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Diversity of free-living nitrogen-fixing microorganisms in wastelands of copper mine tailings during the process of natural ecological restoration

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Abstract

Biological nitrogen fixing is an important source of nitrogen input in the natural ecological restoration of mine wastelands. The diversity of *nifH* genes in tailings samples under different plant communities in Yangshanchong and Tongguanshan wastelands in Tongling, was analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) approach. The nitrogen-fixing microorganism community in the upper layer of tailings of Tongguanshan wasteland discarded in 1980 showed higher Shannon-Wiener diversity index than that in Yangshanchong wasteland discarded in 1991. The diversity of *nifH* genes in Yangshanchong wasteland of copper mine tailings did not display a consistent successional tendency with development of plant communities during the process of natural ecological restoration. Phylogenetic analysis of 25 sequences of *nifH* gene fragments retrieved from the DGGE gels indicated that there were mainly two taxa of free-living nitrogen-fixing microorganisms, Proteobacteria and Cyanobacteria living in the wastelands investigated, most of which were unique and uncultured. Canonical correspondence analysis (CCA) based on the relationship between band patterns of DGGE profile and physico-chemical properties of tailings samples showed that the diversity of *nifH* genes in different tailing samples was mainly affected by loss of ignition, water content, pH and available Zn contents of wastelands. The dominant plant species and development period of plant communities by ameliorating pH, reducing the toxicity of heavy metals, increasing organic matter and water content affected the diversity and structure of the free-living nitrogen-fixing microorganisms in wastelands of copper mine tailings.

Key words: *nifH*; nitrogen fixation; PCR-DGGE; natural ecological restoration; copper mine tailings

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Introduction

The availability of nitrogen often limits plant productivity and affects plant community and ecosystem processes at all scales (Tan et al., 2003). For natural ecosystems, nitrogen necessitated by plant growth mainly relies on biological nitrogen fixation (Poly et al., 2001b; Beneduzi et al., 2008) and dry and wet precipitation of atmospheric nitrogen. The major types of biological nitrogen fixation (BNF) include symbiotic and free-living nitrogen fixation (Beneduzi et al., 2008). BNF makes the reduction of atmospheric nitrogen into bioavailable ammonium, which can only be carried out by *Archaea* and bacteria, using molybdenum- or alternative nitrogenases encoded by *nif*, *anf* or *vnf* genes (Joerger et al., 1991; Tan et al., 2003; Coelho et al., 2009).

The *nifH* gene, encoding the iron protein subunit of nitrogenase, is highly conserved among diazotrophs. However, as a functional gene, *nifH* has the advantage that it provides evidence for potential nitrogen fixation (Coelho et al., 2009). In recent years, the *nifH* gene in environmental samples has been widely studied using molecular methods, including clone library analyses (Zhang et al., 2006; Coelho et al., 2008; Chowdhury et al., 2009), PCR-RFLP (Widmer et al., 1999; Soares et al., 2006; Beneduzi et al., 2008), PCR-T-RFLP (Deslippe et al., 2005), PCR-ARDRA (Aquilanti et al., 2004), PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) (Diallo et al., 2004; Wartiainen et al., 2008; Coelho et al., 2009), etc. These study results have provided a more complete picture of the diazotrophic community and identified that the *nifH* gene is present in various environmental samples (Tan et al., 2003; Coelho et al., 2009; Soares et al., 2006).

Compared to ecosystems such as the rhizobium-legums symbiosis, nitrogen fixation by free-living soil microorganisms is sometimes considered a minor source of nitrogen input in soil (Peoples and Craswell, 1992; Kahindi et al., 1997; Unkovich and Baldock, 2008). However, studies have indicated that free-living nitrogen fixation is the dominant source of fixed nitrogen in different soils (Lovell et al., 2000; Poly et al., 2001b; Chowdhury et

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al., 2009). The estimated input of nitrogen fixed by free-living nitrogen-fixing microorganisms can achieve 60 kg N/(ha·yr) in natural ecosystem (Cleveland et al., 1999). Free-living nitrogen-fixers, belonging to Proteobacteria, Cyanobacteria, Firmicutes and Archaea have been found in soil and rhizosphere and play an important role in the development of natural ecosystem by increasing nitrogen content of substrates, promoting plant growth and regulating species composition (Kahindi et al., 1997; Aquilanti et al., 2004; Deslippe et al., 2005; Soares et al., 2006). Many factors, such as physico-chemical properties of soil, plant species or genotype, soil management, etc., can affect diversity and composition of nitrogen-fixing bacteria community (Widmer et al., 1999; Héry et al., 2003; Tan et al., 2003; Diallo et al., 2004; Deslippe et al., 2005; Soares et al., 2006; Wartiainen et al., 2008; Chowdhury et al., 2009; Coelho et al., 2009), and free-living nitrogen-fixers are sensitive to heavy metal contamination (McGrath et al., 1995; Oliveira and Pampulha, 2006; Oliveira et al., 2009).

The mining tailings, powder wastes produced in the flocculate flotation of copper ore, are generally piled up in the tailings impoundment leading to the formation of wasteland of copper mine tailings. In order to reduce or prevent pollution of the heavy metals and powders from wastelands of copper mine tailings, and to beautify landscape of wastelands area, the wastelands are often restored by revegetation (Bradshaw, 1997; Néel et al., 2003). The ultimate goal of this kind of restoration is to establish the stable and productive ecosystems (Bradshaw, 1997). However, wastelands usually have very low nitrogen content, which limits plant colonization and growth (Bradshaw, 1997; Néel et al., 2003; Sun et al., 2004, 2005).

