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ORIGINAL PAPER

Ectomycorrhizal fungal communities associated with Masson pine (*Pinus massoniana* Lamb.) in Pb–Zn mine sites of central south China

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Abstract To advance our understanding of ectomycorrhizal fungal communities in mining areas, the diversity and composition of ectomycorrhizal fungi associated with Masson pine (*Pinus massoniana* Lamb.) and soil chemistry were investigated in Taolin lead–zinc (Pb–Zn) mine tailings (TLT), two fragmented forest patches in a Huayuan Pb–Zn mineland (HY1 and HY2), and a non-polluted forest in Taolin in central south China. Ectomycorrhizal fungal species were identified by morphotyping and sequence analyses of the internally transcribed spacer regions of ribosomal DNA. The two study sites in the Huayuan mineland (HY1 and HY2) were significantly different in soil Pb, Zn, and cadmium (Cd) concentrations, but no significant difference was observed in ectomycorrhizal colonization, ectomycorrhizal fungal richness, diversity, or rank–abundance. In addition, the similarity of ectomycorrhizal fungal communities between HY1 and HY2 was quite high (Sørensen similarity index=0.47). Thus, the concentration of heavy metals may not be determining factors in the structure of these communities. In the tailings, however, significantly lower ectomycorrhizal colonization and ectomycorrhizal fungal richness were observed. The amounts of Pb and Zn in the tailing sand were higher than the non-polluted forest but far lower than in HY1. Thus, these heavy metals did not account for the reduced colonization and ectomycorrhizal fungal richness in TLT. The ectomycorrhizal fungal community in TLT was dominated by four pioneer species (*Rhizopogon buenoi*, *Tomentella ellisii*, *Inocybe curvipes*, and *Suillus granulatus*), which collectively accounted for 93.2 % of root tip colonization. The immature soil conditions in tailing (low N and P, sand texture, and lack of organic matter) may only allow certain pioneer ectomycorrhizal fungal species to colonize the site. When soil samples from four sites were combined, we found that the occurrences of major ectomycorrhizal fungal taxa were not clearly related to the concentrations of Pb, Zn, and Cd. In conclusion, our results suggest that ectomycorrhizal fungal communities in mining areas are not necessarily affected by heavy metals themselves but could be largely determined by soil maturity.

Keywords Ectomycorrhizal fungal community · Heavy metal contamination · Mineland · Mine tailing · *Pinus massoniana* Lamb

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Introduction

Mining activities destroy vegetation and remove surface soil to reach buried ore deposits. Mineral extraction processes are often accompanied by pulverization of rocks and

chemical use. The residue of the extracted substrates is stored in tailings, producing large areas of bare ground and piles of tailings. Ideally, these mine areas should be restored to the original vegetation (Bradshaw and Johnson 1992). However, the toxicity of heavy metals, lack of macronutrients, and abnormal soil structures in mining areas make restoration difficult without careful planning (e.g., storage of surface soil to cover the bare ground after mining activities). In China, many mining areas have been abandoned, causing severe ecological and environmental problems (Liu et al. 2005; Shu et al. 2005; Li 2006). Soils in these abandoned mine areas are highly contaminated by heavy metals, which are dispersed by wind or dissolved in water, causing human health disorders in the surrounding regions.

Many of the abandoned mine areas in China were originally covered by forests dominated by Pinaceae and Fagaceae. These tree species depend on ectomycorrhizal fungi (EMF) for nutrient absorption and cannot grow in their absence. Some reports have also demonstrated that EMF help trees survive in soils contaminated by heavy metals (Dickinson et al. 1992; Wilkinson and Dickinson 1995) by alleviating the toxicity of the metals (Jentschke and Godbold 2000; Meharg and Cairney 2000; Adriaensen et al. 2004; Adriaensen et al. 2005; Adriaensen et al. 2006; Colpaert et al. 2011). Thus, application of EMF in abandoned mine areas may help with forest restoration. Unfortunately, our knowledge about the function of EMF in heavy metal tolerance is largely from in vitro experiments using a few easily culturable strains.

In the field, EMF always exist and function as a community composed of a diverse range of species. Although ectomycorrhizal fungal communities have been studied in many different forests throughout the world, we know little about these communities in mining areas. A few reports document significantly reduced diversity of ectomycorrhizal fungal communities in heavy metal-contaminated areas (Staudenrausch et al. 2005; Ruotsalainen et al. 2009). In contrast, other studies have shown highly diverse ectomycorrhizal fungal communities in heavy metal-contaminated sites, with no strong indication of selection for heavy metal-tolerant species (Blaudez et al. 2000; Cripps 2003; Krpata et al. 2008; Colpaert et al. 2011; Hui et al. 2011). Because we do not know the reasons for the inconsistent results in previous studies, more research is needed to identify the determinant factors that structure ectomycorrhizal fungal communities in heavy metal-contaminated soils.

Masson pine (*Pinus massoniana* Lamb.) has been frequently planted in deforested areas in central southern China because of its tolerance to drought and arid conditions (Zhu et al. 2010). Many natural forests and plantations of Masson pine are distributed in this region. Even in mining areas, we can find some remaining forest patches or naturally established trees, as well as planted trees. Masson pine is a typical ectomycorrhizal conifer species and maintains symbioses with a diverse range of ectomycorrhizal fungal species in natural forests (Chen 1989; Ke and Liu 2005). To develop an effective reforestation strategy in the mining areas, we need better understanding of EMF on Masson pine in mining areas.

In this study, ectomycorrhizal fungal communities on Masson pine were studied in the largest abandoned lead–zinc (Pb–Zn) mine (Taolin Pb–Zn mine) and an operating Pb–Zn mine (Huayuan Pb–Zn mine) in Hunan Province in central southern China. The goals of this study were to characterize ectomycorrhizal fungal communities on Masson pines growing in two Pb–Zn mining areas and to evaluate the effects of heavy metals and other soil factors on these communities.

Materials and methods

Sampling sites

The study areas are located in Linxiang City and Huayuan County (about 700 km apart) in Hunan Province, China. Three distinct habitats [fragmented forest patches (two sites, HY1 and HY2) in excavated Huayuan Pb–Zn mineland, Taolin Pb–Zn mine tailing (TLT), and non-polluted forest (TLC) in Linxiang City] were selected for present study. The position of Taolin mine and Huayuan mine are shown in Fig. 1. The study sites are described in detail in the following text.

Taolin Pb–Zn tailing (TLT; 29°22′ N, 113°28′ E) of Taolin Pb–Zn mine is located in a low mountain and hill region with elevations ranging from 200 to 300 m and slopes between 25 ° and 35 °. The average annual temperature and precipitation are 16.4–16.8 °C and 1,325 mm, respectively. The mine was operated from 1901 to 2002. The tailing dam with a top surface area of approximately 79 ha, which was built in 1960, stores 5,000 million tons of

Fig. 1 The position of two Pb–Zn mining areas in Hunan Province, China

processed residues and reaches an ultimate height of about 30 m from its foot. The residue is silver white tailing sand mainly composed of SiO_2 (about 70 %). The tailing surface soils were mainly composed of sand and silt, with lack of macronutrients and organic matter. The tailing surface is often drought because of poor water holding capacity and high evaporation (1,424.2 mm/year) on tailing surface (Guo et al. 2007). Most parts of the top surface of this huge tailing were still completely bare except for about 200 individuals of planted 7-year-old Masson pine (*P. massoniana*) and a few naturally established herbaceous plant species. The pine trees showed no symptoms of disorder and were selected for the present research.

