Phylogenetic diversity of non-nodulating *Rhizobium* associated with pine ectomycorrhizae

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**Abstract**

Most *Rhizobium* species described are symbionts that form nodules on legume roots; however, non-nodulating strains of *Rhizobium* are also widespread in nature. Unfortunately, knowledge of non-nodulating *Rhizobium* is quite limited compared with nodulating *Rhizobium*. Here, we studied the phylogenetic diversity of *Rhizobium* species that inhabit Japanese red pine roots (*Pinus densiflora*). Because fine roots of pine trees are usually colonized by ectomycorrhizal fungi in nature, we mainly used ectomycorrhizal root tips for bacterial isolation. Out of 1195 bacteria isolated from 75 independent root samples from the field and greenhouse experiments, 102 isolates were confirmed to be *Rhizobium* following partial 16S rRNA gene analysis. *Rhizobium* species were occasionally dominant in culturable bacterial communities, whereas no *Rhizobium* species were isolated from the soil itself. Molecular phylogenetic analyses using 16S rRNA, *atpD*, and *recA* gene sequences revealed that isolated *Rhizobium* strains were phylogenetically diverse and that several were distantly related to known *Rhizobium* species. Considering that a single species of pine is associated with unique and phylogenetically diverse *Rhizobium* populations, we should pay more attention to non-nodulating strains to better understand the diversity, ecology, and evolution of the genus *Rhizobium* and plant–*Rhizobium* associations.

**Introduction**

Plant–microorganism interactions appear to be the most important symbiotic relationships in terrestrial ecosystems. In fact, the roots of most land plants are colonized by mycorrhizal fungi, which supply host plants with soil-derived nutrients in exchange for photosynthetic products (Smith & Read, 2008). Indeed, up to nearly 90% of the phosphorus and nitrogen in plant tissues are derived from mycorrhizal fungi (Hobbie & Hobbie, 2006; van der Heijden et al., 2006). The origins of this functionally important plant–microorganism interaction date back to the first land plants (c. 450 Ma) as indicated by the fossil record and molecular clock evidence (Remy et al., 1994; Heckman et al., 2001).

The fine roots of plants are associated not only with mycorrhizal fungi but also with numerous bacteria, including *Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Firmicutes*, and *Actinomycetes* (Beattie, 2007; Hawkes et al., 2007; Offre et al., 2007, 2008 and references therein). These bacteria are found in the rhizosphere, on the surface of roots (rhizoplane), inside root tissues (endorhizosphere), and in association with mycorrhizal fungal hyphae, although exact colonization sites have not been examined microscopically in most microbiological ecological studies. In fact, those habitats affected by roots form a continuum that is difficult to distinguish in practice, and therefore they often harbor many of the same bacteria (Nehl & Knox, 2006; Timonen & Marschner, 2006). Thus, bacteria found in these habitats are inclusively described as rhizobacteria in many studies.

Although the functions of most rhizobacteria remain unknown, some promote plant growth and are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR increase plant growth through various direct mechanisms, for example, producing phytohormones (Zimmer & Bothe, 1988), solubilizing phosphorus (Piccini & Azcon, 1987), and iron (Vansuyt et al., 2007). Some PGPR enhance plant growth indirectly by promoting mycorrhizal functions and mycorrhizal infection (Linderman, 1992; Garbaye, 1994; Marschner & Timonen, 2006; Frey-Klett et al., 2007; Pivot
et al., 2009), and are specifically called mycorrhiza helper bacteria. The most notable and extensively studied example of bacteria beneficial to plants are the rhizobia, which form nodules on legume roots, fix gaseous nitrogen in the air, and provide the fixed nitrogen to plants (Sprent & Sprent, 1990). Thus, nodule formation often increases host plant growth, especially in nitrogen-deficient soils (Sprent & Sprent, 1990). In addition, nodule-fixed nitrogen inputs a considerable amount of nitrogen to ecosystems, thereby facilitating vegetation development in early successional areas (Walker & del Moral, 2003). Global nitrogen fixation by cultivated legumes is estimated to be as much as 40 × 10^6 ton year−1 and to provide about 20% of the available nitrogen in agricultural soil (Crews & Peoples, 2004). Therefore, rhizobia are of great importance in agricultural, ecological, and global nitrogen cycles.

The word ‘rhizobia’ is a conventional and generic term for bacteria that form N-fixing root nodules. Therefore, rhizobia are taxonomically paraphyletic and include many different genera such as Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, and several genera in Alpha- and Betaproteobacteria. These genera, which are potentially rhizobial, include many strains that do not form nodules. These strains are often called non-nodulating strains (e.g. Winarno & Lie, 1979). The exact meaning of non-nodulating strains varies slightly in the literature. In this study, we use this term for strains that live and proliferate without forming nodules but phylogenetically pertain to rhizobial genera, to contrast with the strains that form and inhabit the nodules. Non-nodulating strains are not rare and are rather widespread in nature. For example, non-nodulating strains coexist with nodulating strains in nodules (Denison & Kiers, 2004) and even predominate in the rhizosphere of leguminous plants (Segovia et al., 1991; Pongsilp et al., 2002). Non-nodulating strains have also been found in various nonleguminous plants, including rice (Singh et al., 2006), maize (Gutiérrez-Zamora & Martínez-Romero, 2001), carrot (Surette et al., 2003), and poplars (Doty et al., 2005; Ulrich et al., 2008). These non-nodulating strains can directly interact with the nodulating strains through competition (Sachs & Simms, 2008). Furthermore, genes that encode nodule formation and nitrogen-fixation processes are clustered on plasmids or on symbiosis islands, which are easily transferred horizontally between different species (Sullivan et al., 1995; Moulin et al., 2004), possibly allowing nodulating and non-nodulating strains to be interchangeable. Although little is known about the abundance and diversity of non-nodulating strains belonging to potentially rhizobial genera in nature, they may have significant positive or negative effects on agricultural applications and ecosystem functions.