There are many wastelands of copper mine tailings in Tongling, China. Most of them were restored by natural way (Sun et al., 2004, 2005), and a distinct succession series of plant communities in wastelands of copper mine tailings was presented, i.e., bare wastelands (with algal crust in humid seasons, without vascular plants) → cryptogamic crusts (algal + moss crust, moss crust) → vascular communities (*Hippochaete ramosissimum* community, *Zoysia sinica* community and *Imperata cylindrica* var. *major* community, etc) (Sun et al., 2004). The previous studies indicated that dominant vascular plants naturally colonizing on these wastelands of copper mine tailings, including Gramineae, Compositae, Cyperaceae, Polygonaceae, etc., were non-legumes. Only few legumes with symbiotic nitrogen fixation, such as *Vicia sativa*, *Vicia hirsute*, *Crotalaria sessiliflora*, *Kummerowia striata*, *Indigofera pseudotinctoria*, *Medicago lupilina*, etc., were found naturally colonizing on wastelands of copper mine tailings (Tian et al., 2005). We presume that the nitrogen fixed by free-living microorganisms, living in wastelands or on surface of wastelands, is an important nitrogen source for supporting the growth of non-legume vascular plants.

So far, the information about free-living nitrogen-fixing microorganism in mine wastelands is absent. The major objectives of this study were: (1) to investigate the diversity of free-living nitrogen-fixers in wastelands of copper mine tailings during the process of natural ecological restoration using PCR-DGGE method, and (2) to explore the factors resulting in diversity changes of free-living nitrogen-fixers in wastelands. Research on the change of diversity of free-living nitrogen-fixers during the process of natural ecological restoration may provide further theoretical support for the fixation and transformation of nitrogen in wastelands, and the natural restoration of wastelands of copper mine tailings.

1 Material and methods

1.1 Study area

The sites studied, the Yangshanchong wasteland (30°54′N, 117°53′E) and Tongguanshan wasteland (30°54′N, 117°49′E) of copper mine tailings (6.08 km between two wastelands) were located in the Tongling Copper Mine area, Anhui Province, East China. The average annual rainfall in this area is 1346 mm, and the rainy season is from May to September. The average annual temperature is 16.2°C. The frost-free period is 237–258 days (Sun et al., 2004). Substrate of copper mine tailings is a sandy loam and easily eroded by water and wind.

The Yangshanchong and Tongguanshan wastelands of copper mine tailings were discarded in 1991 and 1980, respectively. The development of plant communities on wastelands displays a following series: bare wastelands of copper mine tailings → cryptogamic crusts → herb communities → wood plant communities → ..... (Sun et al., 2004). Presently, main plant community on the Tongguanshan wasteland is *I. cylindrica* community. Successional series of plant communities in Yangshanchong wasteland of copper mine tailings include bare wastelands, cryptogamic crusts and vascular communities (*H. ramosissimum* community, *Z. sinica* community and *I. Cylindrica* community). Other plant communities, such as *Cynodon dactylon* community, *Miscanthus floridulus* community, *Miscanthus sinensis* community and *Phragmites australis* community, are rare and distribute in little patches.

1.2 Collection of tailing samples

Substrate samples of bare wasteland and that under four plant communities growing on Yangshanchong mine wasteland were collected on the natural horizon in October 2008, including bare wastelands (0–2 cm (YBL1) and 2–10 cm (YBL2), distinguished by the color of substrate), *Bryum pallescens* community (A-horizon (YBA) and C-horizon (YBC)), *H. ramosissimum* community (A-horizon (YHA) and C-horizon (YHC)), *Z. sinica* community (A-horizon (YZA) and C-horizon (YZC)), *I. cylindrica* community (A-horizon (YIA) and C-horizon (YIC)). Substrate samples under *I. cylindrica* community (A-horizon (TIA), C1-horizon (TIC1) and C2-horizon (TIC2)) in Tongguanshan mine wasteland were also collected on the natural horizon, and C1 and C2 were distinguished by color difference of substrate.

Three replicates of each substrate sample were collected. Every replicate was mixed by six random sampling

points and 39 samples in total were taken for physico-chemical measurement. Thirteen samples for microbial diversity measuring were collected in this study and each sample was mixed by three replicates from each substrate. In each random sampling point substrate sample of 100 cm^2 (length × width = 10 cm × 10 cm) were taken using a thin shovel in each horizon. All samples were placed in plastic bags on ice in a cooler for transport to the laboratory. Samples used for microbial analysis were stored at –70°C.