The non-polluted mature forest (TLC) on the slope of a hill is about 7 km far from the Taolin tailing. The forest was mainly composed of regenerated Masson pine (about 30 years old), Chinese fir (*Cunninghamia lanceolata* Lamb.), and *Quercus* spp. The forest land is in the typical red soil hilly regions in southern China.

Huayuan Pb–Zn mine (28°31′ N, 109°22′ E) is located in Huayuan County in western Hunan Province and is the largest Pb–Zn ore deposit processed in this province. The region has an average altitude of 800–1,200 m with subtropics mountainous moist climate; the average annual temperature and precipitation in this area are 16 °C and 1,418 mm, respectively. Vegetative cover in the mining area was severely destroyed by mining activities. Two fragmented secondary forest patches (HY1 and HY2) in the mining area, about 500 m apart, were selected as sampling sites. The HY1 forest patch is composed of regenerated Masson pine trees (about 15 years old) and Chinese fir (*C. lanceolata* Lamb.). The HY2 patch, a secondary Masson forest (about 30 years old), was located 200 m away from an extraction plant. Many unprocessed ore rocks were dispersed around the forest patch.

Sampling

Sampling at the Huayuan Pb–Zn mining sites (HY1 and HY2) and Taolin sites (TLT and TLC) was carried out in March 2009 and April 2010, respectively. Because of the limited number of pine trees in HY1 and HY2, only ten Masson pine trees were selected for sampling. From each of the selected trees, three root systems (each approximately 15 cm in length) and three corresponding rhizospheric soil samples (each 50 ml) were collected within approximately 3-m distance from the focal tree. In the Taolin area, we selected 40 trees in the tailing (TLT) and 24 trees in the control forest (TLC) sites and collected one root and one soil sample from each tree. All root systems were traced from the trunk to confirm the identity of the roots. Pine needles were also sampled from each selected tree for analysis of heavy metal contents.

Ectomycorrhizal root tip morphotyping and molecular analysis

Root samples were gently washed in tap water to remove soil particles and debris, cut into approximately 9-cm-long sections, and placed in a glass plate filled with tap water. Root tips that were colonized by EMF were counted to determine percent colonization and classified into ectomycorrhizal fungal morphotypes based on their mantle color, surface texture, branching patterns, rhizomorph characteristics, and emanating hyphae using a dissecting microscope (Agerer 1987–1993). About one tenth of the ectomycorrhizal root tips for each morphotype from each root system were randomly selected, individually placed in 2.0-ml tubes, and vacuum-dried for DNA extraction.

Crude genomic DNA was extracted from dried ectomycorrhizal root tips using a modified cetyl-trimethylammonium bromide (CTAB) method (Lian et al. 2003). A single root tip sample was homogenized in a 2.0-ml tube containing one zirconia ball and 50 μl of CTAB (2 % CTAB, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 NaCl, and 0.5 % β-mercaptoethanol) solution using a beadbeater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After confirming that the sample was completely pulverized, 350 μl of CTAB solution was added to the tube and then incubated at 65 °C for 1 h. DNA was isolated using chloroform–isoamyl alcohol mixture (24:1) extraction, precipitation in isopropanol, and washing in 75 % ethanol. The extracted DNA was dissolved in 30 μl sterilized water and stored at –30 °C until use.

The fungal internal transcribed sequence (ITS) regions were amplified by polymerase chain reaction (PCR) using Ampli *Taq* Gold (Applied Biosystems, Foster City, CA, USA) and fungal-specific primers ITS1-F and ITS4 (White et al. 1990; Gardes and Bruns 1993; Lian et al. 2003). When the PCR products were faint, absent, or multibanded, three alternative methods were tested. For the multiband samples with a morphotype like *Cenococcum* sp., ITS1 and ITS4 primers were used in the PCR reaction (White et al. 1990). For the other multiband samples, the basidiomycete-specific primer pair, ITS1-F and ITS4B (Gardes and Bruns 1993), was used, and the ITS fragments were amplified by MightyAmp Polymerase (Takara, Dalian City, Japan) or Ampli *Taq* Gold. For samples of faint and absent PCR products, a nested-PCR system was used with two primer sets (first PCR step: NLA3 and NLC2 primers; second PCR step: NSI1 and NLB4 primers) using Ampli *Taq* Gold (Martin and Rygiewicz 2005). This nested-PCR system was also employed for the unsuccessful samples after applying ITS1-F and ITS4B primers.

To decrease the number of sequencing samples, restriction fragment length polymorphism (RFLP) was used to identify molecular types within each root sample. Five microliters of each PCR product was digested with *Alu*I or *Hinf*I (1.5 U; Takara Otsu, Shiga, Japan) in the reaction

mixture for 8 h at 37 °C. Restriction fragments were separated by electrophoresis on 1.2 % agarose gels. One representative sample with a unique RFLP pattern within a root sample was used for direct sequencing using ITS1-F, ITS1, ITS4, and ITS3 primers, respectively. The PCR products were purified using a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) according to the manufacturer's instructions. After a sequencing reaction with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), sequences were determined in Applied Biosystems 3130xl Genetic Analyzers. When direct sequencing resulted in failure, PCR products were subcloned using pT7 Blue Perfectly Blunt Cloning kit (Novagen, Madison, WI, USA). Successful inserts were sequenced using T7 and U19 primers as described earlier.

The obtained sequences were edited and manually corrected in BioEdit 7.0.8 and then clustered into species-level operational taxonomic units (OTUs) by BLASTclust with 97 % sequence similarity for species delimitation (http://toolkit.tuebingen.mpg.de/blastclust) (Peay et al. 2009). Sequences from OTUs were identified by querying GenBank and UNITE (Koljalg et al. 2005) online databases using the Blastn search option. Sequences having ITS similarities ≥97 % to known fungal species were given the same species name (Tedersoo et al. 2003; Krpata et al. 2008; Tedersoo et al. 2008; Peay et al. 2009). Sequences with 90–97 % similarities to known species were identified at the genus level (Pestana Nieto and Santolamazza Carbone 2009). When Blast results showed poor matches (<90 % ITS similarities), we treated the OTUs as unknown species. All sequences, except a few short ITS sequences, were submitted to the DNA Data Bank of Japan (DDBJ).

