Phytoremediation is one of most innovative technologies that utilize the natural properties of plants to remediate hazardous waste sites (Salt et al. 1998, Krämer and Chardonnens 2001, McGrath and Zhao 2003). For more cost-effective phytoremediation, hyperaccumulators play an important role of phytoremediation of heavy metals and it is an attractive option to utilize a hyperaccumulating plant after phytoremediation such as the recovery of valuable metals and the production of useful materials (Baker et al. 2000).

In Europe and North America, many studies have been conducted to find more effective plants for phytoremediation of various pollutants (e.g., Tarnau et al. 2001). In Japan, on the other hand, studies have been published only relatively recently (e.g., Saito et al. 2004). The fern *Athyrium yokoscense*, which is native to the Far East including Japan, often flourishes in mine sites and areas that are highly polluted with heavy metals. Moreover, it is well known that the fern accumulates a large amount of metals in the tissues, particularly in the roots (Nishizono et al. 1987). Nishizono et al. (1988, 1989) studied the mechanisms involved in metal detoxification and accumulation of this fern, and suggested that a 9.5-kDa cysteine-rich peptide, which was induced by the exposure of the fern to copper, may contribute to the copper-tolerance of the fern growing in copper-contaminated soil. In addition, Xian (1990) reported that the compartmentalization of excess metals in the root cell wall has been believed to be important for the metal tolerance and accumulation of *At. yokoscense*.

The arbuscular mycorrhizal (AM) fungi of *Athyrium yokoscense* was investigated in the Ikuno mine site of Japan, where a silver mine was active until 1973. We sampled four localities in the Ikuno mine site to identify the AM fungi on *At. yokoscense* using molecular phylogenetic analysis of the 18S ribosomal DNA. These analyses indicated that at least four types of AM fungi colonized the root of *At. yokoscense* in the Ikuno mine site. Among AM fungi, we found *Acaulospora* species from the high arsenic (As) areas of the Ikuno mine site and non-*Acaulospora* species from other localities. It is possible to identify the AM fungi of *At. yokoscense* by using this method.

**Key Words:** 18S ribosomal DNA (rDNA), arbuscular mycorrhizal fungi, *Athyrium yokoscense*, phylogeny, *rbcL*.
The bacteria and fungi living in the rhizosphere of these plants also may play an important role in phytoremediation, but relatively few studies have focused on the effects of these microorganisms on the metal remediation efforts (Pawlowska and Charvat 2004, Vidas et al. 2005, Regvar et al. 2006, Wu et al. 2006). Among them, arbuscular mycorrhizal (AM) fungi can form symbiotic relationships with the vast majority of land plants including Athyrium ferns (Lee et al. 2001, Zhang et al. 2004, Matsuda et al. 2005), and are known to benefit the phosphorus nutrition of host plants by increasing phosphorus acquisition (Smith et al. 2003). Although there are some studies concerned with symbiotic relationships between AM fungi and ferns, only one study has been conducted on At. yokoscense (Matsuda et al. 2005).

As for the identification of AM fungi, Gerdemann (1965) indicated that it is difficult to identify AM fungi to species using various morphological approaches. Moreover, identification of AM fungi spores holds difficulties due to alterations of morphological features during spore ontogeny (Morton 1993). Additionally, diversity assessments of AM fungi are debatable due to the lack of correlation between root colonization and spores (Clapp et al. 1995, Renker et al. 2005, Hempel et al. 2007). Recent advances in molecular biology and their application have enabled the exploration of the extent and distribution of genetic variation among or within particular taxa. Some studies have managed to identify AM fungi using molecular phylogenetic approaches. For example, Whitfield et al. (2004) indicated that Glomus is the predominant AM genus colonizing in Thymus polytrichus (Lamiaceae) by using restriction fragment length polymorphism (RFLP) and DNA sequencing of 18S ribosomal DNA (rDNA), moreover, Zarei et al. (2008) also reported that the root of Veronica rechingeri (Scrophulariaceae) was colonized by some Glomus species by sequencing of the internal transcribed spacer (ITS) region, suggesting that molecular data are an effective tool to infer AM fungi and the combining with morphological data and molecular ones is the best to identify them.

The Ikuno mine site (Fig. 1) was one of the most famous mines of Japan and active more than four hundred years, the operation is stopped now. Mizuno and Fujimura (2003) reported that this area had a deep environmental impact on soil quality of farms downstream, indicating that the removal or reduction of the existing pollution hazards is still urgently required including remediation programs. Therefore, we identified the AM fungi on At. yokoscense using molecular phylogenetic approaches.

Materials and Methods

Sampling

We collected Athyrium yokoscense from the Ikuno mine sites of Hyogo Prefecture of Japan in July 2008. We designated the following four localities; Material, Kanamori, Keiju and Daimaru. These localities are shown in Fig. 1.

