Applied and Environmental Microbiology, Jan. 2010, p. 294–302 Vol. 76, No. 1
0099-2240/10/$12.00 doi:10.1128/AEM.02191-09

Traceability of Marketable Japanese Shoro in New Zealand: Using Multiplex PCR To Exploit Phylogeographic Variation among Taxa in the *Rhizopogon* Subgenus *Roseoli*▽†

Sandra B. Visnovsky,[1] Alexis Guerin-Laguette,[1] Yun Wang,[1] and Andrew R. Pitman[1,2]*

The New Zealand Institute for Plant & Food Research Limited, Private Bag 4704, Christchurch, New Zealand,[1] and Bioprotection Research Centre, Lincoln University, P.O. Box 84, Canterbury, New Zealand[2]

Received 10 September 2009/Accepted 28 October 2009

***Rhizopogon roseolus* Corda (synonym *Rhizopogon rubescens* Tul.), an economically important edible mushroom associated with the Pinaceae (mostly *Pinus* sp.), has a global distribution resulting from the introduction of exotic trees into the Southern Hemisphere for plantation forestry. However, the marketability of *R. roseolus* varies with the place of origin. *R. roseolus* strains cultivated in New Zealand from local carpophores for the Japanese market are morphologically and biologically distinct from those produced in Japan and are consequently considered less valuable. In this study, the ITS1-5.8S-ITS2 rRNA (internal transcribed spacer [ITS]) region was used to examine the phylogenetic relationships of *R. roseolus* and other closely related fungi belonging to *Rhizopogon* subgenus *Roseoli* to determine the genetic basis for phenotypic differences among *R. roseolus* isolates from different geographic regions. Phylogenetic comparison revealed phylogeographic variation within *Rhizopogon* subgenus *Roseoli*. Collections from the United States and Europe grouped into four distinct clades. *Rhizopogon roseolus* isolates found in New Zealand were closely related to those from the United States, likely due to introduction of *Pinus radiata* from its native California in the United States. In contrast, Japanese *R. roseolus* isolates clustered closely with European collections. Phylogenetic differences between Japanese and New Zealand *R. roseolus* isolates may explain the morphological and biological properties attributed to these geographical variants. The ITS region was subsequently used to design a multiplex PCR for the simultaneous identification of Japanese and New Zealand *R. roseolus* isolates to track the establishment of ectomycorrhiza on *P. radiata* seedlings inoculated with commercially valuable *R. roseolus*. This diagnostic demonstrated the first fruiting of Japanese shoro cultivated on *P. radiata* in the Southern Hemisphere.**

Since the latter half of the 19th century, Northern Hemisphere species of exotic trees, in particular *Pinus radiata*, have been planted in the Southern Hemisphere (e.g., Chile, Argentina, South Africa, Australia, and New Zealand). Indeed, over 1,000,000 ha of exotic trees have been established in New Zealand alone (32, 9). Over 200 nonnative basidiomycete and ascomycete ectomycorrhizal (ECM) fungal species are associated with *Pinus* or *Eucalyptus* plantations in the Southern Hemisphere from the introduction of plants with intact root systems (46).

The genus *Rhizopogon* Fries (Basidiomycota, Boletales) contains more than 100 species of hypogeous fungi (24), which form ECM associations mostly with members of the Pinaceae (44). The greatest diversity of *Rhizopogon* can be found in the coniferous forests of the Pacific northwestern United States (40, 30), although a number of species are known to occur in Europe (40) and Asia (3). At least four introduced species of *Rhizopogon* have also been reported in New Zealand, including *R. clelandii* Cunn., *R. luteolus* Fr., *R. roseolus* (7), and *R. vinicolor* A. H. Smith (5). At least two species, *R. luteolus* and *R. roseolus*, have been associated with *P. radiata* seedlings in forest nurseries in the North Island of New Zealand (6).

In Japan, *R. roseolus* is known locally as shoro. Shoro is considered a delicacy (20), and its production is dependent on the collection of the carpophores in the field. In fact, only 200 years ago, shoro was the fourth most commonly consumed mushroom in Japan (34). However, the number of natural shoro has declined in the second half of the 20th century (15), resulting in its cultivation in forestry plantations since the late 1980s (50). To fulfill Japanese demand, plantations of *P. radiata* artificially inoculated with *R. roseolus* have been established in New Zealand since 1999 using spores from fruiting bodies collected locally (47). Three of the four plantations have subsequently produced fruiting bodies, but the crop has been deemed unsuitable due to consumer sensitivity in Japan to the origin of the products and doubts surrounding the authenticity of the fruiting bodies as Japanese shoro. The quality and market price of other edible fungi differ not only with species but also with their origin. In Japan, domestic *Tricholoma matsutake* is considered the premium source of matsutake, traded at $500/kg, while South Korean matsutake and those from China are considered less valuable ($250/kg and $100/kg, respectively) (31). Highly prized edible fungi have also been found to be contaminated with less valuable species. *Tuber rufum* Pico is a truffle species that is found alongside other valuable species in countries where truffles are commer-

* Corresponding author. Mailing address: The New Zealand Institute for Plant & Food Research Limited, Private Bag 4704, Christchurch, New Zealand. Phone: 64 3 325 6400. Fax: 64 3 325 2074. E-mail: andrew.pitman@plantandfood.co.nz.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 13 November 2009.

cially important. However, it is considered to be a poorly flavored species with no marketable value and is deemed a "contaminant" in truffières (4).

Unfortunately, the taxonomy of the genus *Rhizopogon* is surrounded by many unresolved issues, which makes differentiation of shoro-like fungi found in different geographical locations difficult. In 1966, Smith and Zeller (40) completed the first taxonomic study of the genus *Rhizopogon*, dividing it into two subgenera, *Rhizopogonella* (subsequently transferred to *Alpova* [45]) and *Rhizopogon*. The subgenus *Rhizopogon* was further divided into four sections based on differences in the host plant, sporocarp morphology, and color of the peridium. All species associated with *Pinus* spp. were classified in *Rhizopogon* section *Rhizopogon*. *Rhizopogon roseolus* and *R. rubescens* were placed in subsection *Angustispori*, stirps *Rubescens*. A third species, *Rhizopogon vulgaris* Vittad., was placed in subsection *Angustispori*, stirps *Vulgaris*, due to differences in its spore morphology.

Similarities between species in stirps *Rubescens* and stirps *Vulgaris* were recorded, with stirps *Vulgaris* considered a continuation of stirps *Rubescens* into the narrow-spore species. However, classification was based only on examinations of North American collections even though these species were originally described in Europe in the 19th century (40). Taxonomic reexamination of *Rhizopogon* using phylogenetic analyses of internal transcribed spacer (ITS) DNA sequences showed that *Rhizopogon burlinghamii*, *R. roseolus*, and *R. vulgaris* formed *Rhizopogon* section *Rhizopogon* clade C, separate from the other species sampled from section *Rhizopogon* (clades A and B). Their ITS sequences lacked insertions and deletions that are diagnostic of other section *Rhizopogon* clades. *Rhizopogon roseolus* and *R. vulgaris* were placed together under *Rhizopogon* subgenus *Roseoli* (13). Unfortunately, collections classified as *R. rubescens* were not included in the phylogenetic study. More recently, species concepts in the *R. roseolus* species group were examined by Martín and García (25). ITS sequence analyses separated the collections into five possible phylogenetic species.