Chemical analyses of soil and pine leaves

Soil samples were air-dried, ground, and passed through a 2-mm mesh screen. Pine needles were washed with tap water, rinsed with deionized water (DW), oven-dried at 105 °C for 5 min and at 60 °C for 24 h, and finally milled. Soil pH value was determined by HM-30 G pH meter (TOA Electronics Ltd., Kobe, Japan) after mixing the soil sample with DW at 1:2 ratio by volume. Electrical conductivity (EC) was measured with a B-173 compact conductivity meter (Horiba, Ltd., Tokyo, Japan) using soil suspensions (soil/DW=1:5). After digestion by sulfuric acid and perchloric acid, total nitrogen was determined by the indophenol blue method (Dora 1976) and total phosphorus was determined by the molybdenum blue method (Olsen and Sommers 1982) using a U-2010 spectrophotometer equipped with an AS-1000 auto sampler (Hitachi Instruments, Inc., Tokyo, Japan). The total amounts of potassium (K), Pb, cadmium (Cd), copper (Cu), and Zn in soil and pine needle samples were determined by a Z-6100 Polarized Zeeman atomic absorption spectrometer (Hitachi, Co., Tokyo, Japan) after wet digestion with HNO_3 and $HClO_4$ mixture (4:1, *v*/*v* for soil; 7:1, *v*/*v* for leaves). We also analyzed plant-available heavy metal (Pb, Cd, Cu, and Zn) concentrations by extracting metal ions from the soil with DTPA solution (5 mM DTPA, 10 mM $CaCl_2$, and 100 mM triethanolamine at pH 7.3) (Lindsay and Norvell 1978).

Data analysis

The relative abundance of an ectomycorrhizal fungal species in a tree or site was defined as the ratio of ectomycorrhizal tips colonized by that species within a tree or site, respectively. Frequency was shown by the number of trees colonized by each EMF. Observed species richness was the total number of detected ectomycorrhizal fungal species in a site. Because detecting all EMF at a site is impossible, the sufficiency of sampling effort was evaluated by species accumulation curves produced with EstimateS version 8.0 (Colwell 2006). Species richness estimators (i.e., Jackknife1, Jackknife2, and Chao2) and diversity indices [i.e., Simpson's index (1/*D*) and Shannon–Wiener Index (*H*′)] were calculated for each study site using EstimateS, and then one-way ANOVAs were used to compare the difference in those indices among different sites. Sørensen's similarity index was used to compare the ectomycorrhizal fungal composition between the study sites.

One-way ANOVA followed by Tukey's test at 5 % significance level was used to compare soil and plant chemical data among the four sites. By pooling all of the soil and plant heavy metal data from the four sites, correlation between soil and leaf heavy metals was analyzed. To further analyze the effect of each soil factor on ectomycorrhizal fungal diversity after alleviating a large variance within a site and difference in sample sizes among the sites, we created another dataset by grouping 12 soil subsamples from TLC and ten samples from TLT according to the level of each soil factor. The resultant dataset included two subsample groups from TLC and four groups from TLT, each of which was represented by ten or 12 soil samples with similar levels of the soil factor. Then, the correlation between each soil factor and ectomycorrhizal fungal diversity was analyzed among HY1, HY2, and the subsample groups from TLT and TLC (eight data points in total). All of the above statistical analyses were conducted with SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Tukey's test at 5 % significance level was used for comparing the distribution range of nitrogen (N), Cu, Cd, and Zn content in soils for each major EMF taxa. A non-parametric test (Steel–Dwass test) was performed online (http://www.gen-info.osaka-u.ac.jp/MEPHAS/s-d-e.html) to compare the Pb concentration of soil samples in which the major ectomycorrhizal fungal taxa were found.

Results

Rhizosphere soil characteristics and heavy metal concentrations in Masson pine leaves

The results of soil analyses are summarized in Table 1. Soils were slightly acidic to neutral at the three mining sites, HY1 (mean ± stand error, 6.4±0.2) and HY2 (6.9±0.3) in Huayuan and the tailing site TLT (6.4±0.6) in the Taolin area, while the soils of the control forest (TLC) adjacent to the Taolin tailing were acidic (pH 4.7±0.4). Soil EC values were within the nonsaline range in all study sites. The concentrations of macronutrients in HY1 and HY2 soils were within the normal range, but the concentrations of heavy metals in HY1, especially Pb, Zn, and Cd, were high. Tailing (TLT) soils contained slightly higher levels of Pb, Zn, and Cu, accompanied by very low concentrations of total N and phosphorus (P). Soils in the control forest (TLC) were characterized by the lowest concentrations of heavy metals and the highest amounts of N among the four study sites. The concentrations of Zn in HY1, HY2, and TLT, Pb in HY1, and Cd in HY1 and HY2 exceeded grade III levels of the China Environmental Quality Standard (Zn, 500 mg kg^{-1}; Pb, 500 mg kg^{-1}; Cd, 1 mg kg^{-1}). In addition, Cu in TLT exceeded grade II levels (Cu, 50 mg kg^{-1}; indicating the threshold of pollution) but did not reach the grade III level (Cu, 400 mg kg^{-1}). The amount of DTPA-extractable heavy metals was significantly correlated with the total amount in soil (R=0.986, P<0.0001 for Cd; R=0.776, P<0.0001 for Cu; R=0.905, P<0.0001 for Zn; R=0.659, P<0.0001 for Pb) and tended to be high in TLT and HY1.

In Masson pine leaves, the highest concentrations of Zn, Pb, and Cu were observed at TLT, reaching up to 652.1, 55.9, and 6.5 mg kg^{-1}, respectively (Table 2). With the exception of Cu, the lowest concentrations of heavy metals in leaves were recorded in the control forest. The correlation between soil heavy metal concentrations and their concentration in leaves was not significant for any heavy metal examined (Pb: R^2=0.06, P=0.1; Zn: R^2=0.003, P=0.8; Cu: R^2=0.04, P=0.2; Cd: R^2=0.02, P=0.4).

Mycorrhizal colonization

The ectomycorrhizal colonization rate of Masson pine root tips in TLT (25.9±21.0 %) was significantly lower than that in TLC (61.4±29.0 %), HY1 (53.4±19.8 %), and HY2 (61.1±15.6 %) with P<0.05. The values of the latter three sites were not significantly different. At the TLT site, 11 of 40 root samples had no ectomycorrhizal root tips.