1.3 Physico-chemical properties analysis

Fresh subsamples were dried at (105 ± 2)°C in an oven and water content (C_w, %) was calculated by Eq. (1):

$$C_w = \frac{(m_w - m_d) \times 100}{m_w} \quad (1)$$

where, m_w (g) is the mass of fresh substrate, m_d (g) is the mass of dry substrate. Electric conductivity (EC) was measured by electric conductivity method (tailings (mass): water (volume) = 1 g: 5 mL). Samples ovened at (105 ± 2)°C were ignited at (600 ± 5)°C for 6 hr in a muffle furnace and loss of ignition (LOI, %) was calculated by Eq. (2):

$$\mathrm{LOI} = \frac{(m_b - m_a) \times 100}{m_b} \quad (2)$$

where, m_b (g) is the mass before ignition, m_a (g) is the mass after ignition. Total nitrogen (TN) in samples was determined with Kjeldahl method (Nanjing Institute of Soil Science, 1978). Air drying samples were extracted by 0.5 mol/L $NaHCO_3$ for 30 min at room temperature and the available phosphorus (AP) in filtrate was determined with phosphomolybdate blue colorimetric method. Substrate pH and EC was measured in water (tailings (mass): water (volume) = 1 g: 5 mL) (Nanjing Institute of Soil Science, 1978).

The total metals (Pb, Zn, Cu, As, Cr and Cd) in wastelands samples were measured by Indictive coupled plasma-atomic emission spectroscopy (ICP-AES) (XSP Intrepid II, USA) after the samples were digested with a mixture of hydrofluoric (40.0%), nitric (68.0%), and perchloric acid (72.0%). The available metals (Pb, Zn, Cu, As, Cr and Cd) were determined by ICP-AES after extraction with 1 mol/L NH_4OAc for 2 hr at 25°C (tailings (mass): water (volume) = 1 g: 2 mL)

1.4 Diversity analysis of nitrogen fixing microorganisms

1.4.1 Nucleic acid extraction

Pretreatment was essential to the extraction of DNA from wastelands to reduce extracellular DNA and soluble organic contaminants. Before the extraction of DNA, the wastelands samples were washed with phosphate buffer (pH 8.0) and TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) (Shanghai Sangon, China) centrifuging for 10 min at 6000 ×*g*. Tris and EDTA of the buffer were useful to protect the DNA from nuclease activity produced (Picard et al., 1992). Total DNA was extracted by SDS-based DNA extraction method (Zhou et al., 1996). Wasteland samples of 1 g were mixed with 540 μL of DNA extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 100 mmol/L sodium EDTA (pH 8.0), 100 mmol/L sodium phosphate (pH 8.0), 1.5 mol/L NaCl, 1% CTAB) and 10 μL of proteinase K (10 mg/mL) (Shanghai Sangon, China) in sterilized centrifuge tubes by horizontal shaking at 225 r/min for 30 min at 37°C. Then, 60 μL of 20% SDS (Shanghai Sangon) was added and the samples were incubated in a 65°C water bath for 2 hr with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6000 ×*g* for 10 min and transferred into new sterilized centrifuge tubes. The soil pellets were extracted two more times by adding 180 μL of the extraction buffer and 20 μL of 20% SDS, vortexing for 10 sec, incubating at 65°C for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of phenol-chloroformisoamyl alcohol (25:24:1, *V*/*V*/*V*) (Shanghai Sangon), and the aqueous phase containing nucleic acids were separated by centrifugation at 6000 ×*g* for 10 min. Then, an equal volume of chloroformisoamyl alcohol (24:1, *V*/*V*) (Shanghai Sangon) was added. The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol (Shanghai Sangon) at room temperature for 1 hr. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 ×*g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in Tris-EDTA buffer (10 mmol/L Tris base and 1 mmol/L EDTA, pH 8.0), to give a final volume of 50 μL. Extracted DNA was visualized on 0.8% (*W*/*V*) agarose gel to assess its integrity. The diluent aliquot was then used directly for subsequent PCR amplification.

1.4.2 PCR amplification

Fragments of *nifH* gene were amplified using a nested PCR as described by Diallo et al. (2004). The first PCR was carried out with the forward primer FGPH19 and the reverse primer PolR (Table 1; Simonet et al., 1991; Poly et al., 2001a) generating a product of 429 bp. The second PCR was carried out with the forward primer PolF containing a GC clamp and the reverse primer AQER (Table 1; Poly et al., 2001a), yielding a product of 320 bp (including the GC clamp sequence). The 50 μL reaction mix contained: 2 μL of a 1:50 dilution of extracted DNA (50–100 ng), 5 μL of 10× PCR buffer (with 2 mmol/L $MgCl_2$), 0.4 mmol/L of each primer, 200 mmol/L of each dNTPs and 2.5 U of Taq DNA polymerase (Transgen, Beijing, China). For the second PCR, 1 μL of the first PCR product was used as a template. The amplification

Table 1 Oligonucleotide primers used in this study

Primers	Sequence (5′–3′)	Reference
FGPH19	TACGGCAARGGTGGNATHG	Simonet et al., 1991
PolR	ATSGCCATCATYTCRCCGGA	Poly et al., 2001a
AQER	GACGATGTAGATYTCCTG	Poly et al., 2001a
PolF-GC	CGCCCGCCGCGCCCCGCG CCCGGCCCGCCGCCCCCGCC CCTGCGAYCCSAARGCBGACTC	Poly et al., 2001a

conditions used were: 94°C for 3 min, 30 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min at 55°C for the first PCR and at 48°C for the second PCR, primer extension at 72°C for 2 min, with a final extension at 72°C for 5 min. Negative controls (without DNA) were run in all amplifications. PCR products were analyzed by 1.5% agarose gel electrophoresis followed by staining with SYBR Green I.