The continued taxonomic instability of *Rhizopogon* subgenus *Roseoli* has created ongoing confusion, and the correct species names are still not clear. In Japan, until now, shoro has been referred to as *R. rubescens*; however, *R. rubescens* is used widely as a synonym for *R. roseolus*. In this study, *R. roseolus* will be used to describe all collections unless specifically stated, as this taxonomic name appears to have precedence in previous phylogenetic studies of *Rhizopogon* (13, 19, 40) and since Mycobank (http://www.MycoBank.org/) considers *R. rubescens* to be a synonym of *R. roseolus*.

Molecular diagnostic tools capable of distinguishing genetic differences in ECM fungi have been developed, allowing the differentiation of commercially important species from contaminants or similar species of less economic value. There are many studies where PCR primers designed for the amplification of the ITS region have been used to identify basidiomycetes (12, 18). Species-specific primers were created to identify and differentiate marketable boletes (28), to detect black truffle species (37), and to distinguish Asiatic black truffles from *Tuber melanosporum* in commercialized products (22). A multiplex PCR has also been developed to simultaneously detect different white truffle species and one of the most aggressive contaminant fungi for monitoring the persistence of a selected truffle in inoculated seedlings (1).

Due to the sensitivity of consumers to the origins of shoro and the existing taxonomic complexity of the genus *Rhizopogon*, morphological and molecular methods were used to establish the diversity and genetic structure of *Rhizopogon* subgenus *Roseoli*. Phylogenetic relationships between shoro-like species (originally classified as *R. vulgaris*, *R. rubescens*, and *R. roseolus*) from different geographical locations were investigated to verify previously observed differences between shoro grown in Japan and New Zealand. A multiplex PCR was then developed for the rapid identification of ECMs and fruiting bodies grown from Japanese shoro in New Zealand to track the commercial production of this economically valuable edible fungus.

MATERIALS AND METHODS

Collecting fruiting bodies and ectomycorrhizae. A total of 32 collections of *Rhizopogon*, including 18 fruiting bodies and 14 ECM root tips, were obtained between 2004 and 2009 from different regions in New Zealand (Table 1). After removing soil debris, fruiting bodies were stored as air-dried samples. Mycorrhizal root tips were washed repeatedly in water and selected using a dissection microscope. For storage, ECMs were frozen at −20°C. Japanese strains AT630 and AT632 (49) were provided as pure mycelial cultures after introduction into New Zealand in 2004 for commercialization. Collections were obtained from nurseries, plantations, and natural ecosystems.

DNA isolation. Total DNA was extracted from approximately 20 mg of a fruiting body or from a single infected root tip using the DNeasy plant mini kit (Qiagen). The procedure was performed according to the manufacturer's instructions with the following modification: for each sample, the fungal tissue was disrupted in AP1 lysis buffer using a sterile micropestle. After extraction, genomic DNA was eluted in 50 μl of EB buffer.

PCR amplification and DNA sequencing of the ITS region. Amplification of the ITS1-5.8S-ITS2 region from each DNA sample was performed by PCR using the universal primer pairs ITS5 and ITS4, ITS1 and ITS4 (48), and ITS1-F and ITS4-B (12). Each reaction was carried out in a total volume of 25 μl containing 1 μl of each primer (5 μM), 2 μl of deoxynucleoside triphosphates (dNTPs) (2 mM), 2.5 μl of 10× Roche buffer (containing 1.5 mM $MgCl_2$), 0.1 μl of Roche *Taq* polymerase (5 U/μl), and 1 μl of template DNA (1 to 10 ng/μl) in sterile water. Amplification was performed in a GeneAmp PCR system 9700 (Applied Biosystems) thermocycler using the following conditions: 2 min for initial denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and a final extension step of 72°C for 7 min. PCR products were purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. All DNA sequencing of the ITS region was performed by Macrogen Ltd. (Seoul, South Korea) using a combination of the primers listed above. DNA sequence output for each sample was assembled using Sequencher 3.1.1 (Gene Codes).

Phylogenetic analysis of the ITS DNA sequences. Partial ITS1-5.8S-ITS2 DNA sequences obtained for *Rhizopogon* in this study were compared with those retrieved from the GenBank database (National Center for Biotechnology Information, Bethesda, MD) to identify relationships between isolates from New Zealand, Japan, and other geographical locations. Sequences were aligned using ClustalX v1.83 (43), and each DNA sequence was edited to 642 positions in the resulting multiple-sequence alignment. GenBank accession numbers for the sequences used in our phylogenetic studies are listed in Table 1.

Phylogenetic relationships were estimated from the aligned DNA sequences using PAUP v 4.0 (41). Distance analyses were performed, excluding uninformative characters, using maximum-likelihood and general time-reversible (GTR) models of evolution. Unweighted and weighted parsimony analyses were also carried out on the data set, excluding uninformative characters, using the heuristic search option. Weighting was conducted to account for different substitution rates among ITS1, ITS2, and the 5.8S subunit. Initially, JMT22516, a genetically characterized collection of *R. luteolus* associated with *Pinus*, was selected as the outgroup for rooting of neighbor-joining and parsimonious trees. However, given the distance of the outgroup from the *R. roseolus* collections, unrooted phylogenies were generated without JMT22516 to illustrate more effectively the discrimination among clades within the *R. roseolus* collections. Support for the phylogenetic groupings was obtained with bootstrap analyses

TABLE 1. *Rhizopogon* collections obtained from nurseries, plantations, and other sources in New Zealand as well as foreign isolates used in this study