Among rhizobial genera, Rhizobium is the largest genus, with > 25 species formally described (Euzéby, 2008). These include nodulating species associated with many important leguminous plants such as various beans, peas, and grass pastures. Plant pathogenic bacteria that were once included in the genus Agrobacterium are also included in Rhizobium due to the monophyletic clade formed when constructed with nodulating Rhizobium species (Young et al., 2001; Sawada et al., 2003). Although this revision in the nomenclature continues to be controversial (Farrand et al., 2003), we adopted the definition of Rhizobium by Young et al. (2001), as used in many recent studies. Bacteria belonging to Rhizobium/Agrobacterium (hereafter Rhizobium unless otherwise specified) have also been isolated from various nonleguminous plant taxa such as rice (Singh et al., 2006), cotton and sweet corn (McInroy & Klopper, 1995), potato (Sturz et al., 1998), and wheat (Sharma et al., 2005). Among these plant taxa known to be associated with non-nodulating Rhizobium, Pinaceae is the most ancient group (Burke et al., 2008; Kataoka et al., 2008). Although we have very limited information about the Rhizobium species associated with pine roots, this association is potentially important for understanding the origins of plant–Rhizobium interactions including both nodulating and non-nodulating colonization.

In the present study, we focus on non-nodulating Rhizobium species associated with Japanese red pine (Pinus densiflora). Given that most fine roots of pine trees are usually colonized by ectomycorrhizal fungi in nature, we examined non-nodulating Rhizobium species associated with ectomycorrhizal root tips, which were collected from field-grown trees and seedlings cultivated in a greenhouse. The frequency and dominance of non-nodulating Rhizobium bacteria were analyzed in relation to field locations, coexisting ectomycorrhizal fungal species, age of pine trees, and soil types. Molecular phylogenetic analyses were also conducted using three different loci (16S rRNA gene, atpD, and recA coding regions) to elucidate the diversity of the non-nodulating Rhizobium species associated with ectomycorrhizal pine roots and their phylogenetic relationships with known Rhizobium species. To our knowledge, this study is the first to focus on the diversity and phylogeny of non-nodulating Rhizobium species colonizing gymnosperm roots.

Materials and methods

Plant materials

In August 2007, we collected ectomycorrhizal root tips from three different sites. Site 1 is located in scoria desert (c. 1400 m above sea level, c. 2 ha in area, 35°20′09″N 138°47′42″E, at the southeastern end) on Mount Fuji, Japan, where vegetation is patchily distributed and primary succession occurs. At this site, pine trees (P. densiflora) were very
rare and only found in association with pioneer shrubs
(Nara, 2006b). From each of the five saplings, we sampled
one small root system that consisted of an c. 10-cm-long
main root and < 100 ectomycorrhizal root tips that were
traced from the trunk. Sites 2 (c. 900 m above sea level,
c. 1 ha in area, 35°28′29″N 138°45′60″E, at the southeastern
end) and 3 (c. 1000 m above sea level, c. 1.5 ha, 35°27′18″N
138°45′45″E) were closed forests dominated by P. densiflora
established on an old lava flow. At both of these sites, small
pine root systems were randomly sampled at points > 10 m
apart. Although naturally established saplings of P. densiflora
were almost absent in closed forests because of its poor
shade tolerance, there were some saplings in open habitats.
Thus, we also sampled the root systems of five saplings
(> 10 m apart from each other) established along the forest
edge at site 3. In total, 5, 10, and 15 independent root
systems were sampled from sites 1, 2, and 3, respectively.
There were no leguminous plants near the sampling points.
From each root sample we assessed and selected seven
ectomycorrhizal tips that belonged to the most dominant
ectomycorrhizal morphotype using a dissecting microscope
(S8 APO, Leica Microsystems, Wetzlar, Germany). Of the
seven ectomycorrhizal tips, five were grouped together and
used as an independent replicate unit for bacterial isolation.
The remaining two tips were used to identify the ectomycor-
rhizal fungal species by sequencing the internal transcribed
spacer regions of rRNA; see Nara (2006b). From each of the five saplings,
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spacer regions of rRNA; see Nara et al. (2003) for details of
the DNA extraction, PCR amplification, cloning, sequen-
cing, and homology analysis.

It is impossible to control for ectomycorrhizal fungal
species in field samples because > 100 ectomycorrhizal
fungi usually coexist in a small forest (Ishida et al., 2007).
The various soil conditions encountered in the field also
make data evaluation difficult. To understand the effects
of ectomycorrhizal fungal species on ectomycorrhiza-asso-
ciated bacterial communities, we also used ectomycorrhizal
roots of pine seedlings that were experimentally inoculated
with known ectomycorrhizal fungal species under con-
trolled conditions. The inoculation method is the same as
that used in Nara (2006a). Briefly, each fungal culture grown
on modified Melin–Norkrans agar was placed directly on
2-month-old nonmycorrhizal pine seedlings raised in a
rectangular polystyrene rhizobox containing autoclaved soil.
In this inoculation experiment, we used three different soil
media: mixture 1 (nursery soil: sieve-selected loam = 1:1),
mixture 2 (sieve-selected loam: sand: peat = 4:1:1), and
pure scoria collected from site 1 to determine the possible
effect of soil types on ectomycorrhiza-associated bacterial
communities. Three ectomycorrhizal fungal species, Cen-
coccum geophilum, Pisolithus sp. (Pisolithus sp. sensu,
Martin et al., 2002), and Suillus granulatus, were used
separately in the inoculations. Uninoculated controls
were also prepared for the three soil treatments. For each fungus/
soil treatment, three replicates were performed. All inocu-
lated seedlings were kept in a temperature-controlled green-
house (25°C/23°C day/night) for 8 months. Because the
greenhouse is not aseptic, many bacteria can naturally
colonize seedlings from the air. However, for the ease of
bacterial colonization from the original soils, 20 mL of soil
suspension (0.1 g scoria mL⁻¹ suspension for treatments
with the scoria; 0.1 g nursery soil mL⁻¹ suspension for treat-
ments with the other two soil mixtures) was added to each
rhizobox 3 months after ectomycorrhizal inoculation. To
avoid ectomycorrhizal fungal contamination by spores and
mycelia in soil suspensions, each suspension was filtered
through 5-μm membrane filters that can pass most bacteria
before use. Ten ectomycorrhizal root tips were sampled from
each rhizobox, grouped together, and used as an indepen-
dent replicate unit for bacterial isolation.