1.4.3 DGGE analysis

The PCR products (20–30 μL) were loaded onto 8% (*W*/*V*) polyacrylamide-bisacrylamide (37.5:1) (Amresco, USA) gels having denaturation gradients from 45% to 70% where 100% is 7 mol/L urea (Amresco, USA) and 40% (*V*/*V*) deionized fonnamide (Amresco, USA) in 1× TAE electrophoresis buffer. Electrophoresis was carried out at 100 V at 60°C for 17 hr (Bio-Rad, Richmond, USA). Gels were then stained with SYBR Green I in 1 × TAE for 20 min at room temperature and observed by Gel Image System. Bands of interest were excised, and DNA was eluted with 30 μL of Tris-EDTA buffer (pH 8.0) for 24 hr at 4°C. The resulting solution (5 μL) was used as target DNA for subsequent PCR amplification with primers PolF and AQER. The purity and correct running position of each fragment was confirmed by further DGGE analysis.

1.4.4 Cloning, sequencing and phylogenetic analysis

Purified PCR products from DGGE bands were cloned into PEASY T1 Cloning vector using a rapid ligation kit according to the instructions of the manufacturer (Transgen, Beijing, China). Ligation mixtures were transformed into Trans1-T1 Phage Resistant chemically competent *Escherichia coli* cells (Transgen, Beijing, China). The transformed cells were plated onto Luria-Bertani agar plates in the presence of ampicillin. After the incubating about 14 hr at 37°C, white clones were obtained, followed by the sequencing of single clones (Shanghai Sangon).

The nucleotide sequences were compared with those in the GenBank using BLAST on the NCBI's homepage. According to the similarities in the BLAST hits and alignments from all the sequences obtained, the aligned sequences were used to construct a phylogenetic tree using the neighbor-joining method with the MEGA package version 4.0. The topology of this distance tree was tested by resampling data with 5000 bootstraps to provide confidence estimates for tree topologies.

1.5 Statistical analysis

All data were subjected to statistical analysis of variance using SPSS which was used for evaluating differences between separate means. Digitized images of DGGE fingerprints were used to quantify diversity as enabled by Tanon Image System (Tanon 1600, China) which detects bands and quantities relative contents of DNA from cumulative pixel intensities within a given lane. Shannon-Wiener diversity indices of wasteland samples were calculated from the number of bands present and the relative intensities of bands in each lane. In order to determine the relationship between nitrogen-fixing microorganism diversity (based on the band patterns of DGGE profile) and abiotic environmental factors (including pH, EC, water content, LOI, TN, AP and the contents of heavy metals (Cu, Zn, Cd, As, Cr and Pb)), canonical correspondence analysis (CCA) was performed with CANOCO 4.5. A value of $p < 0.05$ was considered significant in the Monte Carlo test.

1.6 Nucleotide sequence accession numbers

The nucleotide sequences of 25 DGGE bands have been deposited in the Genbank Data Library under accession numbers GU362100 to GU362124.

2 Results

2.1 Physical and chemical properties of wastelands of copper mine tailings

The properties of 13 wastelands samples of copper mine tailings are given in Table 2. Tongguanshan wasteland showed lower pH (7.35–7.64) than Yangshanchong wasteland (8.53–8.82). The EC of Tongguanshan wasteland samples was higher than that of Yangshanchong wasteland, and increased with sampling depth. The LOI, TN and AP of samples from Tongguanshan wasteland (except TIC2) were higher than that of samples from Yangshanchong

Table 2 Physical and chemical properties of wastelands of copper mine tailings

Name of copper mine tailings	Sample	pH	EC (μS/cm)	Water content (%)	LOI (g/kg)	TN (mg/kg)	AP (mg/kg)
Tongguanshan wasteland	TIA	7.35 ± 1.10	140.67 ± 10.07	18.37 ± 0.49	59.31 ± 3.74	1366.14 ± 184.35	3.4 ± 0.77
	TIC1	7.19 ± 0.92	280.67 ± 44.12	14.90 ± 0.81	39.49 ± 6.18	350.07 ± 48.20	1.99 ± 0.41
	TIC2	7.64 ± 0.30	1809.67 ± 41.04	11.13 ± 4.77	21.43 ± 3.85	12.44 ± 2.98	ND
Yangshanchong wasteland	YBL1	8.73 ± 0.16	156.00 ± 47.84	5.20 ± 1.53	8.09 ± 2.86	40.89 ± 4.11	1.03 ± 0.42
	YBL2	8.82 ± 0.17	119.00 ± 28.62	6.03 ± 1.90	1.96 ± 0.12	22.49 ± 5.33	1.03 ± 0.45
	YBA	8.57 ± 0.09	133.33 ± 7.57	5.76 ± 0.63	7.33 ± 0.16	97.92 ± 26.73	2.08 ± 1.07
	YBC	8.77 ± 0.02	137.00 ± 13.08	4.94 ± 0.30	1.02 ± 0.68	27.43 ± 1.95	1.35 ± 0.94
	YIA	8.57 ± 0.19	115.67 ± 28.29	9.32 ± 3.27	14.81 ± 7.03	493.13 ± 123.91	2.41 ± 0.59
	YIC	8.74 ± 0.02	132.67 ± 13.43	14.64 ± 2.95	10.26 ± 2.25	88.50 ± 20.16	1.44 ± 1.31
	YHA	8.53 ± 0.10	183.33 ± 9.29	12.72 ± 2.13	15.63 ± 6.50	282.52 ± 77.17	1.65 ± 1.07
	YHC	8.56 ± 0.08	210.33 ± 31.56	14.10 ± 3.74	7.53 ± 0.84	59.29 ± 14.78	0.18 ± 0.11
	YZA	8.69 ± 0.09	124.67 ± 15.57	6.35 ± 1.74	9.81 ± 0.92	202.25 ± 44.17	1.61 ± 1.22
	YZC	8.75 ± 0.03	140.33 ± 20.03	8.59 ± 2.40	7.73 ± 0.69	70.99 ± 13.61	0.69 ± 0.34