Isolate	Original species designation	Accession no.[a]	Tissue[b]	Location	Host or tree species present	Source or reference
Collections						
NZRr1	*R. roseolus*	GQ179934	FB	New Zealand	*P. radiata*	This study
NZRr2	*R. roseolus*	GQ179935	FB	New Zealand	*P. radiata*	This study
NZRr3	*R. roseolus*	GQ179936	FB	New Zealand	*P. radiata*	This study
NZRr4	*R. roseolus*	GQ179937	FB	New Zealand	*P. radiata*	This study
NZRr5	*R. roseolus*	GQ179938	FB	New Zealand	*P. radiata*	This study
NZRr6	*R. roseolus*	GQ179939	FB	New Zealand	*P. radiata*	This study
NZRr7	*R. roseolus*	GQ179940	FB	New Zealand	*P. radiata*	This study
NZRr8	*R. roseolus*	GQ179941	FB	New Zealand	*P. radiata*	This study
NZRr9	*R. roseolus*	GQ179942	FB	New Zealand	*P. radiata*	This study
NZRr10	*R. roseolus*	GQ179943	FB	New Zealand	*P. radiata*	This study
NZRr11	*R. roseolus*	GQ179944	FB	New Zealand	*P. radiata*	This study
NZRr12	*R. roseolus*	GQ179945	ECM	New Zealand	*P. radiata*	This study
NZRr13	*R. roseolus*	GQ179946	FB	New Zealand	*P. radiata*	This study
NZRr14	*R. roseolus*	GQ179947	ECM	New Zealand	*P. radiata*	This study
NZRr15	*R. roseolus*	GQ179948	FB	New Zealand	*P. radiata*	This study
NZRr16	*R. roseolus*	GQ179949	FB	New Zealand	*P. radiata*	This study
NZRr17	*R. roseolus*	GQ179950	FB	New Zealand	*P. radiata*	This study
NZRr18	*R. roseolus*	GQ179951	FB	New Zealand	*P. radiata*	This study
NZRr19	*R. roseolus*	GQ179952	ECM	New Zealand	*P. radiata*	This study
NZRr20	*R. roseolus*	GQ179953	ECM	New Zealand	*P. radiata*	This study
NZRr21	*R. roseolus*	GQ179954	FB	New Zealand	*P. radiata*	This study
AT630	*R. roseolus*	GQ179955	Mycelium	Japan	*P. thunbergii*	This study
AT632	*R. roseolus*	GQ179956	Mycelium	Japan	*P. densiflora*	This study
ER48	*Rhizopogon* sp.	GQ179957	FB	Spain	*P. halepensis*	This study
NZJS1	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS2	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS3	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS4	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS5	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS6	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS7	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZRr22	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZECM1	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZECM2	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZRr23	*R. roseolus*	ND	ECM	New Zealand	*P. radiata*	This study
NZJS8	*R. roseolus*	GU123601	FB	New Zealand	*P. radiata*	This study
GenBank submissions used for phylogenetic analyses						
PALRHI-1	*R. rubescens*	AJ810043	FB	USA	NF	Martín, 2004 (GenBank submission)
3RUB-JMV990612_3	*R. rubescens*	AMO85528	NF	Spain	NF	Martin, 2005 (GenBank submission)
MA-Fungi 47689	*R. roseolus*	AJ419211	NF	Spain	NF	26
MA-Fungi 47687	*R. roseolus*	AJ419210	NF	Spain	NF	26
MA-Fungi 47688	*R. roseolus*	AJ419209	NF	Spain	NF	26
K253	*Rhizopogon* sp.	AB505221	FB	Japan	*P. thunbergii*	Nara, 2009 (GenBank submission)
K259	*Rhizopogon* sp.	AB505222	FB	Japan	*P. thunbergii*	Nara, 2009 (GenBank submission)
K291	*Rhizopogon* sp.	AB505223	FB	Japan	*P. thunbergii*	Nara, 2009 (GenBank submission)
Tottori-10	*R. roseolus*	AB274244	FB	Japan	NF	Matsumoto, 2006 (GenBank submission)
SP1	*R. roseolus*	EU379678	ECM	Poland	*P. sylvestris*	Hilszczanska and Gaszczyk, 2008 (GenBank submission)
JMT8227	*R. roseolus*	AF058315	FB	USA	*P. coulteri*[c]	13
Unnamed	*R. roseolus*	AF158018	ECM	USA	*P. muricata*	42
R19.1	*R. rubescens*	AJ277644	ECM	France	*P. pinea*	10
B.S.1	*R. rubescens*	AJ307681	FB	France	*P. nigra*[c]	11
NS182	*R. rubescens*	DQ068965	ECM	Lithuania	*P. sylvestris*	29
PSERHI-1	*R. pseudoroseolus*	AJ810040	FB	USA	*P. resinosa*[c]	Martín, 2004 (GenBank submission)
PSERHI-2	*R. pseudoroseolus*	AJ810041	FB	USA	*P. resinosa*[c]	Martín, 2004 (GenBank submission)
PSERHI-3	*R. pseudoroseolus*	AJ810042	FB	USA	*P. resinosa*[c]	Martín, 2004 (GenBank submission)
KGP38	*R. vulgaris*	DQ822823	ECM	USA	*P. muricata*	35
JMT19154	*R. vulgaris*	AF062934	FB	USA	*Tsuga heterophylla*, *P. contorta*[c]	13
JMT22516	*R. luteolus*	AF062936	FB	Sweden	*P. sylvestris*[c]	13
SNFD32-2	*R. species*	AJ515415	NF	USA	*P. muricata*[c]	21

[a] ND, not determined.
[b] FB, fruiting body; NF, no details found.
[c] Fruiting bodies and ECMs collected from under a canopy, but host species not confirmed.

using 1,000 replicates. Only bootstrap values greater than 70% were included in the trees.

Multiplex PCR detection of marketable shoro in New Zealand. Four primers containing a total of 12 polymorphic nucleotides were designed using an alignment of ITS sequences from the 46 collections of *Rhizopogon* to simultaneously detect and differentiate shoro from New Zealand and Japan by multiplex PCR. Primer pairs NZRhrF4 (5′-TCTCAACCCCTCTCGATTTTT-3′)-NZRhrR4 (5′-CGGTATTAGAGGCACGGAAC-3′) and Fjap (5′-CATATACCACTTCG TGTAGA-3′)-Rjap630 (5′-CAAGCTCCCCCTCGAAACA-3′) were used to amplify a PCR product of 203 bp from *R. roseolus* from New Zealand and an amplicon of 292 bp from Japanese *R. roseolus*, respectively. DNA amplification was carried out in a total volume of 25 μl containing 1 μl of each primer (5 μM), 2 μl of dNTPs (2 mM), 2.5 μl of 10× Roche buffer (containing 1.5 mM $MgCl_2$), 0.1 μl of Roche *Taq* polymerase (5 U/μl), and 1 μl of template DNA (1 to 10 ng/μl) in sterile water. PCR conditions were optimized as follows: 2 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min and a final extension step at 72°C for 7 min. A total of 15 μl of PCR product was electrophoresed in a 1.5% agarose gel for separation of the bands. In addition, an ITS PCR was carried out using primers ITS1 and ITS4 to check the quality of DNA in each sample.

Mycelial inoculation and mycorrhiza formation of Japanese shoro. Seeds of *P. radiata* D. Don were surface sterilized for 15 min in 30% H_2O_2 and thoroughly rinsed in reverse osmosis (RO) water before germination on vermiculite. The fungal inoculum of isolate AT632 was produced in nonagitated liquid m+p medium (60 ml/flask, pH 5.6) (14). The m+p medium results from the combination (1:1, vol/vol) of MMN medium (with glucose replacing sucrose) (27) augmented with 0.5 g/liter of yeast extract and PDA medium (Difco). Eight-week-old seedlings were then inoculated with one-third of the mycelium growing in a single flask (rinsed with nonsterile RO water) by gently tying the mycelium directly against the short roots using cotton thread. Inoculated seedlings were potted in Masrac olive pots (Carann, New Zealand) using vermiculite-perlite-peat-pine bark (4:2:1:1) previously sterilized by autoclaving (30 min at 121°C, twice at 24-h intervals) and watered with RO water as required. Seedlings were raised in the same potting mix (increasing the pot size from 420 ml to 900 ml after 6 months) for the subsequent 13 months and then transferred to 2.7-liter WM containers (Thermoflan, France) using the previous substrate mixed with autoclaved sandy soil (2:1, vol/vol). The soil was collected in Tara Hills, New Zealand. Sixteen months later, seedlings were either out-planted in an agricultural field or kept in the greenhouse. Prior to out-planting, seedlings were raised under controlled greenhouse conditions (16/8-h light/dark cycle at 23/18°C with a photosynthetic active radiation of about 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ [400 to 700 nm]). Two months after inoculation, seedlings were fertilized twice a week using a 1,000-fold dilution of the A+B solution described by Nylund and Wallander (33).