To further examine the impact of host plant age, we raised
c. 8-year-old ectomycorrhizal saplings by transplanting three
preinoculated ectomycorrhizal seedlings (current year) with
c. 100 surface-sterilized pine seeds in garden planters
(18 cm × 37 cm × 15 cm) filled with autoclaved soil mixture
1, and cultivated them for c. 8 years in the greenhouse.
Mycelia spreading from preinoculated seedlings usually
infect the fine roots of all surrounding germinants within a
few months as described by Nara (2006a). In this long-term
experiment, we used nine different strains of ectomycor-
rhizal fungi found in pine forests in Japan, i.e., C. geophilum,
two strains of Pisolithus sp., Rhizopogon rubescens, two
strains of Suillus bovinus, S. granulatus, Suillus luteus, and
an unknown ectomycorrhizal basidiomycete T1 (Wu et al.,
1999). Most uninoculated control and R. rubescens-treated
seedlings died of poor growth during the 8 years (data of
seedling growth will be available in another study). A
sufficient amount of control plants survived for subsequent
isolation of bacteria, but the mortality of those treated with
R. rubescens was too high; thus, they were excluded from the
study. Although unknown ectomycorrhizal fungal contami-
nants were found at the bottom of the planters in some
treatments during this long-term experiment, their relative
abundance was so low as to be negligible. Using a dissecting
microscope, 30 ectomycorrhizal roots of inoculated ectomy-
corrhizal fungal species were selected from a given planter.
These ectomycorrhizal root tips were grouped together for
each fungal species (30 ectomycorrhizae each) and used for
bacterial isolation.

**Bacterial isolation and *Rhizobium* screening**

Soil particles and adhering debris were carefully removed
from ectomycorrhizal root tips (or nonmycorrhizal root
tips) under the dissecting microscope. The ectomycorrhizal
root tips were further cleaned by washing with 1 mL of
sterilized water in a 2-mL microtube, which was stirred on a
vortex mixer for several seconds. This cleaning step was repeated five times for each sample. Each cleaned ectomycorrhizal sample was then homogenized with a micropestle in a sterilized 2-mL microtube and suspended with 1 mL (2 mL for the long-term experiment) of sterilized water in the same microtube. A 1/10–1/10 000 dilution series of the original homogenized suspension was prepared. One hundred microliters of each suspension was spread on YG agar medium [yeast extract 1 g, glucose 1 g, K₂HPO₄ 0.3 g, KH₂PO₄ 0.2 g, MgSO₄ · 7H₂O 0.2 g, agar 15 g in 1 L distilled water (DW), pH 7.0]. In the long-term experiment, nitrogen-free (NF) agar medium (glucose 10 g, K₂HPO₄ 0.25 g, MgSO₄ · 7H₂O 0.125 g, CaCO₃ 0.1 g, NaCl 0.125 g, FeSO₄ · 7H₂O 5 mg, MnSO₄ · 4H₂O 5 mg, Na₂MoO₄ · 2H₂O 5 mg, agar 15 g in 1 L DW, pH 7.2) was also used for plating. Three different series of YG and NF plates were used for each sample in the long-term experiment. Because saprophytic fungal contamination was frequently observed in the short-term treatments, the initial suspension was filtered through 5-μm membranes to remove fungal contamination on the isolation plates. All plates were kept in the dark at 25 °C for 1–2 weeks.

To determine the frequency of occurrence of Rhizobium in the soil itself, bacteria were isolated from soil samples that were collected from uninoculated controls in the short-term experiment. Soil-inhabiting bacteria in inoculated treatments were not examined because it is impossible to exclude all extramatrical mycelia of ectomycorrhizal fungi and bacteria colonizing these mycelia. We also collected nine scoria samples from vegetation islands and three scoria samples from bare ground in the scoria desert. Serial dilutions were made from the soil suspension (0.1 g soil mL⁻¹) of each soil sample, and 100 μL of each dilution was spread on a YG plate.

For the screening of Rhizobium species, we randomly selected 10 colonies/plate using the most appropriate dilution level for each sample. Selected bacterial colonies were subjected to colony PCR to amplify 16S rRNA gene regions. We used a Qiagen® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany) and the primer pair 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1400R (5'-GGGTTGCGCTGTTG-3') for colony PCR following the manufacturer’s instructions. PCR products were treated and sequenced as described above.

**atpD and recA sequences**

Most of the unique Rhizobium isolates from the 16S rRNA gene screening were subcultured in YG medium. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen GmbH). We amplified the two protein-coding regions, atpD and recA, using AmpliTaq Gold (Applied Biosystems, CA) and primer sets from Gaunt et al. (2001) following the manufacturer’s instructions. PCR products were treated and sequenced as described above.

**Phylogenetic analysis**

All sequence data for the Rhizobium strains obtained in this study were submitted to the DNA Database of Japan (accession nos AB456584–AB456647, Table 1). Related sequences of all the described Rhizobium/Agrobacterium species (type strains were used whenever possible) and some undescribed species in GenBank were also included in the phylogenetic analyses. Sequences of other genera of Rhizobiales (e.g. Ensifer and Sinorhizobium) were not included, because they were distant from sequences of the obtained Rhizobium strains and belonged to apparently different phylogenetic clades. Sequences were aligned with CLUSTAL_W ver. 1.8 (Thompson et al., 1994) and manually edited at misaligned sites. Each of the atpD and recA data sets was too small to accurately calculate the suitable DNA substitution models and related parameters; therefore, both data sets were combined in the phylogenetic analyses (Vinuesa et al., 2005). Both the hierarchical likelihood ratio test and the Akaike information criterion implemented in MMRMODELTEST 2.3 (Nylander, 2004) indicated that the general time reversible model (GTR) combined with rate heterogeneity estimated from a proportion of invariant sites (I) and gamma
distribution (Γ) was the best-fit nucleotide substitution model for each of the 16S rRNA gene and atpD+recA sequence data sets. Thus, the GTR+Γ model was used for Bayesian phylogenetic analyses with MrBayes 3.1.2 (http://mrbayes.csit.fsu.edu/index.php). Average SDs of split frequency values were far < 0.01 after 5 M Metropolis-coupled Markov chain Monte-Carlo generations in both analyses, indicating convergence. Thus, we sampled every 100 generations from 1.25 to 5 M generations to construct a 50% majority rule consensus trees. The Bayesian trees were visualized using MEGA4 (Kumar et al., 2004).

Statistical analysis

To determine the effects of ectomycorrhizal fungal species and soil types on the occurrence of Rhizobium, the relative abundance of Rhizobium (Rhizobium colonies/total bacterial colonies from each independent replicate unit) was compared among ectomycorrhizal fungal species and soils in the short-term experiment using Kruskal–Wallis testing. We also examined the effect of pine age on Rhizobium occurrence using the Mann–Whitney U-test by comparing the frequency of Rhizobium inhabiting samples between saplings (estimated at 4–10 years old) and mature trees (probably > 50 years old) collected from the same pine forest. All statistical analyses were conducted using SPSS ver. 11.0.1J (SPSS Japan Inc., Tokyo, Japan).