ND: not detected.
Samples are referred to Section 1.2.
EC: electric conductivity; LOI: loss of ignition; TN: total nitrogen; AP: available phosphorus.

wasteland, decreasing with depth in all sampling sites. The samples under vascular plant communities (except *Z. sinica* community) presented higher water content than that under *B. pallescens* community (YBA and YBC) and bare lands (YBL1 and YBL2).

Total and available contents of heavy metals in wastelands samples are presented in Table 3. The contents of available Cd, As, Cr and Pb were less than 1 mg/kg in wastelands of copper mine tailings, which were not presented in Table 3. The contents of total Cu and Zn ranged in 740–1670 mg/kg and 317–741 mg/kg, respectively. The contents of available Cu and Zn ranged from 0.259 to 26.921 mg/kg and 0.341 to 14.984 mg/kg, respectively. Tongguanshan wasteland showed lower contents of available heavy metals than Yangshanchong wasteland in A-horizon. The bare wastelands (YBL1 and YBL2) displayed high contents of available heavy metals compared to samples under vascular plant communities in Yangshanchong wasteland.

2.2 Comparison of DGGE profiles and biodiversity indices

All DNA samples were successfully amplified using the nested PCR, and the resulting products were separated on DGGE gels. Examination of DNA band profile in the DGGE gel revealed that the communities consisted of different banding patterns with a total of 48 bands distributed in different electrophoretic positions (Fig. 1). From Fig. 1, it can be found that the samples of TIA and YBL1 had more band richness and higher band intensity than other samples, which indicated that there was a difference of *nifH* gene among wasteland samples.

Figure 2 presents the changes of Shannon-Wiener diversity indices for 13 wastelands samples. The Shannon diversity indices of TIA, TIC1 and TIC2 from Tongguanshan wasteland showed a decrease with increasing sampling depth. No consistent tendency in Shannon diversity indices was found among the samples from Yangshanchong wasteland. The samples taken under vascular plant communities and moss crust in Yangshanchong wasteland had lower Shannon diversity indices (2.069–2.879), compared to YBL1 and YBL2 without moss and vascular plants (3.245 and 2.928). The samples (TIA, TIC1 and TIC2) taken under *Imperata cylindrica* var. *major* community from Tongguanshan wasteland with a discarded period about 30 years showed higher Shannon

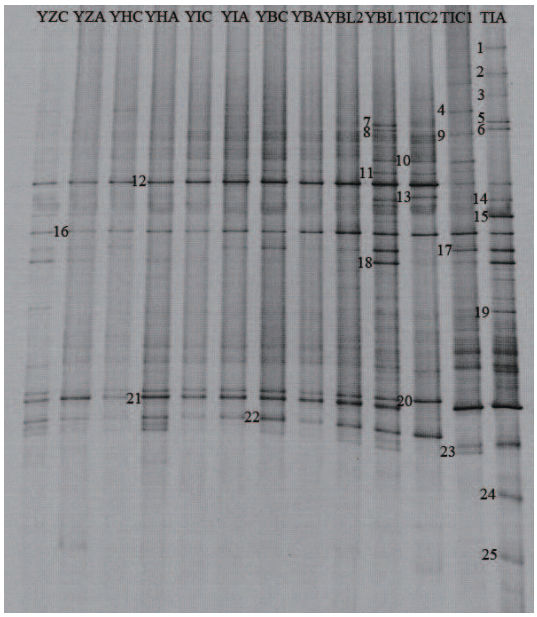

Fig. 1 DGGE profile of free-living nitrogen-fixing microorganisms in wasteland samples.

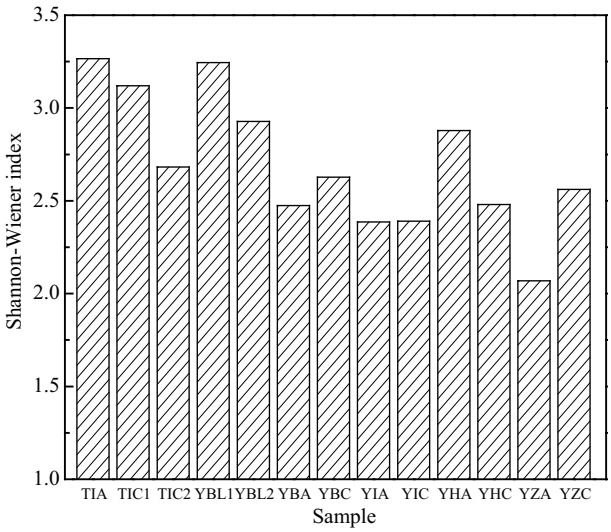

Fig. 2 Shannon-Wiener indices of nitrogen-fixing microorganisms in wasteland samples.