Ectomycorrhizal fungal communities

In total, 1,610, 1,065, 999, and 913 of ectomycorrhizal tips were morphotyped in HY1, HY2, TLT, and TLC samples, respectively. By selecting one-tenth of the root tips from each

Table 1 Rhizosphere soil characteristics at Huayuan and Taolin mining areas in Hunan Province, China

Soil properties	Site				GB15618-95 grade III	Soil background values in Hunan Province
	HY1 (n=10)	HY2 (n=10)	TLT (n=40)	TLC (n=24)		
pH	6.4±0.2 a	6.9±0.3 a	6.4±0.6 a	4.7±0.4 b		
Electrical conductivity (μS cm^{-1})	209.0±43.8	209.3±17.9	186±99.7	147.0±89.8		
Total N (mg kg^{-1})	500.0±171.0 a, b	587.0±162.3 a	114.0±79.2 c	646.7±236.0 a		
Total P (mg kg^{-1})	668.3±248.2 b	784.1±306.7 a	167.1±33.9 d	302.1±105.9 c		
Total K (mg kg^{-1})	11,430.0±3,015.0 b	13,649.5±840.5 a	8,216.3±3,505.4 c	10,590.3±1,673.2 b		
Total metals (mg kg^{-1})						
Pb	11,304.0±10,538.8 a	162.6±12.8 b	212.5±75.3 b	59.0±28.0 b	500	29.7
Zn	6,855.0±3,000.2 a	549.0±32.5 b	558.9±213.7 b	137.2±43.5 c	500	94.4
Cd	14.3±22.0 a	1.8±0.7 b	0.9±0.6 b, c	0.3±0.2 c	1	0.17
Cu	28.7±5.5 b	30.9±6.6 b	149.0±49.2 a	11.1±6.0 c	400	27.3
DTPA-extractable metals (mg kg^{-1})						
Pb	2,215.0±793.7 a	23.7±4.4 c	68.1±26.6 b	23.9±21.7 c		
Zn	225.4±113.1 a	15.1±33.1 b, c	26.6±13.9 b	13.2±10.4 b, c		
Cd	3.4±4.3 a	0.6±0.2 b	0.2±0.1 b	0.2±0.2 b		
Cu	1.2±0.5 b	0.8±0.6 b, c	9.1±3.7 a	1.4±0.7 b		

The values are means ± standard errors. Values within each row followed by the same letter are not significantly different according to Tukey's test at the 5 % significance level

GB15618-1995 grade III indicates the toxic levels of heavy metals for crops and forest tree growth, *HY1* Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

Table 2 Metal concentrations (mg kg^{-1}) in needles of *P. massoniana* growing in the Huayuan and Taolin mining areas in Hunan Province, China

Metal elements	Site			
	HY1 (n=10)	HY2 (n=10)	TLT (n=40)	TLC (n=24)
Pb	19.8±15.8 a, b	9.2±12.5 b	26.5±13.5 a	7.8±6.0 b
Zn	156.0±40.0 b	139.2±65.3 b	389.5±126.1 a	102.1±44.1 b
Cd	1.6±0.7 b	2.9±1.5 a	1.7±0.6 b	0.7±0.3 c
Cu	3.4±4.6	4.2±3.0	46.2±73.3	25.9±32.5

The values are means ± standard errors. Values within each row followed by the same letter are not significantly different according to Tukey`s test at the 5 % significance level

HY1 Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

morphotype, 126, 188, 265, and 291 samples of ectomycorrhizal root tips from sites HY1, HY2, TLT, and TLC, respectively, were used in RFLP analysis. All of the unique RFLP patterns in each root sample were subjected to sequencing, totaling 212 (HY1: 38, HY2: 43, TLT: 66, TLC: 65). After pairwise alignment, we obtained 88 unique sequences, which were deposited in DDBJ with accession numbers AB634253–AB634284 and AB636414–AB636468. Sequences of non-ectomycorrhizal taxa were excluded from the following analyses. By applying 97 % ITS sequence similarity for species delimitation, 47 molecular OTUs were identified and a representative sequence from each OTU were listed in Table 3. Of the 47 OTUs, 41 belonged to Basidiomycetes and six to Ascomycetes. Thelephoraceae was the most species-rich family, represented by 14 OTUs, followed by Russulaceae with 10 OTUs. All other fungal families had less than three OTUs.

In total, 17, 13, 8, and 23 OTUs were found at HY1, HY2, TLT, and TLC, respectively. OTU richness accumulation was significantly lower in TLT than the other three sites (Fig. 2). The accumulation curves for HY1 and HY2 were not significantly different (P>0.05); both were located in the middle between the curves for TLT and TLC. Sørensen similarity indices between three root samples from the same pine tree were 0.58±0.39 in HY1 and 0.53±0.33 in HY2, both of which were significantly higher than the values between root samples from different trees at each site (HY1: 0.14±0.20, P=0.0004; HY2: 0.26±0.22, P=0.0008). This result indicates that three root samples from the same tree could not be treated as independent samples. Thus, to avoid violating sample independence, we pooled the three root samples in the following analyses. OTU accumulations for HY1 and HY2 sites after the pooling were not significantly different from the curve for the control forest (P>0.05). Species richness estimators (i.e., Chao2, Jackknife1, and Jackknife2) and diversity indices (i.e., Shannon's H' and Simpson's $1/D$) were lowest in TLT among the four sites. Estimators and diversity indices for HY1 and HY2 were not significantly different from the values for the control forest (Table 4).

Of the 47 OTUs, 35 occurred at only one site. Only one OTU, Atheliaceae sp., occurred at all of the study sites. Three OTUs (Ascomycota sp., *Phialocephala fortinii*, and *Suillus granulatus*) were shared between the Huayuan and Taolin areas. Two sites (HY1 and HY2), located a close distance apart in the Huayuan area, shared seven OTUs, resulting in very similar communities (0.47 in the Sørensen index; Table 5), although soil heavy metal concentrations were significantly different between the two sites (Table 1).

Rank–abundance relationships revealed that the ectomycorrhizal fungal community in TLT was dominated by a few dominant species (Fig. 3); specifically, *Rhizopogon buenoi* (34.1 % in relative abundance), *Tomentella ellisii* (32.4 %), *Inocybe curvipes* (17.1 %), and *S. granulatus* (9.6 %) accounted for 93.2 % of the total relative abundance (Table 3). They were found in 28 of 29 ectomycorrhizal root samples. In contrast, the ectomycorrhizal fungal community in TLC was represented by many less dominant species, in which six OTUs of Thelephoraceae (30.3 % relative abundance in total) and six OTUs of Russulaceae (22.3 %) were relatively abundant at the family level and Atheliaceae sp. (16.0 %) was most abundant at the species level. The rank–abundance relationships for HY1 and HY2 were almost similar to each other and also to the curve for TLT, represented by the higher dominance of a few ectomycorrhizal fungal species.

After grouping similar soil samples (i.e., similar level of soil N, P, K, Pb, Zn, Cd, or Cu concentration) within each of TLT and TLC, we analyzed the correlation between individual soil factors and ectomycorrhizal fungal diversity among the subgroups, HY1 and HY2. This analysis revealed that the Chao2 richness estimator and Simpson diversity index were significantly correlated with soil N of the site/subgroups (R=0.874, P=0.005 for Chao2 vs. N; R=0.729, P=0.04 for Simpson vs. N, Fig. 4a, b). Although the correlation between the Chao2 richness estimator or Simpson value and soil Cu amount were also significant (R=–0.771, P=0.025 for Chao2 vs. Cu; Fig. 4c), ectomycorrhizal fungal diversity or richness was not significantly correlated with other soil factors such as Pb, Zn, or Cd.