Identifying ECMs and fruiting bodies associated with cultivated *P. radiata* using morphology and multiplex PCR. Root tips of *P. radiata* seedlings inoculated with *R. roseolus* AT632 were subsequently monitored for the development of ECM structures using a dissecting microscope (Leica MZ16), and micrographs were obtained with a Leica DFC420 digital camera. Upon observing ECMs, root tips were collected and the identity of ECM fungi associated with the root tips was investigated using morphological identification and multiplex PCR. In April 2009, a single fruiting body was also collected underneath an inoculated tree. To confirm its identity as Japanese shoro, it was morphologically identified and tested using multiplex PCR. Fruiting bodies and ECMs of *R. roseolus* originating from New Zealand (either from the field or as greenhouse contaminants) were compared with those obtained from isolate AT632. Their ultrastructures were compared using a compound microscope (Zeiss Axio Imager.A1).

Nucleotide sequence accession numbers. The DNA sequences determined in the study have been submitted to GenBank using the accession numbers listed in Table 1.

RESULTS

Phylogenetic analysis of *Rhizopogon* subgenus *Roseoli*. The ITS DNA sequences of *Rhizopogon* isolates obtained from New Zealand, Japan, and other geographical regions (including GenBank accession numbers) totaled 46 collections. The complete data set for these collections included 642 characters, 68 of which were parsimony informative. Collections were divided into 21 genotypes defined by sequence identity across the ITS region tested (Table 2).

TABLE 2. Genotypes of *Rhizopogon* collections assigned using their ITS rRNA sequences

Clade and ITS genotype[a]	Collection(s)
A	
ITS-JAP1	K253, K259, K291, AT630, AT632, Tottori-10
ITS-PLD1	SP1
ITS-SPN1	MA-fungi 47689, 3RUB-JMV990612_3
ITS-SPN2	MA-fungi 47687
B	
ITS-NZ1	NZRr6, NZRr8, NZRr9, NZRr10, NZRr21
ITS-NZ2	NZRr13
ITS-NZ3	NZRr1, NZRr2, NZRr3, NZRr4, NZRr5, NZRr7, NZRr11, NZRr12
ITS-NZ4	NZRr14, NZRr17, NZRr18, NZRr19, NZRr20
ITS-USA1	PSERHI-2, PSERHI-3
ITS-USA2	PSERHI-1
ITS-USA3	JMT19154, PALRHI-1
ITS-USA4	SNFD32-2
C	
ITS-NZ5	NZRr15, NZRr16
ITS-USA5	Unnamed (accession no. AF158018)
ITS-USA6	KGP38
ITS-USA7	JMT8227
D	
ITS-FRA1	B.S.1
ITS-FRA2	R19.1, NS182
ITS-SPN3	MA-fungi 47688
Other	
ITS-SPN4	ER48
Outgroup	
ITS-luteolus	JMT22516

[a] The abbreviations for the ITS genotypes are based on the geographic origin of the collections represented by each group.

Phylogenetic analyses using both unweighted and weighted parsimony grouped the ITS genotypes into four distinct and strongly supported clades within *Rhizopogon* subgenus *Roseoli* (Fig. 1). The homoplasy, consistency, and rescaled indices were 0.23, 0.78, and 0.89 and 0.21, 0.80, and 0.90 for the unweighted and weighted parsimony analyses, respectively. The structure of the parsimony tree generated using unweighted and weighted characters was congruent with neighbor-joining analyses using both maximum-likelihood and GTR analyses (Fig. 1). Parsimony-informative nucleotide polymorphisms associated with each genotype of *Rhizopogon* subgenus *Roseoli* are described in Table S1 in the supplemental material.

The collections represented by the 21 ITS genotypes did not cluster according to their original classifications as *R. vulgaris*, *R. rubescens*, or *R. roseolus*. Instead, collections grouped into clades based on their geographical origin (Table 2; Fig. 1). All Japanese shoro tested were found to have an identical ITS genotype (*ITS*-JAP1) and were clustered in clade A along with collections originating from Europe (*ITS*-PLD1 and *ITS*-SPN1). The bootstrap values supporting clade A were high (98 to 96% using parsimony). *ITS*-SPN2 was also closely related to clade A in all distance and parsimony analyses, although boot-

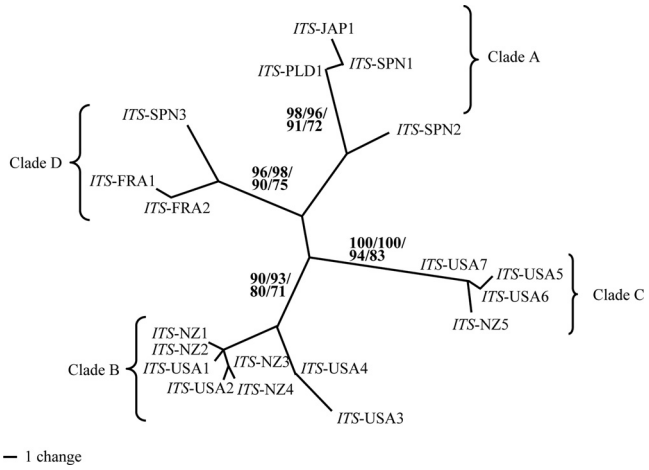

FIG. 1. One of 18 unrooted, equally parsimonious trees for all genotypes of *R. roseolus* and other closely related fungi defined by the ITS1, ITS2, and 5.8S subunit DNA sequences in this study (Table 2). The tree was constructed using weighted parsimony, excluding all uninformative characters. Bootstrap values of >70% from 1,000 replicates are indicated above the nodes for the following parsimony and distance analyses: unweighted parsimony/weighted parsimony/maximum likelihood/general time reversible. The operational taxonomic units used at the end of each node represent the names of each ITS genotype.

strap support for this relationship was lower (53 to 59% using parsimony).

Clade D contained a second group of collections of European origin. The bootstrap values supporting clade D were 96 to 98% using parsimony. In rooted trees, the relationship of these taxa to the other members of *Rhizopogon* subgenus *Roseoli* appeared to be more distant, suggesting the presence of two distinct genotypic groups of *R. roseolus* in Europe (data not shown).

Rhizopogon isolates originating from the United States or from locations in New Zealand were clustered into clades B and C. Clade B was composed of eight ITS genotypes, *ITS*-USA1 to *ITS*-USA4 and *ITS*-NZ1 to *ITS*-NZ4, supported by bootstrap values of between 90 and 93% using parsimony. This clade contained the previously characterized collection *R. vulgaris* JMT19154 (*ITS*-USA3) as well as three paratypes of *R. pseudoroseolus* (*ITS*-USA1 and -2). The majority of *Rhizopogon* isolates collected from *P. radiata* plantations in New Zealand also clustered within clade B (*ITS*-NZ1 to *ITS*-NZ4).