Table 3 Concentration of total and available heavy metals in wastelands samples

Sample	Total As (mg/kg)	Total Cd (mg/kg)	Total Cr (mg/kg)	Total Cu (g/kg)	Total Pb (mg/kg)	Total Zn (mg/kg)	Available Cu (mg/kg)	Available Zn (mg/kg)
TIA	532.88 ± 561.67	5.51 ± 2.61	33.32 ± 11.02	0.96 ± 0.26	335.84 ± 312.78	741.90 ± 506.68	0.259 ± 0.071	0.314 ± 0.100
TIC1	307.57 ± 66.49	3.23 ± 0.39	40.24 ± 5.00	1.05 ± 0.49	116.38 ± 94.24	381.84 ± 169.10	0.032 ± 0.027	0.456 ± 0.274
TIC2	159.32 ± 15.77	2.70 ± 0.41	27.01 ± 2.82	0.95 ± 0.27	34.48 ± 20.23	302.09 ± 47.71	3.783 ± 2.672	0.921 ± 0.239
YBL1	359.38 ± 79.84	2.58 ± 0.14	54.14 ± 16.27	1.67 ± 0.60	14.67 ± 7.97	435.09 ± 72.85	14.776 ± 4.269	12.983 ± 6.440
YBL2	283.60 ± 132.50	2.60 ± 0.65	47.63 ± 12.95	1.49 ± 0.24	9.45 ± 5.83	461.35 ± 163.47	12.438 ± 0.586	8.452 ± 3.776
YBA	309.10 ± 93.47	2.53 ± 0.25	46.10 ± 10.94	1.19 ± 0.37	22.54 ± 10.81	394.68 ± 64.18	13.316 ± 2.657	9.499 ± 2.720
YBC	281.63 ± 80.80	2.48 ± 0.37	47.00 ± 10.44	1.05 ± 0.27	30.20 ± 14.33	390.48 ± 83.40	26.921 ± 4.198	14.984 ± 4.446
YIA	227.67 ± 30.01	2.20 ± 0.21	49.08 ± 10.21	0.92 ± 0.29	17.45 ± 10.60	317.34 ± 72.42	3.830 ± 1.475	9.709 ± 5.873
YIC	296.25 ± 86.23	2.54 ± 0.34	52.05 ± 9.29	0.74 ± 0.16	35.14 ± 17.07	378.69 ± 77.88	10.462 ± 3.360	11.076 ± 4.746
YHA	285.76 ± 42.71	2.51 ± 0.30	50.19 ± 9.20	0.92 ± 0.18	21.88 ± 11.50	367.86 ± 37.27	6.236 ± 0.037	4.973 ± 1.079
YHC	264.74 ± 40.15	2.49 ± 0.32	52.07 ± 9.47	0.91 ± 0.42	39.92 ± 14.31	346.10 ± 39.74	7.297 ± 2.910	5.839 ± 1.123
YZA	363.57 ± 221.31	2.82 ± 0.35	30.98 ± 8.06	1.26 ± 0.42	36.17 ± 13.17	458.41 ± 74.17	2.465 ± 2.414	5.800 ± 5.050
YZC	281.91 ± 64.33	2.28 ± 0.18	36.26 ± 10.04	1.06 ± 0.27	36.29 ± 20.09	375.77 ± 52.52	8.733 ± 3.828	5.711 ± 3.519

diversity indices (3.266, 3.120 and 2.682, respectively) than the samples (YIA and YIC, 2.386 and 2.390, respectively) taken under the same type of plant community from Yangshanchong wasteland with a discarded period about 20 years. This result indicated that the diversity of *nifH* genes was affected by discarded period of mine tailings.

The canonical correspondence analysis (CCA) showed that diversity of *nifH* genes in different wasteland samples was significantly affected by LOI, water content, pH and the contents of Zn of samples ($p < 0.05$), but not by EC, TN and AP. The relationship between wasteland samples and correlative environmental variables on the basis of differences of nitrogen-fixing microorganism communities is shown in Fig. 3. It appears that the pH had a strong negative relationship with the first and second component axes, and the samples from Yangshanchong wasteland closely related to the environmental factor of pH. Samples from Tongguanshan wasteland (TIA, TIC1 and TIC2) with high LOI, water content, low pH and contents of available heavy metals displayed a significantly distinctive diversity of *nifH* genes compared with that from Yangshanchong wasteland (Fig. 3).

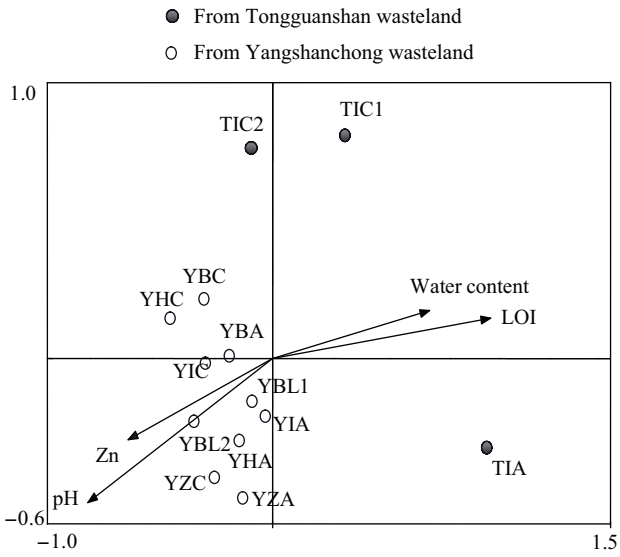

Fig. 3 Canonical correspondence analysis of the wasteland samples and correlative environmental variables on the basis of differences of nitrogen-fixing microorganism communities. Environmental factors are presented by arrows showing direction of increasing values.