The occurrence of each of the major fungal taxa revealed some biased occurrence to a certain soil condition. For example, the occurrence of *R. buenoi* and *I. curvipes* was restricted to lower soil N conditions and never appeared in soil containing >229 mg N kg^{-1} soil (Fig. 4a). ANOVA revealed that the N concentrations of soil samples that harbored *R. buenoi*, *I. curvipes*, *T. ellisii*, and *S. granulatus* were significantly lower than those with Atheliaceae sp., *T. terreum*, Thelephoraceae (excluding *T. ellisii*), and Russulaceae (Fig. 5a). Conversely, the Cu content of soil samples associated with those four fungi was significantly higher than those for Atheliaceae sp., *T. terreum*, Thelephoraceae

Table 3 Identification of ectomycorrhizal fungal OTUs associated with *P. massoniana* growing at the study sites in the Huayuan and Taolin mining areas in Hunan Province, China

Code	OTUs	Accession number	Closest Blast match accession in Genbank/EMBJ/DDBJ/Unite[a]	Query/aligned portion length (bp) (similarity, %)[b]	Relative abundance (%)/relative frequency (%)[c]			
					HY1 (n=10)[d]	HY2 (n=10)	TLT (n=40)	TLC (n=24)
1	Ascomycota sp.	AB636442	EU046087 uncultured ascomycete	485/484 (99)	0.75/10.00			4.50/8.33
2	Atheliaceae sp.	AB634282	FJ876186 Atheliaceae sp.	594/585 (97)	0.75/10.00	3.69/10.00	3.58/7.50	16.04/8.33
3	*Cenococcum geophilum*	AB636435	EU346870 *Cenococcum geophilum*	483/466 (97)			1.88/12.50	5.10/16.67
4	*Clavulina* sp.	AB636463	FN669173 *Clavulina* sp.	587/587 (92)	35.82/50.00			
5	*Coltriciella* sp.	AB636461	JF273521 *Coltriciella* sp.	800/786 (97)				7.65/29.17
6	*Helotiales* sp.	AB636433	AB598109 *Helotiales* sp.	550/525 (99)				0.60/8.33
7	*Inocybe curvipes*	AB636458	AM882813 *Inocybe curvipes*	649/628 (98)			17.14/20.00	
8	*Inocybe* sp.	AB636457	HQ604204 *Inocybe suaveolens*	570/542 (93)				0.45/4.17
9	*Lactarius akahatsu*	AB636431	EF141544 *Lactarius akahatsu*	698/696 (99)	1.96/10.00	0.40/10.00		
10	*Lactarius quieticolor*	AB636429	UDB000880 *Lactarius quieticolor*	712/702 (99)		5.54/40.00		
11	*Lactarius* sp.	AB636424	HM189806 *Lactarius subdulcis*	653/625 (93)				10.04/16.67
12	*Phialocephala* sp.	AB636440	HM190136 *Phialocephala fortinii*	497/474 (95)			0.75/2.50	
13	*Phialocephala fortinii*	AB636438	AY394921 *Phialocephala fortinii*	523/520 (99)	0.84/10.00			1.65/4.17
14	*Pseudotomentella* sp.	AB636446	AJ889968 *Pseudotomentella tristis*	713/557 (94)		0.79/10.00		
15	*Rhizopogon buenoi*	AB636450	AJ297263 *Rhizopogon buenoi*	509/447 (98)			34.09/27.50	
16	*Rhizopogon* sp.	AB636449	AF071440 *Rhizopogon occidentalis*	688/538 (91)				1.35/4.17
17	*Russula densifolia*	AB636427	AB291758 *Russula densifolia*	586/560 (98)				2.25/4.17
18	*Russula nauseosa*	AB636420	UDB001716 *Russula nauseosa*	672/579 (97)		0.92/10.00		
19	*Russula* sp.1	AB636419	UDB001634 *Russula sanguinea*	677/636 (95)	0.09/10.00	0.79/10.00		
20	*Russula* sp.2	AB636423	FJ876170 *Russula* sp.	611/553 (90)				1.95/8.33
21	*Russula* sp.3	AB636425	UDB000343 *Russula amoenolens*	657/617 (95)				4.95/4.17
22	*Russula* sp.4	Too short						0.30/4.17
23	*Russula amoenolens*	AB636426	UDB000343 *Russula amoenolens*	586/574 (97)				2.85/4.17
24	*Suillus granulatus*	AB636448	UDB000666 *Suillus granulatus*	688/648 (97)		0.40/10.00	9.60/22.50	
25	*Thelephora* sp.1	AB634264	EU819444 *Thelephora terrestris*	721/598 (90)				1.05/4.17
26	*Thelephora terrestris*	AB634267	HM189964 *Thelephora terrestris*	660/653 (99)	0.93/10.00			
27	*Tomentella ellisii*	AB634268	HQ406823 *Tomentella ellisii*	568/564 (99)			32.39/30.00	1.2/8.33
28	*Tomentella* sp.1	AB634253	AJ534916 *Tomentella* sp. N44	664/618 (96)	0.37/10.00			
29	*Tomentella* sp.2	AB634255	AB211278 *Tomentella* sp. Nara 2-01	664/628 (99)	7.65/10.00			
30	*Tomentella* sp.3	AB634256	AJ534916 *Tomentella* sp. N44	664/602 (93)	0.28/10.00			
31	*Tomentella* sp.4	AB634257	UDB003294 *Tomentella stuposa*	663/615 (96)	0.75/20.00			
32	*Tomentella* sp.5	AB634260	FM244909 *Tomentella* sp. TU 103663	564/517 (92)			0.56/2.50	
33	*Tomentella* sp.6	AB634262	JF273546 *Tomentella* sp. EMF45	560/539 (99)				19.04/16.67
34	*Tomentella* sp.7	AB634265	GQ900537 *Tomentella* sp. ECM5	621/549 (96)				1.65/8.33

Table 3 (continued)

Code	OTUs	Accession number	Closest Blast match accession in Genbank/EMBJ/DDBJ/Unite[a]	Query/aligned portion length (bp) (similarity, %)[b]	Relative abundance (%)/relative frequency (%)[c]			
					HY1 (n=10)[d]	HY2 (n=10)	TLT (n=40)	TLC (n=24)
35	*Tomentella* sp.8	AB634266	UDB000033 *Tomentella sublilacina*	600/525 (93)				1.05/8.33
36	*Tomentella* sp.9	AB634270	UDB003347 *Tomentella* sp.	664/567 (94)	6.9/20.00	0.66/20.00		
37	*Tomentella stuposa*	AB634259	UDB002429 *Tomentella stuposa*	625/601 (97)				6.3/25.00
38	*Tricholoma terreum*	AB636451	AF377212 *Tricholoma terreum*	695/690 (99)	1.87/10.00	42.48/60.00		
39	*Trichophaea* sp.	AB636444	FM206477 *Trichophaea hybrida*	595/546 (91)	25.09/50.00			
40	Unknown 1	AB634276	AB587734 uncultured ectomycorrhizal fungus	651/632 (97)		15.44/30.00		
41	Unknown 2	AB634277	AJ410861 ectomycorrhizal isolate	612/548 (98)	0.56/10.00	24.14/40.00		
42	Unknown 3	AB634284	FJ554011 uncultured Agaricomycetidae	601/583 (94)		0.66/10.00		
43	Unknown 4	AB636415	FJ553461 uncultured Agaricomycetidae	593/572 (96)	14.55/20.00	4.09/30.00		
44	Unknown 5	AB636455	FN550898 *Inocybe vulpinella*	663/590 (84)	0.84/10.00			
45	Unknown 6	AB636462	EU862207 *Clavulina* cf. *rugosa*	631/534 (84)				1.05/8.33
46	Unknown 7	Not deposited	FJ266725 uncultured Agaricales	477/472 (99)				8.55/4.17
47	Unknown 8	Not deposited	AB587742 uncultured ectomycorrhizal fungus	638/567 (98)				0.45/4.17

