all samples was based on morphological and ecological characters. In addition, we confirmed that all of rDNA-internal transcribed spacer (ITS) sequences from the 44 isolates used in this study showed more than 99.4% similarity to that from the isolate, Tottori-10 (Accession no. AB274244), which has been used as one of Japanese *R. roseolus* ITS sequences by Visnovsky et al. (2010).

Fungal isolates were cultured for three weeks at 25 °C on MYG liquid medium (2% malt extract, 0.2% yeast extract, 2% glucose). Mycelia were harvested, washed with distilled water, and lyophilized. Total DNA was extracted from approximately 10 mg powdered lyophilized mycelium using GenElute Plant Genomic DNA kit (Sigma, Toronto, Canada) according to the manufacturer's instructions. DNA isolated from each of the 45 isolates was dissolved in 100 μl 1xTE buffer and stored at −20 °C until further use.

The AFLP procedure followed Vos et al. (1995) and Terashima et al. (2002a, 2002b) with minor modifications. Total DNA was digested with the endonucleases *Eco*RI and *Mse*I, and double-stranded *Eco*RI and *Mse*I adaptors were ligated to the sticky ends of the fragments using an AFLP Core Reagent Kit (Invitrogen, Carlsbad, CA). A preselective primer pair with no selective bases (*Eco*RI + 0 and *Mse*I + 0) and three selective primer pairs with two selective bases (*Eco*RI + AC/*Mse*I + CA, *Eco*RI + AC/*Mse*I + CC and *Eco*RI + CA/*Mse*I + CA) were used for amplification. Two selective primers, *Eco*RI + AC and *Eco*RI + CA, were labeled with 6-FAM dye. Preliminary experiments showed that these primer pairs produced banding patterns that were variable and easy to score reproducibly. Electrophoresis and detection of amplified fragments were performed using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). Fragments in the range 100–500 bp were scored using GeneScan software (Applied Biosystems). AFLP analysis was performed on each DNA sample at least twice and non-reproducible fragments, which may have constituted artifacts, were excluded.

From AFLP electropherograms, the presence or absence of reproducible polymorphic bands was scored as 1 and 0, respectively. A dissimilarity distance (D) matrix based on a modification of the Dice coefficient (Sneath and Sokal 1973) was calculated between pairs of different AFLP phenotypes as $D = 1-2(n_{xy})/(n_x + n_y)$ where n_{xy} is the number of shared fragments and n_x and n_y are the number of fragments in the fingerprints x and y, respectively. Trees based on this matrix were constructed using an unweighted pair-group with arithmetic averaging (UPGMA) method (Sokal and Michener

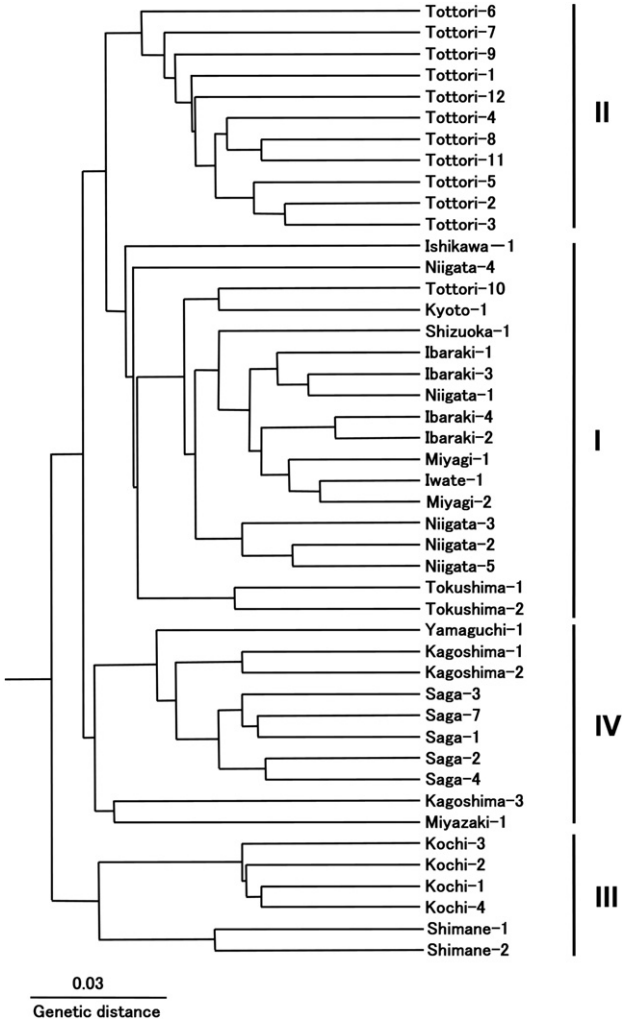

Fig. 1 – UPGMA tree generated using genetic dissimilarity coefficient data from AFLP profiles of 45 *Rhizopogon roseolus* isolates from Japan. Roman figures, I–IV, show major clusters (details provided in text).