2.3 Phylogenetic analysis

Twenty-five prominent bands were retrieved from the DGGE gel, reamplified and sequenced. The protein database using a translated nucleotide query revealed that the 25 sequences showed between 93% and 100% similarity indicating the function of nitrogen fixing (Table 4). A neighbour-joining phylogenetic tree based on *nifH* nucleotide sequences among the characteristic DGGE bands and their closest relatives was constructed. Two different clusters, Proteobacteria and Cyanobacteria, were distinguished in the phylogenetic tree (Fig. 4). Blast-N analyses revealed that 23 bands were closely related to *nifH* genes of uncultured bacteria or cyanobacteria. Sixteen of 25 sequences clustered in Proteobacteria, and remaining sequences grouped within the Cyanobacteria. Within the Protobacteria, fourteen bands were affiliated with uncultured bacteria, and only two *nifH* sequences were affiliated with known species (Table 4). Within the Cyanobacteria, nine bands were affiliated with uncultured Cyanobacteria (Fig. 4). These results showed that most of *nifH* gene sequences in this study may be unique and have not been described previously.

Table 4 Sequence similarities to the closest relatives of nitrogen-fixing microorganisms in Genbank

Band	Closest relative in Genbank (Blastn) (Accession no.)	Identity/nucleic acid (%)	Identity/amino acid (%)	Reference
1	Uncultured bacterium (DQ142865)	88	96	Jasrotia and Ogram, unpublished
2	Uncultured bacterium (DQ142865)	88	96	Jasrotia and Ogram, unpublished
3	Uncultured bacterium (DQ520379)	88	97	Izquierdo et al., unpublished
4	Uncultured soil bacterium (FJ008170)	95	99	Hsu and Buckley, 2009
5	Filamentous thermophilic cyanobacterium (DQ471425)	87	98	Ionescu et al., unpublished
6	Uncultured bacterium (DQ142865)	88	98	Jasrotia and Ogram, unpublished
7	Uncultured bacterium (DQ142865)	89	98	Jasrotia and Ogram, unpublished
8	Uncultured bacterium (EU047959)	95	100	Coelho et al., 2008
9	Uncultured bacterium (EU047975)	96	100	Coelho et al., 2008
10	Uncultured bacterium (DQ142865)	89	98	Jasrotia and Ogram, unpublished
11	Uncultured bacterium (AB471118)	96	99	Sato et al., 2009
12	Uncultured bacterium (EF988491)	92	96	Duc et al., 2009
13	Uncultured bacterium (EF988491)	88	95	Duc et al., 2009
14	Uncultured bacterium (AJ716359)	99	99	Héry et al., 2005
15	Uncultured bacterium (DQ520379)	88	97	Izquierdo et al., unpublished
16	Uncultured bacterium (DQ520379)	88	98	Izquierdo et al., unpublished
17	Uncultured bacterium (DQ142865)	89	96	Jasrotia and Ogram, unpublished
18	Uncultured bacterium (AY601064)	92	98	Zhang et al., 2006
19	Agrobacterium tumefaciens (FJ822995)	99	100	Hu et al., unpublished
20	Uncultured bacterium (EF583588)	94	96	Wartiainen et al., 2008
21	Azospira oryzae (U97115)	87	93	Hurek et al., 1997
22	Uncultured bacterium (AJ716230)	89	97	Héry et al., 2005
23	Uncultured bacterium (EF988491)	92	95	Duc et al., 2009
24	Uncultured bacterium (AJ716366)	98	99	Héry et al., 2005
25	Uncultured soil bacterium (FJ008581)	96	99	Hsu and Buckley, 2009