HY1 Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

[a] Closest matched Blast results with informative species and genera were used

[b] Similarity values were computed from the percent match between the portion of the query aligned and its reference sequence

[c] Relative frequency refers to the percentage of trees colonized by OTUs within one site

[d] *n* represents the number of trees in each site

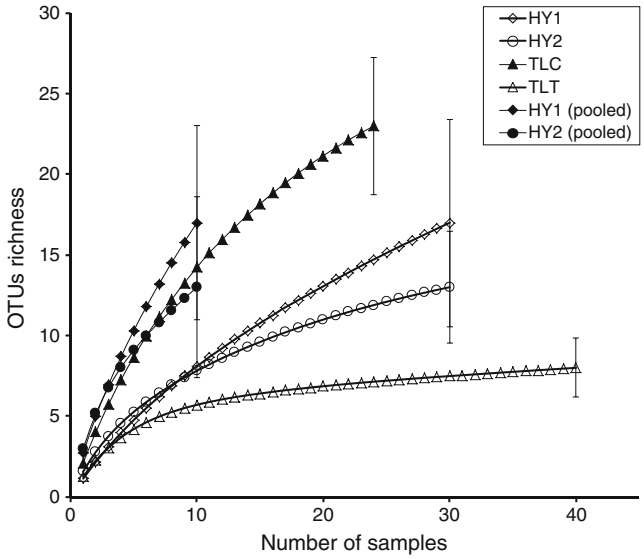

Fig. 2 Accumulation curves for ectomycorrhizal fungal OTUs in the Huayuan and Taolin mining areas. The observed number of OTUs is given as Mao Tau estimates (Sobs) with 95 % confidence intervals. The mean cumulative number of ectomycorrhizal fungal OTUs in each site is plotted after 50 randomizations. *HY1* and *HY2* Sobs were calculated based on 30 samples without combination at Huayuan sites 1 and 2, *TLT* Taolin tailing, *TLC* Taolin control site, *HY1 (pooled)* and *HY2 (pooled)* three samples were pooled for each tree

(excluding *T. ellisii*), and Russulaceae (Fig. 5c). *Cenococcum geophilum* did not differ significantly with the other major taxa except for *R. buenoi* and *I. curvipes*. We found no clear patterns of biased occurrence in other soil factors for those major taxa (Fig. 5b, d, e).

Discussion

Most in vitro studies concerning the effects of heavy metals on EMF symbioses have provided strong evidence of reduced ectomycorrhizal fungal infection in higher heavy metal concentrations (Jones and Hutchinson 1986; Bell et al. 1988; Dixon 1988; Dixon and Buschena 1988; Chappelka et al. 1991; Hartley et al. 1999; Hartley-Whitaker et al. 2000).

Table 5 The number of shared ectomycorrhizal fungi taxa (upper triangle) and Sørensen index (lower triangle) among the study sites in the Huayuan and Taolin mining areas in Hunan Province, China

	HY1	HY2	TLT	TLC
HY1		7	1	3
HY2	0.47		2	1
TLT	0.08	0.19		3
TLC	0.15	0.06	0.19	

HY1 Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

However, these in vitro experiments only used a limited number of ectomycorrhizal fungal species and young seedlings for short experimental periods. Thus, it is inappropriate to extrapolate the results of these in vitro studies to natural settings, which are characterized by high species/genetic diversity of EMF associated mainly with mature trees.

In the field, conflicting results have been found for the effect of heavy metals on EMF associations. Around a former lead/zinc smelter, *Populus tremula* was intensively colonized by EMF (up to 95 %) and was associated with highly diverse (H'=2) ectomycorrhizal fungal communities (Krpata et al. 2008). In a former uranium mining area, birch (*Betula pendula*) growing on the waste heap covered with an organic soil layer was also well colonized (61.5 %) by diverse ectomycorrhizal fungal communities comparable to non-polluted sites, while the colonization rate (27.7 %) and ectomycorrhizal fungal diversity at the bare heap site was significantly reduced (Staudenrausch et al. 2005). Naturally established *Salix caprea* on a former ore with high concentrations of Pb and Cu were poorly colonized (3 to 36 %) by EMF (Hrynkiewicz et al. 2008). No significant changes in diversity and richness of the ectomycorrhizal fungal communities were observed in a shotgun shooting range heavily contaminated with Pb up to 18,780 mg kg^{-1} (Hui et al. 2011). More field research in heavy metal-contaminated areas is required to determine the causes of these conflicting results.

In our study, two forest patches in Huayuan minelands were quite different in Pb, Zn, and Cd concentrations. Pb

Table 4 Richness and diversity indices of ectomycorrhizal fungal OTUs in the Huayuan and Taolin mining areas in Hunan Province, China

Sites	Number of trees	Observed EMF richness	Chao2 ± SD	Jackknife1 ± SD	Jackknife2	Shannon's H'	Simpson's $1/D$
HY1	10	17	41.0±22.7 a	27.8±2.6 a	35.3 a	1.8 a, b	4.5 a, b, c
HY2	10	13	37.5±16.6 a, b	19.3±1.92 a, b	24.2 a, b	1.7 a, b	3.7 b, c
TLT	40	8	9.0±8.1 b	10.0±1.4 b	11.9 b	1.5 a, b	3.8 c
TLC	24	23	29.3±24.5 a	32.6±2.7 a	34.7 a	2.6 a	10.2 a

Different letters refer to significant differences according to Tukey's test at $P<0.05$