1958) implemented in Phylip (Phylogeny Inference Package, version 3.5c, Felsenstein 1994) and the tree was drawn with the Treeview software package (Page 1996). The robustness of the clusters was examined by applying the bootstrap method with 1000 replications using PHYLIP (Felsenstein 1994). Principal coordinate (PCO) analysis was also performed using the GenAIEx program (Peakall and Smouse 2006) based on the binary data generated by the AFLP analysis.

Using three primer pair combinations, *Eco*RI + AC/*Mse*I + CA, *Eco*RI + AC/*Mse*I + CC and *Eco*RI + CA/*Mse*I + CA, a total of 223 AFLP fragments were obtained for 45 isolates (Table 2). The total number of DNA fragments detected by individual primer pairs ranged from 65 to 97. Of these fragments, 168 (75.3%) fragments were polymorphic for two or more isolates. The number of polymorphic DNA fragments for each primer pair varied from 51 to 66, with an average of 56 polymorphic fragments per primer pair. The percentage of polymorphic DNA fragments against the total number of DNA fragments ranged from 68.0% to 83.6%. By comparing the AFLP patterns of each isolates, the range in dissimilarity values was 0.043–0.228.

A UPGMA dendrogram was constructed based on the results of the dissimilarity matrix analysis (Figs. 1 and 2). Four major clusters could be distinguished: Cluster I, including the isolates collected from the seven prefectures (prefs.), Iwate, Miyagi, Ibaraki, Niigata, Ishikawa, Shizuoka, Kyoto and Tokushima, although one isolate from Tottori Pref. was included in it, Cluster II, including the isolates from Tottori Pref., Cluster III, including the isolates from Kochi Pref. and Shimane Pref., and Cluster IV, including the isolates from the four prefs., Yamaguchi, Saga, Miyazaki and Kagoshima. However, these clusters were not supported by bootstrap values (<50%). Therefore, in order to confirm the reproducibility of the UPGMA clustering described above, we performed a PCO analysis using the binary data generated by the AFLP analysis. The first and second PCO axis explained 24.0% and 23.2% of the total variation, respectively. The first two PCO axes separated four groups (dotted circles in Fig. 3), which corresponded to the groups in the UPGMA dendrogram (Fig. 1) except for only five isolates from the three prefs., Kyoto, Tokushima and Shimane. Although there were some contradictions between the results from two analyses as described above, these results suggest the possibility that the *R. roseolus* isolates have accumulated genetic variations that correspond to the geographical distribution of the species in Japan. Further study using more samples from many different local areas in Japan and different properties of genetic markers such as co-dominant markers (e.g. microsatellites) is needed to elucidate the detailed and exact geographical structure of this fungus in Japan.

Basidiospores of hypogeous sporocarp characteristic of *Rhizopogon* species are thought to be dispersed primarily by small mammal mycophagy, and it is generally assumed that their spore dispersal is less effective than that of epigeous sporocarps with wind-dispersed spores. Some surveys on population genetic structure with microsatellite analysis in other *Rhizopogon* species (Kretzer et al. 2003, 2005; Grubisha et al. 2007) estimated both the genetic variation within populations and the genetic differentiation among populations separated by geographic barriers caused by geological history, geographic distances, mountain ranges, and ocean, etc. There are many mountains, rivers, lakes and seas in Japan, and the population of the host plant, Japanese black pine, of this fungus is separated by these geographic barriers. Given these geographical features of Japan in mind, it is considered that the characteristics of spore dispersal of the hypogeous sporocarp as described above might strongly related to the genetic variation observed among natural