Measurement of heavy metal concentration in soils

To compare heavy metal concentration in soils of the Ikuno mine site, we collected soil samples from all localities. Soils were air-dried for 7 days before being powdered. For analysis of concentrations of Cadmium (Cd), Lead (Pb), and Zinc (Zn), the soil samples were microwave-digested in a concentrated HNO₃ and HF. Determinations of Cd, Pb, and Zn were conducted by atomic adsorption spectrometry (AA-6800, Shimadzu, Kyoto, Japan). For analysis of concentrations of As and P, the soil samples were digested in a concentrated HNO₃, HClO₄, HF and 2% KMnO₄. Determinations of As and P were conducted by ICP-Atomic Emission Spectrometry (ICPS-1000IV, Shimadzu, Kyoto, Japan).
Fig. 1. Position of the Ikuno mine site including four localities in the Ikuno mine site and localities of *Athyrium yokoscense* used in phylogenetic analysis. The black box indicates the Ikuno mine site and black circles indicate localities of *Athyrium yokoscense*.

**DNA extraction and sequencing analyses**

Total DNAs were isolated from approximately 300 mg of root from *At. yokoscense* for the phylogenetic analyses of the AM fungi analyses with a Plant Genomic DNA Mini Kit (VIOGENE) used according to the manufacturers’ protocols. The isolated DNA was resuspended in TE and stored at −20 °C until use.

For the phylogenetic analysis of the AM fungi, the 18S rDNA region with primers designed by Saito et al. (2004) and Sato et al. (2005) were used. Double-stranded DNA was amplified by incubation at 94°C for 2 min followed by 40 cycles of incubation at 94°C for 1.5 min, 48°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 15 min. After the amplification, reaction mixtures were subjected to electophoresis in 1% low-melting-temperature agarose gels to purify of the amplified products. The cloning of purified PCR products was performed using the TA cloning kit (Invitrogen). Approximately 100 clones were characterized and sequenced using a BigDye Terminator ver. 3.1 (Applied BioSystems) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer’s instructions. At least two independent samples of each PCR products were cloned, and both strands were sequenced. This process was repeated twice to confirm the same sequences.

**Data analyses**

For phylogenetic analyses of AM fungi, a representative of each OTU was compared phylogenetically to sequences of known species in the GenBank database of the DDBJ/EMBL/GenBank by using the Blast program. Database sequences yielding the greatest percent similarity to the clone sequences were chosen as the best match for each OTU. Moreover, some sequences of closely related species of the best match sequences were retrieved from GenBank (Table 1). Phylogenetic trees were then constructed with *Catenomyces* sp. (AY635830) as use for outgroups.
Table 1. Samples used in the phylogenetic study and corresponding GenBank accession numbers

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<th>Taxa</th>
<th>Accession No.</th>
<th>Reference</th>
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<td>Glomus sp. 6</td>
<td>AJ496109</td>
<td>Opik et al. (2003)</td>
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To construct phylogenetic trees, the 18S rDNA sequences were aligned independently using Clustal W (Thompson et al. 1994), and were edited manually using MEGA 4 (Tamura et al. 2007). The positions of deletions or insertions were determined by eyes and eliminated in all of the phylogenetic analyses. In the phylogenetic analyses, the most appropriate model of DNA substitution was chosen using the Akaike information criteria with PAUP 4.0b10 (Swofford 2002) and Modeltest 3.0 (Posada and Crandall 1998). For the 18S rDNA sequences, the GTR model with gamma shape parameter of 0.2958 (GTR+G) was chosen (Substitution rates: A, 0.3729; C, 0.1552; G, 0.1827; T, 0.2891; A-C, 0.0000; A-G, 1.6392; A-T, 0.1821; C-G, 1.0085; C-T, 3.8909; and G-T, 1.000). The neighbor-joining (NJ) analysis was performed using PAUP 4.0b10 on the most

Results
The results of the comparison of heavy metal
Fig. 2. Heavy metal concentration in soils of four localities of the Ikuno mine site. (A): Cd, (B): Zn, (C): P, (D): Pb, (E): As. Error bars indicate standard. Columns marked by different letters are significantly different according to Kruskal-wallis test (P < 0.05).

Concentration in the Ikuno mine site are shown in Fig. 2. The concentration of As, Cd and Zn in the locality ‘Material’ is highest in any of the localities (Figs. 2A, B, E), and P and Pb of the other locality ‘Keiju’ were highest values of all (Figs. 2C, D).

From the molecular analysis of the AM fungi, four sequences were isolated from each of the four localities of At. yokoscense examined in this study. These sequences isolated in this study were named from Type 1 to Type 4. Type 1 is the AM fungi isolated from the root of At. yokoscense collected from the one locality ‘Material’ and ‘Keiju’, Type 2 is from ‘Daimaru’, Type 3 and Type 4 are from ‘Kanamori’. These four sequences have been also submitted to DDBJ/EMBL/GenBank nucleotide sequence databases under Accession Nos. AB490491, AB490492, AB490494, and AB490495 (Table 1).