HY1 Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

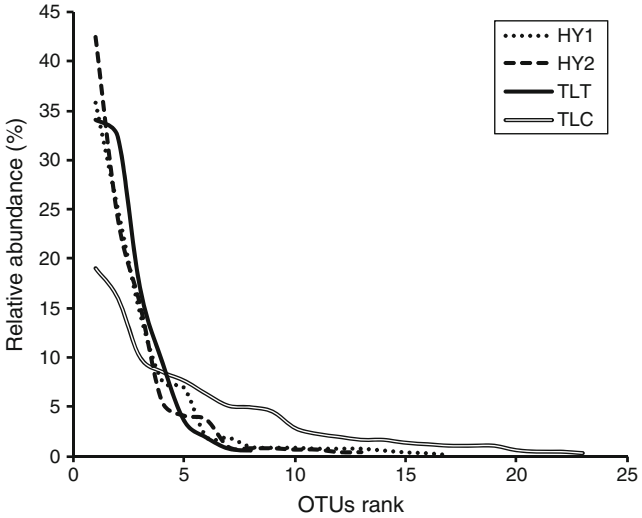

Fig. 3 Rank–abundance curves for ectomycorrhizal fungal OTUs in the Huayuan mineland and in Huayuan and Taolin mining areas in Hunan Province, China. *HY1* and *HY2* Huayuan site 1 and site 2, *TLT* Taolin tailing, *TLC* Taolin control site

was about 70 times higher, Zn 12 times higher, and Cd eight times higher in HY1 soil compared to HY2 soil. However, ectomycorrhizal colonization rates were not significantly different between the forest patches (53.4±19.8 % in HY1, 61.1±15.6 % in HY2). These values were also similar to the colonization level in the non-polluted control forest TLC (61.4±29.0 %). In addition, ectomycorrhizal fungal communities in HY1 and HY2 were similar in species accumulation curves, rank–abundance patterns, diversity indices, and richness estimators. Moreover, the similarity of ectomycorrhizal fungal communities between HY1 and HY2 was higher than any of the other combinations of the four sites (Sørensen index=0.47). These results clearly indicate that Pb, Zn, and Cd amounts are not necessarily major determinants in structuring these fungal communities.

At the tailing site, however, ectomycorrhizal colonization was very poor (25.9±21.0 %) and ectomycorrhizal fungal richness was apparently lower than that in HY1, HY2, and the control forest TLC, as shown in the species accumulation curve (Fig. 2). Although the tailing soil contained higher amounts of Pb, Zn, and Cd than the non-polluted forest, the heavy metal concentrations were significantly lower than at HY1 (Table 1), where the species accumulation curve was significantly higher than at TLT. Thus, heavy metals may not be responsible for the reduced ectomycorrhizal colonization and ectomycorrhizal fungal richness at TLT. On the other hand, the immature tailing soil was composed of heavily processed residues, lack of macronutrients, and contained significantly higher Cu (Table 1) and characterized by sandy texture, low organic matter, and drought (Guo et al. 2007), and

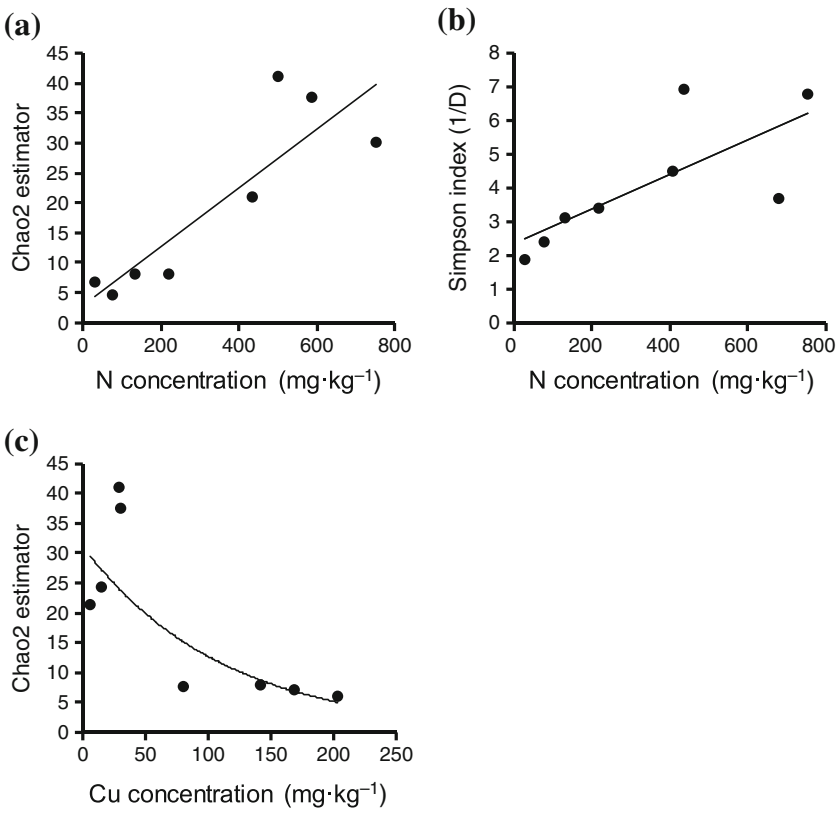

Fig. 4 **a** Chao2 richness estimator and **b** Simpson diversity index (1/*D*) in correlation with N concentration (R=0.874, P=0.005 for Chao2 vs. N; R=0.729, P=0.04 for Simpson index vs. N), and **c** Chao2 richness estimator of ectomycorrhizal fungal species negatively correlated with Cu concentration in rhizosphere soil (R=–0.771, P=0.025 for Chao2 vs. Cu). The dataset included HY1, HY2, two subsample groups from TLC, and four groups from TLT. Twelve and ten soil subsamples from TLC and TLT, respectively, were combined according to the level of each soil factor