Results

Frequency and dominance of Rhizobium bacteria in association with pine ectomycorrhizae

In total, 102 of 1195 bacterial colonies isolated from pine roots were confirmed to be Rhizobium species by partial 16S rRNA gene screening (88 of 1045 colonies from ectomycorrhizal roots and 14 of 150 colonies from nonmycorrhizal roots were Rhizobium). Many other bacteria belonging to Burkholderia, Bradyrhizobium, and Variovorax were also detected from pine ectomycorrhizal roots. In contrast to these ectomycorrhiza-inhabiting bacteria, Paenibacillus, Arthrobacter, and Bacillus were dominant in colonies isolated from the soil in the short-term experiment (control treatments) and from scoria samples from site 1. Furthermore, Rhizobium species were not detected in the 210 selected bacterial colonies (90 from short-term experiments and 120 from the field) isolated from the soil and scoria. Non-Rhizobium bacteria were not the focus of the present study and were thus excluded from further analyses.

Rhizobium was found in six of 30 independent ectomycorrhizal root samples collected from the field (Table 2). Most of these Rhizobium-associated samples were collected from young saplings (several years old) established in open environments (Fig. 1): tree no. 5 from the primary successional volcanic desert and tree nos 27–30 at the edge of the pine forest. Among the samples collected from the same pine forest, the occurrence of Rhizobium differed significantly between saplings and mature trees (Mann–Whitney U-test, P = 0.00023, Fig. 1). In each of the Rhizobium-associated ectomycorrhizal root samples, Rhizobium was

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Ectomycorrhizal fungi</th>
<th>Rhizobium type (strain no.)</th>
</tr>
</thead>
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<td>Site 1</td>
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<tr>
<td>5</td>
<td>Tylospora sp2.</td>
<td>0.20 Type 17-1 (4918)</td>
</tr>
<tr>
<td>16</td>
<td>Toremellata sp1.</td>
<td>0.43 Type 18 (15935)</td>
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<tr>
<td>27</td>
<td>Tuber sp.</td>
<td>0.40 Type 17-2 (26718)</td>
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<td>Site 3</td>
<td></td>
<td></td>
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<tr>
<td>28</td>
<td>Tylospora sp4.</td>
<td>1.00 Type 17-3 (27118)</td>
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<tr>
<td>29</td>
<td>Suillus sp.</td>
<td>0.20 Type 19 (28949)</td>
</tr>
<tr>
<td>30*</td>
<td>Laccaria sp2.</td>
<td>0.50 Type 17-4, 20</td>
</tr>
<tr>
<td></td>
<td>Tylospora sp5.</td>
<td>(29118, 29953)</td>
</tr>
</tbody>
</table>

*See Table S1 for the identification of ectomycorrhizal fungi.
1Relative abundance of Rhizobium colonies to the total bacterial colonies.
2Two ectomycorrhizal fungi were detected from the same sample.

| Type 5 | N19 | AB456592 | AB456593 | AB456594 |
| Type 6 | YG8 | AB456595 | AB456596 | AB456597 |
| Type 7 | YG2 | AB456598 | AB456599 | AB456600 |
| Type 8 | N17 | AB456601 | ND       | AB456602 |
| Type 9 | N10 | AB456603 | AB456604 | AB456605 |
| Type 10| N13 | AB456606 | AB456607 | AB456608 |
| Type 11| N14 | AB456609 | AB456610 | AB456611 |
| Type 12| A15 | AB456612 | AB456613 | AB456614 |
| Type 13| S11 | AB456615 | AB456616 | AB456617 |
| Type 14| N13 | (NBRC 104349) | AB456618 | AB456619 | AB456620 |
| Type 15| A3  | (NBRC 104348) | AB456621 | AB456622 | AB456623 |
| Type 16| S9  | (NBRC 104350) | AB456624 | AB456625 | AB456626 |
| Type 17| 4918| (NBRC 104344) | AB456627 | AB456628 | AB456629 |
| Type 18| 26718| AB456630 | AB456631 | AB456632 |
| Type 19| 27718| AB456633 | AB456634 | AB456635 |
| Type 20| 29118| AB456636 | AB456637 | AB456638 |
| Type 21| 29953| (NBRC 104347) | AB456645 | AB456646 | AB456647 |

Table 1. Rhizobium strains isolated from pine ectomycorrhizae and their sequences in three different DNA regions

<table>
<thead>
<tr>
<th>Rhizobium type</th>
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<th>16S rRNA gene</th>
<th>atpD</th>
<th>recA</th>
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<td>AB456637</td>
<td>AB456638</td>
</tr>
<tr>
<td>Type 18</td>
<td>15935 (NBRC 104345)</td>
<td>AB456639</td>
<td>AB456640</td>
<td>AB456641</td>
</tr>
<tr>
<td>Type 19</td>
<td>28949 (NBRC 104346)</td>
<td>AB456642</td>
<td>AB456643</td>
<td>AB456644</td>
</tr>
<tr>
<td>Type 20</td>
<td>29953 (NBRC 104347)</td>
<td>AB456645</td>
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<td>AB456647</td>
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</tbody>
</table>
The effect of ectomycorrhizal fungal species on *Rhizobium* occurrence and dominance was not clear in the field samples because most samples were colonized by different ectomycorrhizal fungal species. Ectomycorrhizal fungi associated with *Rhizobium* included *Laccaria* sp.2, *Suillus* sp., *Tomentella* sp1., and *Tuber* sp. (Table 2). *Rhizobium* was not isolated from the root tips of the following ectomycorrhizal fungi: *Amphinema* sp., *C. geophilum*, *Clavulina* sp., *Hymenoscyphus* sp., *Piloderma* sp., *Russula* sp., *Thelephora* sp., *Tomentellopsis* sp., *Tricholoma* sp., and *Xerocomus* sp. (see Supporting Information, Table S1).

In the short-term experiment, *Rhizobium* occurrence (frequency) was generally rare (Fig. 1). In fact, *Rhizobium* was not isolated from *S. granulatus* or nonmycorrhizal control treatments from any soil type. *Rhizobium* was found only in two samples from *Pisolithus* sp. treatments and six samples from *C. geophilum* treatments (Table 3). However, *Rhizobium* species were dominant in some of these samples, for example, all bacterial colonies belonged to *Rhizobium* in a sample from *Pisolithus* sp. – scoria treatment. Although the soil type did not affect the relative abundance of *Rhizobium* ($P = 0.583$), the effect of ectomycorrhizal fungal species was found to be significant ($P < 0.001$).