Clade C (supported by a bootstrap value of 100%) was represented by *R. vulgaris* KGP38 and *R. roseolus* JMT8227 (*ITS*-USA6 and -7, respectively) originating from the United States, previously classified using ITS sequencing. The remaining collections of *Rhizopogon* from New Zealand (*ITS*-NZ5) clustered within this clade.

ER48 (*ITS*-SPN4), was originally collected in Spain under a *P. halepensis* canopy and classified morphologically as *R. roseolus*. In our phylogenetic analysis using the ITS region, however, this collection did not cluster with any of the other *R. roseolus* isolates. Nevertheless, further investigation of ER48 using an unrooted phylogeny demonstrated that it was more closely related to *R. roseolus* collections than to the outgroup *R. luteolus* (data not shown).

All collections were found on or under *Pinus*, including *P. thunbergii* and *P. densiflora* in Japan, *P. radiata* in New Zealand, and a multitude of hosts in both Europe and the United States. However, no host specificity was observed between *Rhizopogon* ITS genotypes and host tree species.

Differentiation of *R. roseolus* isolates from Japan and New Zealand by morphology and multiplex PCR. Although the fruiting bodies of *R. roseolus* isolates originating from Japan and New Zealand have very similar overall morphological features (Fig. 2A and B), New Zealand shoro are significantly larger than the Japanese ones and are softer to the touch. At the ultrastructural level, the New Zealand shoro also display a thicker peridium, which stains red-brown in the presence of 5% KOH (Table 3). No morphological differences were seen between the spores from the two geographic origins or between their mycorrhizae on *P. radiata* (Fig. 2C and D).

Once it was established that *R. roseolus* isolates from New Zealand and Japan could be distinguished morphologically and genetically, primers were designed for their specific identification by PCR. Using the multiple alignments of the ITS DNA sequences, the primer pairs NZRhrF4-NZRhrR4 and Fjap-Rjap630 were designed to amplify PCR fragments of 203 bp from New Zealand *R. roseolus* and 292 bp from Japanese shoro, respectively. Upon testing, all DNA samples extracted from New Zealand *R. roseolus* displayed a single band of 203 bp using NZRhrF4 and NZRhrR4, whereas DNA from Japanese shoro produced the expected 292-bp fragment with Fjap and Rjap630 (data not shown).

The four primers were then combined for use in a single diagnostic multiplex PCR. As before, all DNA samples extracted from New Zealand *R. roseolus* amplified a single fragment of 203 bp and the DNA from Japanese shoro produced a 292-bp amplicon (Fig. 3). The specificity of the primers designed for multiplex PCR was also confirmed using DNA extracted from other ECM species introduced into New Zealand, including species of *Tuber*, *Lactarius*, *Amanita*, *Suillus*, *Boletus*, *Hebeloma*, and *Thelephora*. Multiplex PCR using the DNAs of these species as a template produced no amplicon (Fig. 3).

Multiplex PCR confirms production of the first *R. roseolus* fruiting body cultivated in the Southern Hemisphere. The use of multiplex PCR confirmed the establishment of ECMs on *P. radiata* seedlings after artificial inoculation of Japanese shoro on their roots. ECMs of Japanese shoro AT632 were observed as early as 4 weeks following inoculation (data not shown) and continued to form regularly on inoculated seedlings (Fig. 2D). Approximately 3 years following inoculation, multiplex PCR of DNA extracted from root tips showing shoro-like ECM structures confirmed the presence of AT632 on *P. radiata* seedlings, either containerized or after transplantation into the field. Of 11 DNA samples from root tips tested in this study, 7 produced an amplicon of 292 bp indicative of Japanese shoro, whereas two ECMs produced a PCR fragment of 203 bp characteristic of New Zealand *R. roseolus*. Two other samples showing ECM structures distinct from those of shoro mycorrhizae failed to produce an amplicon indicative of either form of *R. roseolus* (data not shown), suggesting that the roots of *P. radiata* had also been colonized by other ECM fungi.

In April 2009 the first fruiting body, NZJS8, was observed under *P. radiata* previously inoculated with a pure mycelial

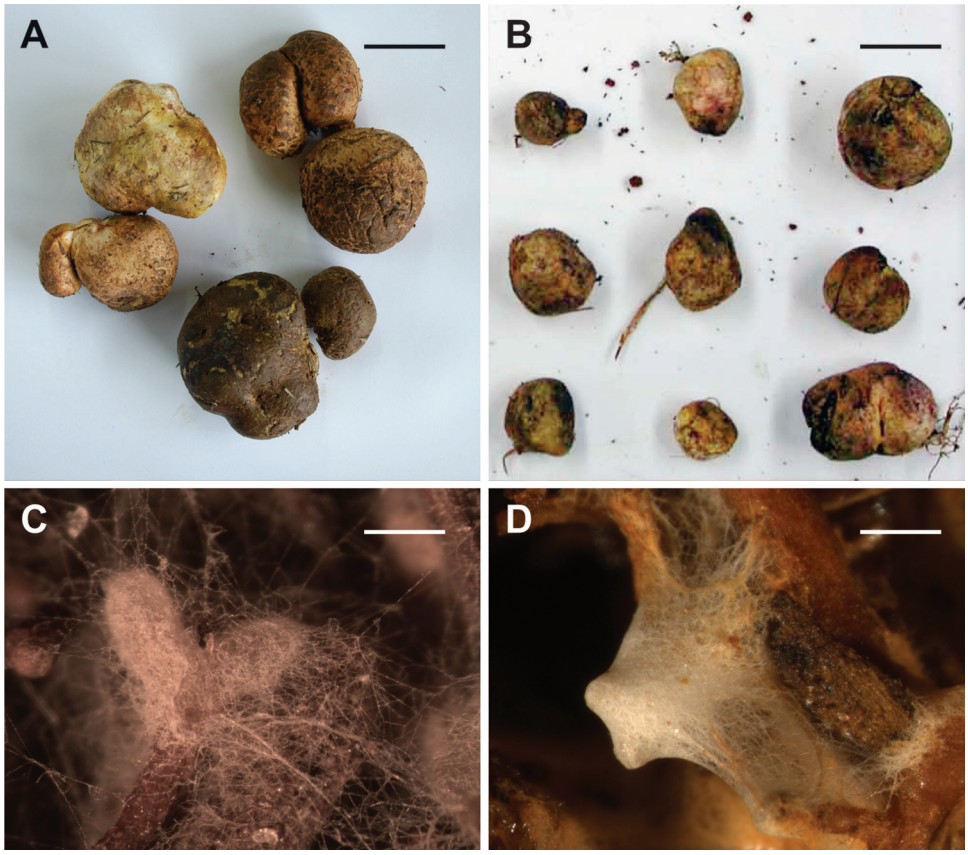

FIG. 2. Fruiting bodies and ECMs produced by New Zealand and Japanese shoros. (A) Fruiting bodies of New Zealand *R. roseolus* collected in Otago, New Zealand, in 2004. Bar, 2.5 cm. (B) Fruiting bodies of Japanese shoro grown in Japan. Bar, 1.7 cm. (Courtesy of Hisayasu Kobayashi.) (C) ECM of New Zealand *R. roseolus* collected from containerized seedlings inoculated with spores of New Zealand shoros in 2004. Bar, 0.8 mm. (D) ECM produced after mycelial inoculation of *P. radiata* with Japanese shoro isolate AT632 in 2006. Bar, 1.2 mm.

culture of Japanese shoro AT632 (see Fig. S1A in the supplemental material). A DNA sample was extracted, and a multiplex PCR was performed to verify the identity of the fruiting body. A PCR product of 292 bp was amplified, indicating that NZJS8 was a Japanese shoro (see Fig. S1B in the supplemental material). Furthermore, the ITS sequence of NZJS8 (Table 1) was identical to that of the original mycelial inoculum (AT632). To our knowledge, these data confirm the first successful cultivation of Japanese *R. roseolus* in the Southern Hemisphere.