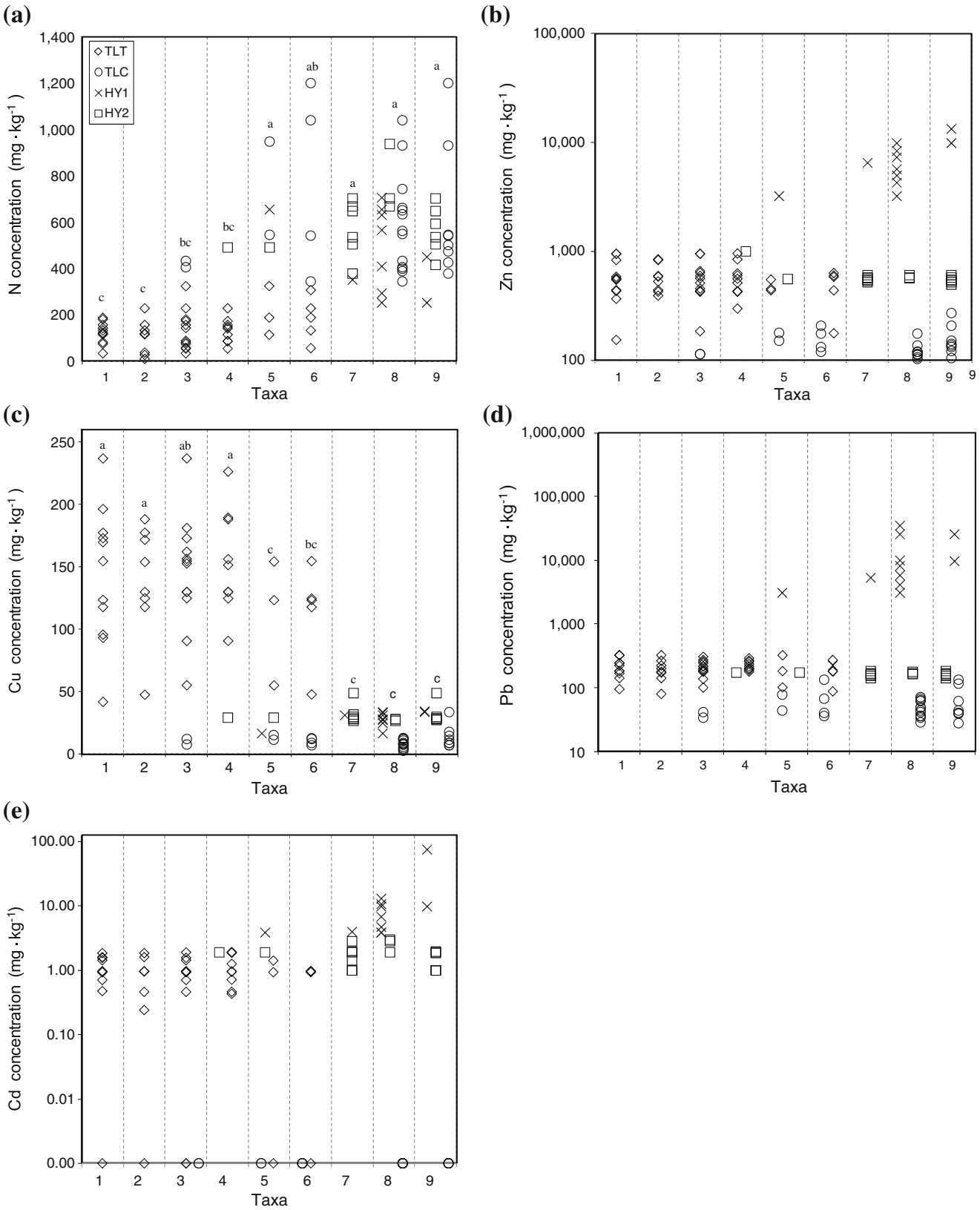

Fig. 5 The occurrence of major ectomycorrhizal taxa in different concentrations of N (**a**), Zn (**b**), Cu (**c**), Pb (**d**), and Cd (**e**). Taxa *1*=*R. buenoi*, *2*=*I. curvipes*, *3*=*T. ellisii*, *4*=*S. granulatus*, *5*=Atheliaceae, *6*=*C. geophilum*, *7*=*Tricholoma terreum*, *8*=Thelephoraceae (excluding *T. ellisii*), *9*=Russulaceae. Different *letters* refer to significant differences according to Tukey's test at $P<0.05$. *HY1* Huayuan site 1, *HY2* Huayuan site 2, *TLT* Taolin tailing, *TLC* Taolin control site

possibly by insufficient EMF propagules. These soil properties would adversely affect ectomycorrhizal fungal colonization and may account for the poor ectomycorrhizal colonization and low EMF richness in the tailings.

Besides, the pine trees in young stage (7-year-old) might be also responsible for the low richness and diversity of EMF in the tailing site compared with the 15- or 30-year-old forest sites. In a rehabilitated bauxite mine site, the numbers of EMF species associated with 7-year-old *Eucalyptus* stands were less than half of those associated with native forest (Gardner and Malajczuk 1988). Low ectomycorrhizal fungal species richness was also observed in 4-year-old jarrah forest in the same mine sites in a recent study by Glen et al. (2008) and in a 5-year-old red oak (*Quercus rubra*) plot in disturbed sites (Gebhardt et al. 2007). Glen et al. (2008) also revealed a clear trend of increasing ectomycorrhizal fungal species richness with increasing time since rehabilitation and found no significant difference in species richness in the rehabilitated mine sites in 12- and 16-year-old classes compared with their unmined reference sites in similar age. Thus, the character of an ectomycorrhizal fungal community in TLT is probably indicative of the early stage of development of the rehabilitated sites. Meanwhile, some other confounding factors (e.g., geographical location, land history) may also account for the observed patterns to some extent.

The ectomycorrhizal fungal community of the non-polluted TLC forest was dominated by Thelephoraceae and Russulaceae, which were represented by six OTUs each and accounted for 30.3 and 22.3 % of the relative abundance, respectively. The dominance of these two families has been frequently reported from many forest ecosystems including boreal Pinaceae forests (Horton and Bruns 2001). Ectomycorrhizal fungal communities in HY1 and HY2 also followed this general pattern and harbored six OTUs of Thelephoraceae (8.8 % in relative abundance) and four OTUs of Russulaceae (4.9 %) collectively. Thus, as in ectomycorrhizal colonization rates and EMF richness, the occurrence of Thelephoraceae and Russulaceae was not strictly affected by higher amounts of Pb, Zn, and Cd (Fig. 5b, d, e).

In contrast, the ectomycorrhizal fungal community of the tailings was devoid of Russulaceae and was less dominated by Thelephoraceae, except *T. ellisii*. Instead, *R. buenoi* (34.1 %), *T. ellisii* (32.4 %), *I. curvipes* (17.1 %), and *S. granulatus* (9.6 %) collectively occupied over 93 % of the ectomycorrhizal root tips. Many *Rhizopogon* spp. and suilloid fungi have been frequently found in association with young trees in early successional sites (Nara 2006; Ishida et al. 2008), even under heavy metal-contaminated conditions (Turnau et al. 1996; Vrålstad et al. 2002; Adriaensen et al. 2005; Johansson et al. 2005; Colpaert et al. 2011). Some *Inocybe* species also frequently occur in early successional sites and mine sites (Kalin and Stokes 1981; Malloch 1982; Cripps 1997, 2003; Nara et al. 2003; Krpata et al. 2008). *T. ellisii* appears after fire disturbance because of its heat-tolerant propagules (Buscardo et al. 2010). Therefore, the ectomycorrhizal fungal community in the tailing can be regarded as an early successional community. Immature soil conditions of the tailing sand, which contained less organic matter, may only allow pioneer EMF to colonize the site.

The importance of soil maturity irrespective of heavy metals in structuring ectomycorrhizal fungal communities in mining areas was also supported by the significant correlation between soil N and ectomycorrhizal fungal diversity/richness (Fig. 4a, b), while soil Pb, Zn, and Cd amounts were not correlated with ectomycorrhizal fungal richness or diversity. Further evidence was found in the results of our analyses based on individual soil samples and occurrence data of each major EMF taxon. Each of the pioneer EMF, which were dominant in the tailings, only appeared in soil samples with lower N (Fig. 5a), and their occurrence was not related to other soil factors (Fig. 5b–e). In contrast, major taxa in non-polluted forests and fragmented forest patches in Huayuan mineland [i.e., Russulaceae and Thelephoraceae (except *T. ellisii*)] were only found in higher N soil samples, while their occurrence was not clearly related to Pb, Zn, and Cd. Therefore, we may be able to conclude that soil immaturity may be a more prevalent determinant than heavy metals in structuring ectomycorrhizal fungal communities.

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