In the long-term experiment, *Rhizobium* species were found in all treatments (Fig. 1) including eight different ectomycorrhizal fungal treatments and one nonmycorrhizal treatment, in at least one of the two isolation media (Table 3). The relative abundance of *Rhizobium* species in isolated colonies ranged from 8% to 47% (mean $\pm$ SD = 19 ± 16%).

**Molecular phylogenetic analyses of *Rhizobium* strains isolated from pine ectomycorrhizae**

Partial sequences of the 16S rRNA gene were obtained from 22 *Rhizobium* strains isolated from ectomycorrhizal pine roots. The aligned sequence matrix for the 16S rRNA gene data of these *Rhizobium* strains, including all formerly described *Rhizobium* species, contained 1246 base sites, of which 277 were phylogenetically informative.

Eleven RFLP types of *Rhizobium* were found from the long-term experiment. Most of these *Rhizobium* strains were included in phylogroup 1-1 by molecular phylogenetic analysis based on 16S rRNA gene sequences regardless of the ectomycorrhizal fungal species (Fig. 2). Partial 16S rRNA gene sequences were obtained from another five isolates that were excluded from the final phylogenetic tree (Fig. 2). When these partial sequences were included in a neighboring phylogenetic analysis, three of the isolates were incorporated into phylogroup 1-1 while the others were more similar to phylogroups 1-2 and 2. The most frequently occurring group in the long-term experiment, phylogroup 1-1, was phylogenetically close to *Rhizobium tropici* and *Rhizobium multihospitium* (Fig. 2 and Table S3).

In the short-term experiment, five RFLP types of *Rhizobium* were found. Type 12, isolated from a *Pisolithus* sp. treatment, was included in phylogroup 1-3 and was phylogenetically close to *Rhizobium rhizogenes* (Fig. 2). Another *Rhizobium* strain, Type 13 from the same treatment, was included in phylogroup 1-2, which was phylogenetically close to *Rhizobium milunense* and phylogroup 1-1 (Fig. 2). Strains from *C. geophilum* treatments in the short-term experiment were phylogenetically different from those in *Pisolithus* sp. treatments, composed of Type 14 (phylogroup 4), Type 15 (phylogroup 3), and Type 16 (phylogroup 4). These *Rhizobium* strains were distinctly different from known *Rhizobium* species with 100% posterior probability in branching support index (Fig. 2, see also Table S3).

*Rhizobium* strains isolated from the field sites were divided into four RFLP types and were eventually grouped into three major phylogenetic groups, i.e., phylogroups 1, 2, and 5 (Fig. 2). The most dominant type was Type 17, which accounted for 57% of all *Rhizobium* isolates from the field. All Type 17 strains belonged to phylogroup 1; however, intratype sequence variations (several nucleotides out of the partially sequenced 16S rRNA gene) among the different samples divided them into two subphylogroups (1-3 and 1-4). Phylogroup 2 was represented by the Type 19 strain.
related to the unknown *Rhizobium* species (DQ100062) isolated from *Albizia julibrissin* in China (Wang et al., 2006). Phylogroup 5, represented by Type 18 and Type 20 strains, was distantly related to the other phylogroups and contained undescribed species that were phylogenetically close to this particular phylogroup (Fig. 2 and Table S3).

Phylogenetic analysis based on 16S rRNA gene sequences identified five phylogroups (and four subphylogroups) among 22 *Rhizobium* strains isolated from ectomycorrhizal pine roots (Fig. 2). Representative strains of all major phylogroups were deposited in the National Institute of Technology and Evaluation, Biological Resource Center (NITE-NBRC) under strain numbers NBRC 104344–104350 (Table 1). These strains are also available from the authors upon request.

We also analyzed phylogenetic relationships among *Rhizobium* isolates and known *Rhizobium* species using *atpD* and *recA* gene sequences (Fig. 3). Because sequence data for these regions were unavailable for many described species of *Rhizobium*, fewer taxa were included in this analysis than in the 16S rRNA gene analysis. The aligned matrix for the combined sequences of the *atpD* and *recA* regions contained 750 sites, of which 297 were phylogenetically informative. Phylogroups identified in the 16S rRNA gene analysis were generally supported by the Bayesian tree based on *atpD* and *recA*. However, closely related phylogroups identified in the partial 16S rRNA gene analysis were not clearly differentiated by the *atpD* and *recA* analysis. For example, the differentiations between subphylogroups in phylogroup 1 and between phylogroups 3 and 4 were not clear (Fig. 3).

Table 3. *Rhizobium* occurrence in ectomycorrhizae of pine seedlings (8-month-old) and pine saplings (c. 8-year-old) inoculated with known ectomycorrhizal fungi under greenhouse conditions