DISCUSSION

The ITS region has been used extensively for exploring the diversity of ectomycorrhizal communities, delimiting fungal species, and identifying their phylogeographical structure. Such studies have revealed cryptic, uncultured, or previously unrecognized fungi not readily detected by other means. For example, phylogeography of the cosmopolitan ECM fungus *Pisolithus tinctorius*, using ITS DNA sequences, identified a complex of cryptic species that exhibited geographical patterns

TABLE 3. Differentiation of *R. roseolus* isolates from Japan and New Zealand by morphology

Characteristic	Description for *R. roseolus* isolates from:	
	Japan	New Zealand
Fruiting body diam (cm)	1–3	Up to 7
Fruiting body surface	White when young; becoming yellow to greenish-yellow, brownish, or reddish in age; staining reddish when bruised; cottony or fibrillose	White when young; becoming yellow to greenish-yellow, brownish, brown, and dark brown in age; staining reddish when bruised; cottony or fibrillose; sometimes scaly
Flesh texture	Firm, rubbery to sponge	Soft, spongy
Peridium	Thin, 80–120 μm, yellowish in the presence of 5% KOH	Thicker, 180–200 μm, brownish with red-brown patches in the presence of 5% KOH

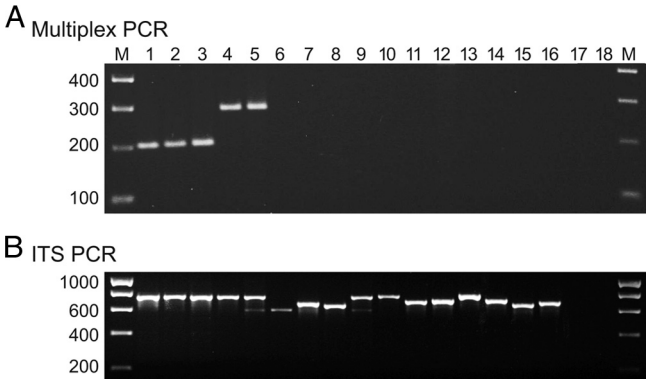

FIG. 3. Specificity of multiplex PCR primers for detection of *R. roseolus* from New Zealand and Japan. (A) Multiplex PCR using NZRhrF4, NZRhrR4, Fjap, and Rjap630. (B) Control PCR using primers ITS1 and ITS4. The agarose gels show amplification products generated by DNA extracted from the following ECM fungi: lanes 1 to 3, *R. roseolus* from New Zealand (NZRr5, NZRr13, and NZRr14, respectively); lanes 4 and 5, *R. roseolus* from Japan (AT630 and AT632, respectively); lanes 6 to 16, *Tuber borchii*, *T. magnatum*, *T. melanosporum*, *T. uncinatum*, *Lactarius deliciosus*, *Amanita muscaria*, *Suillus luteus*, *Boletus edulis*, *Hebeloma* species, *Thelephora terrestris*, and *Tuber maculatum*, respectively; lanes 17 and 18, no-template control. Lane M, size marker (bp).

in relation to the distribution of their host trees (23). In this study, *R. roseolus* isolates from Japan and New Zealand as well as collections of *Rhizopogon* subgenus *Roseoli* from other geographical origins were successfully differentiated by morphological identification and phylogenetic analyses of their ITS DNA sequences. The results supported the hypothesis that collections of *Rhizopogon* subgenus *Roseoli* from different locations have distinct genetic and biological characteristics brought about by global movement of exotic pines and by geographical separation.

The subtle morphological distinction between the fruiting bodies of *R. roseolus* isolates from Japan and New Zealand reported here is supported by previous work (47, 17). Organisms belonging to the *Rhizopogon* subgenus *Roseoli* also clustered into four ITS-defined groups associated with geographical origin using both parsimony and distance analyses. Japanese shoro were represented by a single genotype (*ITS*-JAP1), which clustered in clade A with several *Rhizopogon* isolates originating from Europe. A second group of collections from Europe clustered in a separate clade, clade D, indicating that at least two different lineages of *R. roseolus* exist in Europe. Johannesson and Martín (19) also found that intraspecific variation was evident in *R. roseolus* in Europe; three collections from Italy clustered together and separately from the two collections from Spain using cladistic analysis of ribosomal ITS2 sequences. Furthermore, European collections belonging to the *R. roseolus* species group were resolved into at least two clades using ITS sequence analyses (25). These results, in combination, suggest the possibility of differences in European *R. roseolus* due to geographical origin or global movement of exotic trees.

R. roseolus isolates collected from New Zealand clustered into two strongly supported clades, B and C, closely associated with North American collections. The close relationship between *R. roseolus* from New Zealand and *Rhizopogon* from North America is likely due to the introduction of *P. radiata* from North America, where it is native. Many of the first trees imported into New Zealand were seedlings or small saplings (16), which would have included entire root systems and thus almost certainly ECM fungi (46). Later, symbiotic fungi were deliberately introduced with *P. radiata* to promote forest productivity in plantations (36). The identification of clades B and C is consistent with the discovery of two clades that appear to have a North American affiliation by Martín and García (25).

ER48 (*ITS*-SPN4) was previously identified by morphology as *R. roseolus*. Molecular analysis, however, suggested that this collection was only distantly related to *R. roseolus*. Furthermore, ER48 did not cluster closely with *R. luteolus*, another species associated with *Pinus* belonging to *Rhizopogon* section *Rhizopogon*. ER48 therefore could belong to other subgenera within *Rhizopogon* that are more closely related to *R. roseolus*, such as *Rhizopogon* subgenus *Villosuli* or *Rhizopogon* subgenus *Amylopogon*. The taxonomic classification of this collection would require further comparison using collections from within these subgenera.

In our investigation, the paratypes for *R. pseudoroseolus* (*ITS*-USA1 and -2) and *R. vulgaris* JMT19154 (*ITS*-USA3), previously characterized by morphology and/or DNA sequencing, clustered within the same clade (clade B). *R. roseolus* JMT8227 (*ITS*-USA7), another collection classified morphologically and phylogenetically, and *R. vulgaris* KGP38 (*ITS*-USA6), an ECM previously identified using molecular techniques, also grouped together in clade C. Evidence from prior studies has shown that species names have been applied inconsistently to sporocarp collections and even paratype collections (21). The clustering of these collections together places in doubt their previous identification, which may have been due to the narrow geographical range of collections analyzed in previous studies or to taxonomic concepts that vary from one study to another (25).