We reconstructed phylogenetic trees including our isolates and previous published sequences of AM fungi based on NJ analysis (Fig. 3). In NJ tree, Type1 was identical to previous published sequences of Acaulospora longula and Ac. rugosa, and consisted of monophyletic group containing the following Acaulospora species; Ac. sp. 2, Ac. sp. 16, Ac. sp. 18, Ac. laevis, Ac. spinos, and Ac. scrobiculata. Type 2 was consisted of monophyletic group with following Glomus species, G. sp. 36, G. sp. 37, G. sp. 38, G. sp. 39, and positioned at the most basal node in this monophyletic group. Type 3 and Type 4 consisted of a monophyletic group with Archaeospora leototicha and Ambispora fennica.

Discussion

In this study, some AM fungi colonized Athyrium yokoscense distributed in the Ikuno mine site. Our phylogenetic results of the AM fungi indicated that Type1 isolated from ‘Material’ belonged to a species of Acaulospora because Type1 was included in the monophyletic group of species of Acaulospora. Our phylogenetic analyses could not identify species
or genus for other haplotypes. However, Type 2 was positioned at the most basal node of a monophyletic group which consisted of *Glomus* species. This result suggested that Type 2 was closely related to the genus *Glomus*, probably belonged to the species of *Glomus* (Fig. 3).

The results of the comparison of heavy metal concentration in the Ikuno mine site indicated that all localities of the Ikuno mine site involved higher concentration of all heavy metals than those of previously published standard data (Asami and Chino 1983), indicating that the soils of this area still polluted. In this study, why are the AM fungi different between near localities of the Ikuno mine site? It is not surprising because these areas had different values of metallic concentrations. Our soil data indicated that the soil of both localities ‘Material’ and ‘Keiju’ had higher concentrations of As than other localities (Fig. 2). Therefore, the amount of As might influence the survival of the AM fungi. In fact, Wang et al. (2008) indicated that *Acaulospora* species could survive in high concentrations of As, moreover, some studies have reported that
Glomus and Scutellospora species were the most widespread (Vestberg 1995, Zhang et al. 1998, Oehl et al. 2003), although Acaulospora species dominated in some restricted areas (Zhang et al. 2003, Tao et al. 2004). These studies were supported as Acaulospora species could be isolated only from the localities ‘Material’ and ‘Keiju’ where is a high concentration of As. Thus, the dominance of Acaulospora in the localities ‘Material’ and ‘Keiju’ and non-Acaulospora in other localities most likely reflects the influence of specific soil conditions and may represent differences in tolerance to high As concentration, rather than a spatially dominant individual. Considering these results, we could hypothesize the best way to remove As polluted soils of the Ikuno mine site is to use At. yokoscense colonizing Acaulospora species.

The interest in trying to link At. yokoscense living in various soil types and AM fungi rests on the wide use of such markers for phylogeny as well as on an increasing number of studies. The 18S rDNA analyzed in this work provides a robust hypothesis between At. yokoscense and the AM fungi. This hypothesis, based on molecular information, is a timely contribution that allows unbiased interpretation between hosts and mycorrhizae. Additional molecular markers may lend support to this scenario, and multiple markers should be surveyed within this species to find any variation. This work will help to reinterpret existing 18S rDNA data sets and facilitate the interpretation of new ones. Moreover, the AM fungi predominated in the colonized roots of At. yokoscense at all sites, supporting findings from earlier studies that suggest that they are particularly tolerant of large concentrations of heavy metals. Identification and isolation of the most tolerant strains may have important implications for the future phytoremediation of heavy-metal-contaminated soils (Khan et al. 2000).

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References


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野々村暢仁 a, 川田由紀 a, 南谷幸雄 b, 早川宗志 b, 福田達哉 a, 康 峪梅 a, 櫻井克年 a: 生野鉱山のヘビノネゴザに着生するアーバスキュラー菌根菌の分子同定
ヘビノネゴザ Athyrium yokoscense のアーバスキュラー菌根菌（AM 菌）を、1973 年まで銀山として採鉱されていた生野鉱山で研究した。生野鉱山の 4 地点から、土壤中の重金属蓄積量の解析と、形態学的・分子系統学的手法を用いた AM 菌の同定を行った。分子系統学的解析は、18S ribosomal DNA を用いた。これらの解析から、形態観察により全ての地点のヘビノネゴザの根に AM 菌が着生しており、分子系統学的手法により生野鉱山のヘビノネゴザの根には少なくとも 4 タイプの AM 菌が着生していることが明らかになった。生野鉱山内のヒ素の高濃度蓄積地点から Acaulospora 属の AM 菌が、その他の地点から非 Acaulospora 属の AM 菌が検出された。従って、これらの AM 菌相の違いは、空間的な優占種の違いではなく、土壤の特殊性に影響しており、これはヒ素蓄積量の差に影響されている可能性が高い。

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