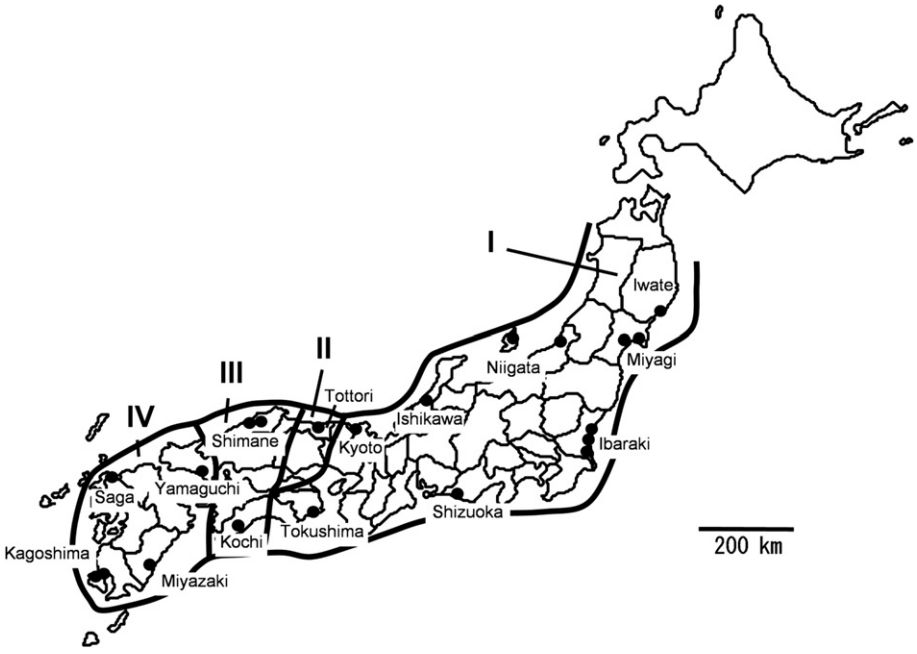

Fig. 2 – Tentative delineation of Japanese *Rhizopogon roseolus* populations speculated from the results of cluster analysis (See Fig. 1). ●: Sampling sites (name of prefectures) corresponding to Table 1.

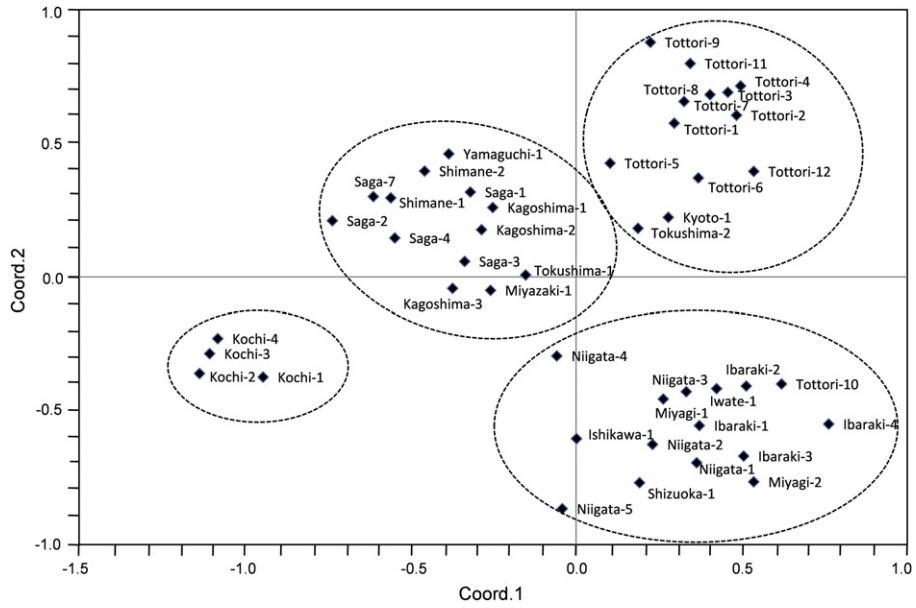

Fig. 3 – Scatter diagram showing relative similarities between 45 *Rhizopogon roseolus* isolates from Japan using principal coordinates (PCO) analysis. The first two PCO axes were plotted, and the first and second PCO axis explained 24.0% and 23.2% of the total variation, respectively. Dotted lines delineate groups identified by PCO analysis.

isolates of *R. roseolus* in this study. On the other hand, human activities are expected to have altered the distribution of this fungus in Japan (e.g. cultivation and reforestation of the host plant, *P. thunbergii*), the cultivation of *R. roseolus* on a national scale has not yet been undertaken. This differs from the situation in *Lentinula edodes* (Berk.) Pegler, *Pholiota nameko* (T. Ito) S. Ito and Imai and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer and other fungal species, which have been extensively cultivated and which there is no longer any relationship between the genetic characteristics of natural populations and their sites of geographical origin (Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995; Obatake et al. 2002). In addition, areas where black pine seedlings can be distributed and planted in Japan is restricted by the Forestry Seeds and Seedlings Act, which means seedlings of black pine grown in particular region have been not widely distributed throughout the country (Miyata 1996).

Thus, the various Japanese isolates of *R. roseolus* could potentially be valuable genetic resources for cultivation programs involving this fungus. This is the first report on the genetic variation among isolates of an ectomycorrhizal hypogeous basidiomycete from different geographical regions in Japan.

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