<table>
<thead>
<tr>
<th>Age of pine</th>
<th>Inoculated ectomycorrhizal fungi</th>
<th>Soil*</th>
<th>Bacteria isolation medium†</th>
<th>Rr,</th>
<th>Rhizobium occurrence§</th>
<th>Isolated Rhizobium type (strain no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c. 8 years</td>
<td><em>Suillus bovinus</em> 1</td>
<td>Mixture 1</td>
<td>YG</td>
<td>0.13</td>
<td>2/3</td>
<td>Type 1 (YG8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture 1</td>
<td>NF</td>
<td>0.35</td>
<td>3/3</td>
<td>Type 2-1, 3-1, 4, 5 (NF1, 2, 13, 19)</td>
</tr>
<tr>
<td></td>
<td><em>S. bovinus</em> 2</td>
<td>Mixture 1</td>
<td>YG</td>
<td>0.53</td>
<td>3/3</td>
<td>Type 6-1 (YG8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture 1</td>
<td>NF</td>
<td>0.10</td>
<td>2/3</td>
<td>Type 7 (NF17)</td>
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<tr>
<td></td>
<td><em>S. granulatus</em></td>
<td>Mixture 1</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture 1</td>
<td>NF</td>
<td>0.08</td>
<td>2/3</td>
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<td><em>S. luteus</em></td>
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<td>YG</td>
<td>0.13</td>
<td>3/3</td>
<td>Type 6-2 (YG3)</td>
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<tr>
<td></td>
<td></td>
<td>Mixture 1</td>
<td>NF</td>
<td>0.34</td>
<td>3/3</td>
<td>Type 8, 9 (NF10, 13)</td>
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<td></td>
<td><em>Pisolithus sp1.</em></td>
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<td>Type 10 (YG8)</td>
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<tr>
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<td></td>
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<td>NF</td>
<td>0.20</td>
<td>2/3</td>
<td>Type 11 (NF14)</td>
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<tr>
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<td><em>Pisolithus sp2.</em></td>
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<td>YG</td>
<td>0.20</td>
<td>2/3</td>
<td>Type 6-3 (YG6)</td>
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<td></td>
<td></td>
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<td>NF</td>
<td>0</td>
<td>0/3</td>
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</tr>
<tr>
<td></td>
<td><em>Cenococcum geophilum</em></td>
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<td>YG</td>
<td>0.20</td>
<td>2/3</td>
<td>Type 6-4 (YG3)</td>
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<td></td>
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<td>NF</td>
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<td>0/3</td>
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<td><strong>T01</strong></td>
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<td>YG</td>
<td>0.20</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td>NF</td>
<td>0.47</td>
<td>3/3</td>
<td>Type 2-3, 3-3 (NF22, 12)</td>
</tr>
<tr>
<td>8 months</td>
<td><em>S. granulatus</em> 2</td>
<td>Mixture 1</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture 2</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scoria</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pisolithus sp2.</em></td>
<td>Mixture 1</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture 2</td>
<td>YG</td>
<td>0.13</td>
<td>1/3</td>
<td>Type 12 (A15)</td>
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<td></td>
<td></td>
<td>Scoria</td>
<td>YG</td>
<td>0.33</td>
<td>1/3</td>
<td>Type 13 (S11)</td>
</tr>
<tr>
<td></td>
<td><em>C. geophilum</em></td>
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<td>YG</td>
<td>0.10</td>
<td>2/3</td>
<td>Type 14 (N13)</td>
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<td></td>
<td></td>
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<td>YG</td>
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<td>1/3</td>
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<td>YG</td>
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<td>3/3</td>
<td>Type 16 (S59)</td>
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<td>YG</td>
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<td>0/3</td>
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<td></td>
<td></td>
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<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scoria</td>
<td>YG</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
</tbody>
</table>

*Three different soil substrates were used to grow ectomycorrhizal pine seedlings for the short-term experiment. See the text for details.

†Two types of nutrient medium were used for bacterial isolation; YG and NF. See the text for details.

§Relative abundance of *Rhizobium* colonies to the total bacterial colonies.

‰The number of *Rhizobium* detected subsamples/total subsamples from each treatment in the long-term experiment and that of *Rhizobium* detected samples/total samples from each treatment in short-term experiment.

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Fig. 2. Bayesian phylogenetic tree of Rhizobium strains isolated from pine ectomycorrhizae with known Rhizobium species using partial 16S rRNA gene sequences. The numbers on the nodes indicate the percentages supporting those branches based on Bayesian posterior probabilities. Strains isolated in this study are shown in bold (Types 2–20) with treatment information; Site 1–3, field sites; Short, short-term experiment; Long, long-term experiment; Sb, Suillus bovinus; Sl, Suillus luteus; Pt, Pisolithus sp.; and Cg, Cenococcum geophilum. We only used strains for which the entire range of the 16S rRNA gene was obtained. Type strain data were used for all described species. Identified phylogroups and subphylogroups are shown on the right. *Former Agrobacterium species.
Discussion

Ectomycorrhizal roots are associated with a wide range of bacterial communities. Although the functions of most ectomycorrhiza-associated bacteria are unknown, a number of studies have demonstrated that some strains (mostly *Pseudomonas* and *Bacillus* in the literature) isolated from ectomycorrhizal roots have positive effects on ectomycorrhizal infection processes (see Garbaye, 1994; Frey-Klett et al., 2007 and references therein). However, these strains of *Pseudomonas* and *Bacillus* are not necessarily dominant in bacterial communities associated with ectomycorrhizal roots. Although the information available on ectomycorrhiza-associated bacteria in nature is very limited, some recent studies using culture-independent or culture-dependent methods have indicated the dominance of *Alpha*-, *Beta*-, (especially *Burkholderia*), and *Gammaproteobacteria* lineages (Chow et al., 2002; Izumi et al., 2006, 2008; Timonen & Hurek, 2006; Burke et al., 2008; Kataoka et al., 2008). Unfortunately, finer taxonomic identities of these *Proteobacteria* are not always available in these studies, partly because of the short sequences obtained, but these ectomycorrhiza-associated bacterial communities are distinctly different from those found in bulk soil, where *Bacillus* and *Paenibacillus* are frequent (Timonen et al., 1998; Bending et al., 2002; Timonen & Hurek, 2006). Although the entire community of ectomycorrhiza-associated bacteria is not the focus of this study, our results are consistent with these recent studies in terms of broader taxonomic groupings. In fact, the most dominant bacterial genus isolated from pine ectomycorrhizal roots in this study was *Burkholderia* (*Betaproteobacteria*), followed by *Rhizobium* (*Alpha-proteobacteria*), *Bradyrhizobium* (*Alphaproteobacteria*), and *Variovorax* (*Betaproteobacteria*), while the bulk soil was dominated by *Paenibacillus* (*Firmicutes*), *Arthrobacter* (*Actinobacteria*), and *Bacillus* (*Firmicutes*).

The presence of *Rhizobium* in association with ectomycorrhizal roots has rarely been documented in the literature.

Fig. 3. Bayesian phylogenetic tree of *Rhizobium* strains isolated from pine ectomycorrhizae with known *Rhizobium* species using *atpD* and *recA* gene sequences. The numbers on the nodes indicate the percentages supporting those branches based on Bayesian posterior probabilities. Strains isolated in this study are shown in bold (Types 2–20) with treatment information; Site 1–3, field sites; Short, short-term experiment; Long, long-term experiment; Sb, *Suillus bovinus*; Sl, *Suillus luteus*; Pt, *Pisolithus* sp.; and Cg, *Cenococcum geophilum*. Type strain data were used for all described species. Phylogroups shown on the right were derived from the phylogenetic analysis based on partial 16S rRNA gene sequences (see Fig. 2). *Former Agrobacterium* species.
Kataoka et al. (2008) described one Rhizobium strain among 12 different ectomycorrhiza-associated bacteria from Pinus thunbergii. Burke et al. (2008) also found Rhizobium sequences in PCR products from one ectomycorrhizal root sample (Pseudotsuga menziesii – Lactarius sp.). Based on these limited examples of Rhizobium detection in single samples, it was not possible to conclude that Rhizobium was an important component of ectomycorrhiza-associated bacterial communities. In this study, however, we found Rhizobium species in 23 of 75 independent root samples collected from the field (six of 30 samples) and greenhouse experiments (eight of 36 samples in the short-term experiment and all nine samples in the long-term experiment). In addition, Rhizobium was the second most dominant genus in terms of relative abundance, dominating nearly one tenth of the bacterial isolates obtained from pine roots in this study (102 out of 1195 isolates). Rhizobium occurrence is not specific to pine ectomycorrhizae and was found more frequently among Salix ectomycorrhizae (M. Tanaka, unpublished data). These results indicate that Rhizobium can be a major component of ectomycorrhiza-associated bacterial communities.