Given the phylogeographic variation of the taxa inferred in our study, geographical isolation may have been important in the divergence of *R. roseolus* globally. However, the use of a single locus provides only limited phylogenetic information on the evolution of these fungi. Kjøller and Bruns (21) found that the ITS sequence was not sensitive enough to reveal genetic variation among geographical regions or within clades of *Rhizopogon* shared among different study sites. Perhaps this was due to their focus on differentiating *Rhizopogon* isolates solely from California, which likely had less genetic diversity than found within our collection. Certainly, in the same study, amplified fragment length polymorphism (AFLP) analysis indicated that populations from the sites could be separated into site-specific groups, suggesting that isolation could be a driving force in the diversification of *Rhizopogon* in forests in the United States.

Other methods have been used to study the interspecific and intraspecific diversity of ECM fungi and the geographical distribution of cryptic species. Cleaved amplified polymorphic sequence (CAPS) markers, designed for single-copy nuclear genes, identified reproductive isolation among cryptic species in the ectomycorrhizal genus *Strobilomyces* (39). Polymorphic microsatellite loci MA2 and MA5 also showed that southernmost and north westernmost populations of *Tuber magnatum*

were significantly differentiated from the rest of the populations in Italy due to geographical separation (38).

The ITS region usually correlates with the phylogenies of other genes (see, e.g., reference 8), although a more reliable phylogenetic framework would be provided by multigene data sets. Presently, there are no phylogenetic markers established for *Rhizopogon* except ITS, the 28S rRNA, and microsatellites. However, phylogenetically based taxonomy using multilocus data sets or using CAPS would resolve further the biogeography of *Rhizopogon* subgenus *Roseoli* inferred from our analysis of the ITS region. Furthermore, the comparison of additional collections of *Rhizopogon* from other areas likely to have a high diversity of Pinaceae and *Rhizopogon* is liable to reveal additional clades or to extend the distribution of the existing genotypes described in this study, leading to the identification of cryptic species.

Cultivation of edible mycorrhizal fungi relies heavily on artificial inoculation of seedlings with fungal mycelium or spores derived from carpophores. Yet, their quality and market price vary not only with species but also with the place of origin. As a result, the identification of edible fungi can be useful for tracing highly prized fungi according to their geographical origin for marketing purposes. The premium species *T. magnatum* Pico is harvested only in Italy and the Balkans (38). PCR primers were designed from the ITS region for the specific detection of *T. magnatum* during the mycelial, ectomycorrhizal, and fruiting phases of the truffle life cycle to verify the identity of mycelial cultures, evaluate the success of inoculations of seedlings, and facilitate characterization of fruiting bodies (2). In this study, multiplex PCR using the phylogeographically variable ITS region proved useful for differentiating marketable Japanese shoro cultivated in New Zealand from previously introduced and less economically valuable *R. roseolus*. The multiplex PCR permitted the unambiguous detection of both Japanese and New Zealand *R. roseolus* by performing a single reaction and comparing the lengths of the amplicons produced. The results showed the importance of this diagnostic tool for tracking the establishment of ECMs on seedlings of *P. radiata* inoculated with Japanese shoro, identifying nursery contaminants, and confirming the identity of fruiting bodies on cultivated trees. Using the multiplex PCR, this study, to our knowledge, demonstrated the first propagation of Japanese *R. roseolus* in the Southern Hemisphere.

ACKNOWLEDGMENTS

We are grateful to Akiyoshi Yamada for providing pure cultures of isolates AT630 and AT632; to Kazuhide Nara for providing DNA extracts and ITS sequences of isolates K253, K259, and K291; to Daniel Mousain for providing DNA extracts of ER48; and to Hisayasu Kobayashi for photographs of fruiting bodies of shoro grown in Japan. We also thank Slaven Kljucanin, Nina Hesom-Williams, and George Strong for technical assistance with inoculation and maintenance of seedlings and Ian Scott for helpful comments on the manuscript.

This work was funded by New Zealand's Foundation for Research, Science & Technology through contract C02X0704; by the New Zealand Institute for Plant & Food Research Limited; and by First Light Mushrooms.

REFERENCES

1. **Amicucci, A., C. Guidi, A. Zambonelli, L. Potenza, and V. Stocchi.** 2000. Multiplex PCR for the identification of white *Tuber* species. FEMS Microbiol. Lett. **189:**265–269.
2. **Amicucci, A., A. Zambonelli, G. Giomaro, L. Potenza, and V. Stocchi.** 1998. Identification of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. Mol. Ecol. **7:**273–277.
3. **Bakshi, B. K.** 1974. Mycorrhiza and its role in forestry. Forest Research Institute and Colleges, Dehera Dun, India.
4. **Ceruti, A., A. Fontana, and C. Nosenzo.** 2003. Le specie europee del genere Tuber. Una revisione storica. Regione Piemonte, Museo Regionale di Scienze Naturali, Turin, Italy.
5. **Chu-Chou, M., and L. J. Grace.** 1981. *Tuber* sp., a mycorrhizal fungus of Douglas fir in New Zealand N. Z. J. Bot. **21:**183–190.
6. **Chu-Chou, M., and L. J. Grace.** 1984. Cultural characteristics of *Rhizopogon* spp. associated with *Pinus radiata* seedlings. N. Z. J. Bot. **22:**35–41.
7. **Cunningham, G. H.** 1942. The Gasteromycetes of Australia and New Zealand. McIndoe Printer, Dunedin, New Zealand.
8. **Douhan, G. W., and D. M. Rizzo.** 2005. Phylogenetic divergence in a local population of the ectomycorrhizal fungus *Cenococcum geophilum*. New Phytol. **166:**263–271.
9. **Dunstan, W. A., B. Dell, and N. Malajczuk.** 1998. The diversity of ectomycorrhizal fungi associated with introduced *Pinus* spp. in the Southern Hemisphere, with particular reference to Western Australia. Mycorrhiza **8:**71–79.
10. **El Karkouri, K., F. Martin, and D. Mousain.** 2002. Dominance of the mycorrhizal fungus *Rhizopogon rubescens* in a plantation of *Pinus pinea* seedlings inoculated with *Suillus collinitus*. Ann. For. Sci. **59:**197–204.
11. **El Karkouri, K., F. Martin, J. P. Douzery, and D. Mousain.** 2005. Diversity of ectomycorrhizal fungi naturally established on containerised *Pinus* seedlings in nursery conditions. Microbiol. Res. **160:**47–52.
12. **Gardes, M., and T. D. Bruns.** 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol. Ecol. **2:**113–118.
13. **Grubisha, L. C., J. M. Trappe, R. Molina, and J. W. Spatafora.** 2002. Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences. Mycologia **94:**607–619.
14. **Guerin-Laguette, A., C. Plassard, and D. Mousain.** 2000. Effects of experimental conditions on mycorrhizal relationships between *Pinus sylvestris* and *Lactarius deliciosus* and unprecedented fruit-body formation of the saffron milk cap under controlled soilless conditions. Can. J. Microbiol. **46:**790–799.
15. **Hall, I., and Y. Wang.** 2002. Truffles and other edible mycorrhizal mushrooms—some new crops for the Southern Hemisphere. *In* Edible mycorrhizal mushrooms and their cultivation. Proceedings of the 2nd International Conference on Edible Mushrooms, Christchurch, New Zealand, 3 to 6 July 2001. (CD-ROM.)
16. **Herriot, E. M.** 1919. A history of Hagley Park, Christchurch, with special reference to its botany. Trans. Proc. R. Soc. N. Z. 1868–1961 **51:**XXXVIII.
17. **Imazeki, R., Y. Otani, and T. Hongo.** 1988. Fungi of Japan. Yama-Kei Publishers Co. Ltd., Tokyo, Japan. (In Japanese.)
18. **Iotti, M., A. Amicucci, G. Bonito, E. Bonuso, V. Stocchi, and A. Zambonelli.** 2007. Selection of a set of specific primers for the identification of *Tuber rufum*: a truffle species with high genetic variability. FEMS Microbiol. Lett. **277:**223–231.
19. **Johannesson, H., and M. P. Martin.** 1999. Cladistic analysis of European species of Rhizopogon (Basidiomycotina) based on morphological and molecular characters. Mycotaxon **71:**267–283.
20. **Kawai, M., M. Yamahara, and A. Ohta.** 2008. Bipolar incompatibility system of an ectomycorrhizal basidiomycete, *Rhizopogon rubescens*. Mycorrhiza **18:** 205–210.
21. **Kjøller, R., and T. D. Bruns.** 2003. *Rhizopogon* spore bank test within and among California pine forests. Mycologia **95:**603–613.
22. **Mabru, D., C. Dupré, J. P. Douet, P. Leroy, C. Ravel, J. M. Ricard, B. Médina, M. Castroviejo, and G. Chevalier.** 2001. Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products: fruiting bodies and mycorrhizal seedlings. Mycorrhiza **11:**89–94.
23. **Martin, F., J. Díez, B. Dell, and C. Delaruelle.** 2002. Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from nuclear ribosomal DNA ITS sequences. New Phytol. **153:**345–357.
24. **Martín, M. P.** 1996. The genus *Rhizopogon* in Europe. Spec. Soc. Catalana Micol. **5:**1–173.
25. **Martín, M. P., and M. A. García.** 2009. How many species in the *Rhizopogon roseolus* group? Mycotaxon **109:**111–128.
26. **Martín, M. P., and S. Raidl.** 2002. The taxonomic position of *Rhizopogon melanogastroides* (Boletales). Mycotaxon **84:**221–228.
27. **Marx, D. H.** 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology **59:** 153–163.
28. **Mello, A., S. Ghignone, A. Vizzini, C. Sechi, P. Ruiu, and P. Bonfante.** 2006. ITS primers for the identification of marketable boletes. J. Biotechnol. **121:** 318–329.
29. **Menkis, A., R. Vasiliauskas, A. F. S. Taylor, J. Stenlid, and R. Finlay.** 2005. Fungal communities in mycorrhizal roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by morphotyping, direct sequencing and mycelia isolation. Mycorrhiza **16:**33–41.