A notable finding of this study was the dominance of Rhizobium species in some ectomycorrhizal samples (up to 100% of isolated bacteria). This may not be due solely to the bias generated by the nutrient medium used in the isolation, because Burke et al. (2008) documented the dominance of Rhizobium in a Pseudotsuga ectomycorrhizal sample using a culture-independent method. It should be noted that Rhizobium species were too rare to be isolated from the soil or scoria in this study, which is in accordance with the observation that Rhizobium form a small proportion of the total soil bacteria (Sadowsky & Graham, 1998). The dominance of Rhizobium in or on ectomycorrhizal roots and their rarity in the soil suggest that Rhizobium are dependent on ectomycorrhiza-derived nutrients. Ectomycorrhizal root tips are a strong sink of photosynthetic products (Wu et al., 2001; Smith & Read, 2008), accounting for nearly half of the net primary production (Fogel & Hunt, 1983). Ectomycorrhizal roots are also rich in mineral nutrients, because ectomycorrhizal fungi absorb them effectively from the soil and translocate them to ectomycorrhizal roots for nutrient exchange with the host plant (Smith & Read, 2008). Thus, ectomycorrhizal roots may be an ideal place for bacteria to obtain organic and inorganic elements, if the bacteria have the ability to access such resources. Rhizobium strains found in this study, as well as other ectomycorrhiza-associated bacteria, may adapt well to this potentially ideal habitat.

Because all Rhizobium strains in this study were obtained in the absence of leguminous plants, these strains may not depend on nodule formation in their life cycle. To confirm this hypothesis, we examined whether the non-nodulating Rhizobium strains had the potential to form nodules. In our inoculation tests of three representative strains, no Rhizobium strain formed nodules on roots of five leguminous plant species examined (Table S2). Only one strain formed a structure resembling crown gall tumors on Medicago sativa roots (Table S2), although this strain was phylogenetically close to R. tropici and R. multithisporium and rather remote from the tumor-forming Rhizobium radiobacter (syn. Agrobacterium tumefaciens). The presence of nodC and nifH genes in four representative Rhizobium strains was not confirmed by PCR amplification using ordinary primer sets (data not shown). Thus, it is likely that the non-nodulating Rhizobium strains isolated in this study actually lack nodule-forming ability. Although the ubiquity and ecological importance of non-nodulating Rhizobium have not been examined in detail, such strains have been isolated from a wide range of plant taxa (Sturz et al., 1998; Surette et al., 2003; Mano & Morisaki, 2008; Ulrich et al., 2008). Even in association with nodule-forming plants, non-nodulating species were 40 times more abundant than nodulating ones (Segovia et al., 1991). Based on these data, Sachs & Simms (2008) noted that the non-nodulating form may be the dominant form of Rhizobium in natural ecosystems. Our results provide additional evidence to support this view.

The methods used to isolate rhizobacteria have often been used to describe forms of rhizobacterial colonization (e.g. ‘endophytes’ when surface sterilization is applied and ‘rhizosphere bacteria’ when using root-adhering soil alone). In the present study, we carefully removed all visible soil particles and organic debris from ectomycorrhizal roots under a stereomicroscope and subsequently washed the ectomycorrhizal roots five times by stirring with sterilized water. Thus, Rhizobium strains in this study did not originate in the rhizosphere soil; rather, they were isolated from the ectomycorrhizal roots themselves. A recent study found that some Rhizobium bind specifically to cell wall proteins of Tuber boehnii (Cerigni et al., 2008), which is an ectomycorrhizal fungus. In our study, Tuber ectomycorrhizal roots collected from field site 3 were also dominated by Rhizobium species. Thus, these Rhizobium bacteria may be tightly attached to the fungal tissues in the ectomycorrhizal root tips, forming a biofilm-like colonization of ectomycorrhizal fungal tissues (mantle and Hartig net), as demonstrated for some other bacterial strains (Frey-Klett et al., 2007 and references therein). On the other hand, in our long-term study, Rhizobium bacteria were detected in all ectomycorrhizal fungal samples as well as from a nonmycorrhizal root sample. Thus, some Rhizobium bacteria may also exist as plant endophytes, as described for other plant species (Surette et al., 2003; Doty et al., 2005; Singh et al., 2006; Ulrich et al., 2008). Because bacterial communities isolated from surface-sterilized and -unsterilized ectomycorrhizal roots are similar and dominated by the same bacterial genera (e.g. Burkholderia and Pseudomonas in Izumi et al.,
2007), the surfaces and interiors of ectomycorrhizal roots may share many common bacterial species. Moreover, there is increasing evidence that many rhizobacteria live in a continuum ranging from the interior of the plant root to the rhizosphere soil (and possibly extending to the bulk soil by extrametrical mycelia of mycorrhizal fungi, as demonstrated by Timonen & Hurek, 2006). Hence, it is difficult to unequivocally categorize rhizobacteria by their theoretically defined colonization sites (Nehl & Knox, 2006; Timonen & Marschner, 2006). It is also likely that the colonization sites of many rhizobacteria change with abiotic and biotic conditions, especially during developmental stages and senescence. Although the exact colonization sites of non-Rhizobium bacteria were not examined microscopically in this study, we assumed that they were located in a continuum ranging from the ectomycorrhizal surface to the interior of the roots, the extent of which varies with diverse factors.

Various factors may affect Rhizobium occurrence in pine ectomycorrhizal roots. Because the occurrence of Rhizobium was significantly higher in saplings than in mature trees at site 3 (Fig. 1, P = 0.00023), it may be reasonable to consider host age as a potential determinant. However, the two age classes were not collected under the same conditions (saplings were located at the forest edge and mature trees were in the closed forest); thus, the effect of age class may be confounded by environmental conditions. A potential effect of host age was also suggested from the long-term experiment, wherein Rhizobium were detected in all treatments, i.e., 8-year-old seedlings inoculated with different ectomycorrhizal fungi. The high frequency of Rhizobium may have also been confounded by the greater number of root tips used for isolation (30 ectomycorrhizal tips) compared with the number of tips from the field samples (five ectomycorrhizal tips) and in the short-term experiment (10 ectomycorrhizal tips). However, these confounding effects do not exclude the possible effect of host age on Rhizobium occurrence. Thus, future studies of ectomycorrhiza-associated bacteria should include host age as a potential factor or variable in sampling and experimental design.

Another possible factor may be ectomycorrhizal fungal species, because Rhizobium occurrence was significantly affected by ectomycorrhizal fungal species in the short-term experiment. Indeed, Rhizobium strains were frequently isolated from C. geophilum treatments, but not from S. granulatus treatments under the same conditions in the short-term experiment. This result, however, does not indicate that S. granulatus ectomycorrhizal roots are always devoid of Rhizobium. In fact, Rhizobium were found in association with S. granulatus and other closely related Suillus species in the long-term experiment and in the field. Moreover, Rhizobium were detected in all treatments irrespective of ectomycorrhizal fungal species in the long-term experiment (Fig. 2). These results indicate that every ectomycorrhizal fungus is likely to have some compatible Rhizobium strains. Therefore, ectomycorrhizal fungal species alone is not the main determinant of Rhizobium occurrence, but the interaction between ectomycorrhizal fungal species and other factors, such as host age, may be an important determinant.

Most Rhizobium strains isolated in this study belonged to phylogroup 1, which was detected in both the greenhouse experiments and the field. However, different treatments and experiments found different Rhizobium types (species) and generally did not share common Rhizobium types (Table 1, Fig. 2). Thus, there may be some specificity between Rhizobium type and treatment (ectomycorrhizal fungal species, soil, and plant age), although this relationship cannot be confirmed using the present data set, because each Rhizobium type was too infrequent (mostly represented by a single or a few samples) to be analyzed statistically. In addition to such deterministic factors, we assumed some stochastic mechanisms. Given that rhizobacteria colonizing an ectomycorrhizal root originate from the outside environment and are not vertically inherited from the host parent, the bacterial composition of the ectomycorrhizal root should be a small subset of an environmental pool of diverse rhizobacteria. Random selection of such a subset would generate considerable variability in bacterial communities between different ectomycorrhizal samples, even without any deterministic factors. The composition of the rhizobacterial pool itself may also be heterogeneous and change spatiotemporally. Therefore, these stochastic mechanisms, in balance with the deterministic mechanisms, may explain the observed variability of the Rhizobium type composition in this study.

Molecular phylogenetic analyses using partial sequences of 16S rRNA gene revealed that Rhizobium strains associated with ectomycorrhizal pine roots were phylogenetically diverse and divided into five major clades (Fig. 2). Phylogroup 5 was especially distant from any described Rhizobium species that belongs to nodule-forming Rhizobium or former Agrobacterium lineages. In addition, many Rhizobium strains isolated from ectomycorrhizal pine roots varied distinctly from the known Rhizobium species with high branching support in the phylogram, indicating the possibility of several new species. Because several studies have suggested that 16S rRNA gene-based phylogenies of rhizobia can be misleading due to chimeric 16S rRNA gene sequences (Eardly et al., 1996; Turner & Young, 2000), we also used atpD and recA gene sequences for phylogeny. Although the two phylogenetic trees (Figs 2 and 3) did not show exactly the same pattern probably because of the differences in the number of taxa included, the major grouping of our Rhizobium strains in atpD+recA tree was generally consistent with that of the 16S tree, indicating that the 16S rRNA
gene-based phylogeny is also supported by other DNA regions (Fig. 3). Therefore, we think it is unequivocal that ectomycorrhizal pine roots are associated with phylogenetically diverse Rhizobium species, most of which vary from known Rhizobium species.

The diversity of non-nodulating Rhizobium strains reported in this study has profound implications in the molecular phylogeny of this particular genus. In most studies on Rhizobium phylogeny, non-nodulating strains, except former Agrobacterium species, are not included. However, our results revealed a single pine species associated with diverse non-nodulating strains, some of which had unique phylogenetic positions. Although the phylogenetic positions of non-nodulating Rhizobium isolated from other plant species are unclear, they are quite likely to broaden the phylogenetic diversity further. To better understand the diversity and phylogeny of the important genus Rhizobium, more attention should be paid to non-nodulating strains.

According to Turner & Young (2000), the divergence time for the split between Rhizobium and Sinorhizobium is estimated at 203–346 Ma. However, the origin of plant–Rhizobium associations remains uncertain. Soltis et al. (1995) demonstrated that all extant nodule-forming plants belong to a clade within Rosid I in angiosperm phylogeny, suggesting a rather recent origin (c. 60 Ma) of nodule association. Pathogenic associations with Rhizobium (syn. Agrobacterium) have been found in a wider plant taxonomic range including a wide range of dicotyledons (Thomashow et al., 1980). Non-nodulating plant–Rhizobium associations have also been reported in various angiosperms as described above and conifers described in this study. Given that Pinaceae was a dominant plant group on the planet before angiosperm diversification (345–130 Ma; Millar, 1998; Wang et al., 2000), our data on Pinus–Rhizobium associations may provide additional information toward understanding the origin of plant–Rhizobium associations.

In conclusion, we demonstrated the occurrence and diversity of non-nodulating Rhizobium bacteria in ectomycorrhizal roots of Japanese red pine. The dominance of Rhizobium in some ectomycorrhizal roots indicates intimate associations between Rhizobium and ectomycorrhizal pine roots under certain conditions. Rhizobium strains isolated from ectomycorrhizal pine roots were phylogenetically diverse and possibly include several new species. These results may substantially advance our understanding of Rhizobium diversity, ecology, evolution of the genus, and plant–Rhizobium associations.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Table S1. Ectomycorrhizal fungi identified in field-collected ectomycorrhizal roots of Pinus densiflora.

Table S2. Nodule formation test using selected Rhizobium strains isolated from pine ectomycorrhizae.
Table S3. Rhizobium strains isolated from pine ectomycorrhizae and their divergence from the closest type strains.

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