30. **Molina, R., J. M. Trappe, L. C. Grubisha, and J. W. Spatafora.** 1999. *Rhizopogon*, p. 129–161. *In* J. W. G. Cairney and S. M. Chambers (ed.), Ectomycorrhizal fungi: key genera in profile. Springer-Verlag, Heidelberg, Germany.
31. **Murata, H., K. Babasaki, T. Saegusa, K. Takemoto, A. Yamada, and A. Ohta.** 2008. Traceability of Asian *Matsutake*, specialty mushrooms produced by the ectomycorrhizal basidiomycete *Tricholoma matsutake*, on the basis of retroelement-based DNA markers. Appl. Environ. Microbiol. **74:**2023–2031.
32. **Nouhra, E. R., L. S. Dominguez, G. G. Daniele, S. Longo, J. M. Trappe, and A. W. Claridge.** 2008. Occurrence of ectomycorrhizal, hypogeous fungi in plantations of exotic tree species in central Argentina. Mycologia **100:**752–759.
33. **Nylund, J. E., and H. Wallander.** 1989. Effects of ectomycorrhiza on host growth and carbon balance in a semi-hydroponic cultivation system. New Phytol. **112:**389–398.
34. **Okumura, A.** 1989. Healthy mushroom dishes. Hoikusha, Osaka, Japan. (In Japanese.)
35. **Peay, K. G., T. D. Bruns, P. G. Kennedy, S. E. Bergemann, and M. Garbelotto.** 2007. A strong species-area relationship for eukaryotic soil microbes: island size matters for ectomycorrhizal fungi. Ecol. Lett. **10:**470–480.
36. **Richardson, D. M., N. Allsopp, C. M. D'Antonio, S. J. Milton, and M. Rejmánek.** 2000. Plant invasions—the role of mutualisms. Biol. Rev. **75:**65–93.
37. **Rubini, A., F. Paolocci, B. Granetti, and S. Arcioni.** 1998. Single step molecular characterization of morphologically similar black truffle species. FEMS Microbiol. Lett. **164:**7–12.
38. **Rubini, A., F. Paolocci, C. Riccioni, G. G. Vendramin, and S. Arcioni.** 2005. Genetic and phylogeographic structures of the symbiotic fungus *Tuber magnatum*. Appl. Environ. Microbiol. **71:**6584–6589.
39. **Sato, H., and N. Murakami.** 2008. Reproductive isolation among cryptic species in the ectomycorrhizal genus *Strobilomyces*: population-level CAPS marker-based genetic analysis. Mol. Phylogenet. Evol. **48:**326–334.
40. **Smith, A. H., and S. M. Zeller.** 1966. A preliminary account of the North American species of *Rhizopogon*. Memoirs N. Y. Bot. Garden **14:**1–178.
41. **Swofford, D. L.** 2002. PAUP*, phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, MA.
42. **Taylor, D. L., and T. D. Bruns.** 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. **8:**1837–1850.
43. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTALX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:**4876–4882.
44. **Trappe, J. M.** 1962. Fungus associates of ectotrophic mycorrhizae. Bot. Rev. **28:**538–606.
45. **Trappe, J. M.** 1975. A revision of the genus Alpova with notes on Rhizopogon and the Melanogastraceae. Beih Nova Hedwigia **51:**279–309.
46. **Vellinga, E. C., B. E. Wolfe, and A. Pringle.** 2009. Global patterns of ectomycorrhizal introductions. New Phytol. **181:**960–973.
47. **Wang, Y., I. R. Hall, C. Dixon, M. Hance-Halloy, G. Strong, and P. Brass.** 2002. The cultivation of *Lactarius deliciosus* (saffron milk cap) and *Rhizopogon rubescens* (shoro) in New Zealand. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3 to 6 July 2001. (CD-ROM.)
48. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York, NY.
49. **Yamada, A., T. Ogura, and M. Ohmasa.** 2001. Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by *in vitro* mycorrhizal synthesis. I. Primordium and basidiocarp formation in open-pot culture. Mycorrhiza **11:**59–66.
50. **Yun, W., and I. R. Hall.** 2004. Edible ectomycorrhizal mushrooms: challenges and achievements. Can. J. Bot. **82:**1063–1073.