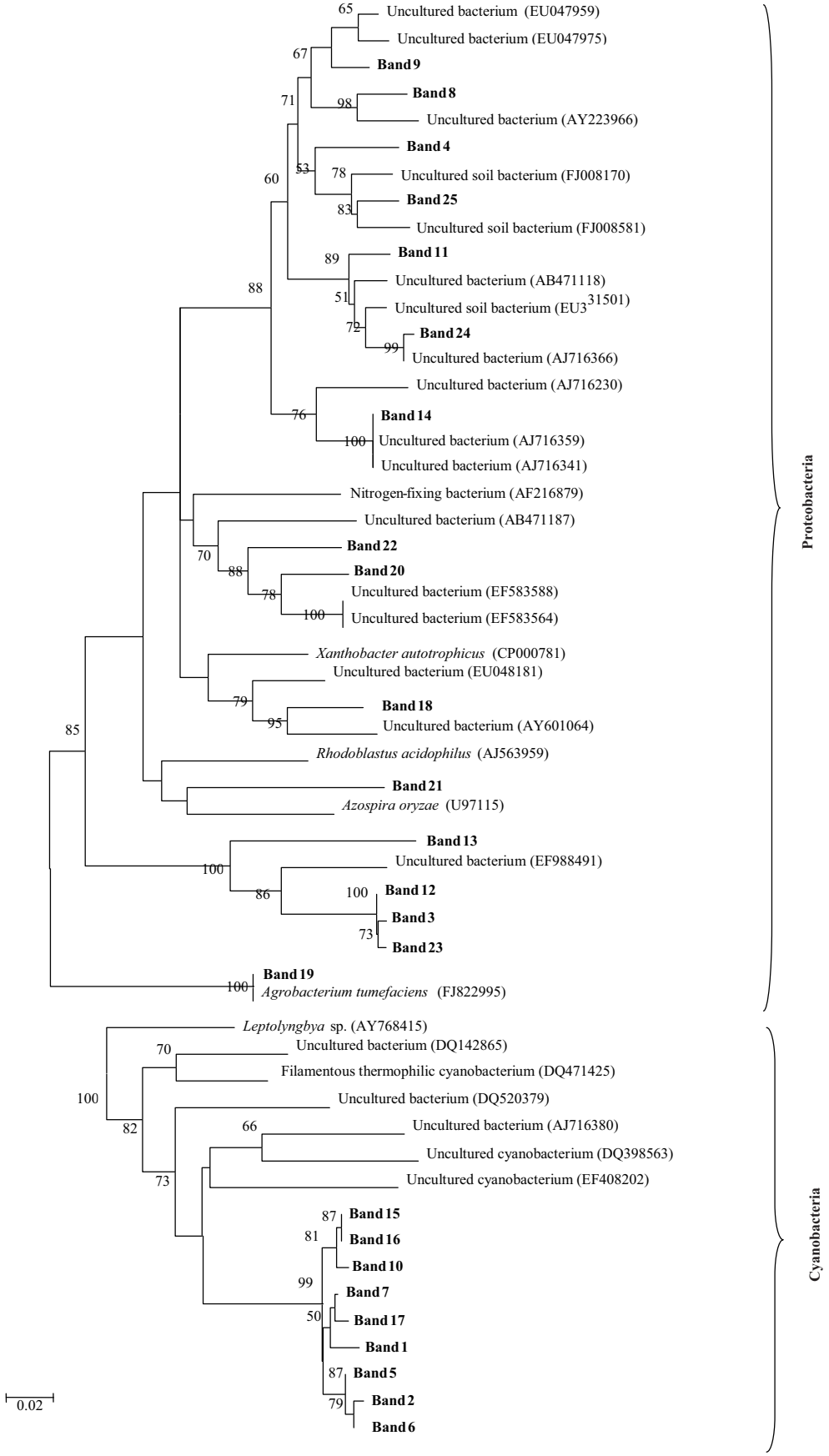

Fig. 4 Neighbour-joining phylogenetic tree based on *nifH* nucleotide sequences among the characteristic DGGE bands and their closest relatives. DGGE bands detected in this study are given in bold. The numbers shown next to each bifurcation are bootstrap percent values based on 5000 pseudo-replications. The values below 50 are not shown.

3 Discussion

3.1 Structure of free-living nitrogen-fixing microorganism community in wastelands of copper mine tailings

Diversity and structure of free-living nitrogen-fixing microorganism community in wastelands of copper mine tailings with different plant communities and discarded periods were investigated. The previous studies indicated that free-living nitrogen-fixers living in soil and rhizosphere generally included Proteobacteria, Cyanobacteria, Firmicutes and Archaea (Hurek et al., 1997; Widmer et al., 1999; Soares et al., 2006; Wartiainen et al., 2008).

In the present study, a total of 48 bands in 13 lanes were distinguished. Phylogenetic analysis of 25 prominent bands sequences indicated that there were mainly two taxa, Proteobacteria and Cyanobacteria, namely, both heterotrophic nitrogen-fixers and autotrofic nitrogen-fixing cyanobacteria detected in wastelands of copper mine tailings during the process of natural ecological restoration. However, this result did not mean that there was no Firmicutes or Archaea living in the wastelands investigated, which could be caused by many factors, such as a low abundance of *nifH* genes from Firmicutes or Archaea, preferential template amplification and DGGE biases, etc. (Wartiainen et al., 2008). Study results indicated that there was a significant difference in diversity and structure of free-living nitrogen-fixing microorganism community under different environmental conditions. The Proteobacteria are known as an abundant nitrogen fixing microorganism and the predominance of Proteobacteria has been reported in nickel mine soils (Héry et al., 2003), high arctic (Deslippe et al., 2005), dead aboveground biomass (Lovell et al., 2001), forest soil and litter (Widmer et al., 1999), rhizosphere soil (Soares et al., 2006; Chowdhury et al., 2009), etc.

In this study, in 16 bands clustered in Proteobacteria, band 19 showed 99% similarity to the corresponding sequence of *Agrobacterium tumefaciens*, belonging to the genera of *Agrobacterium*, while the band 21 showed only 87% similarity to the *Azospira oryzae*, member of *Azospira* (Table 4). Meanwhile, remained 14 bands affiliated with uncultured Proteobacteria and 8 of our sequences had less than 90% similarity to the *nifH* gene of uncultured Proteobacteria (Table 4).

All of nine bands clustered in Cyanobacteria were affiliated with uncultured cyanobacteria (Fig. 4), and presented lower similarities (< 90%) (Table 4). The Cyanobacteria are found contributing significantly to the total nitrogen fixation in the rice paddy (Vaishampayan et al., 2001) and forest soil (Widmer et al., 1999). The study in the Canadian high arctic by Deslippe et al. (2005) suggested that cyanobacteria were responsible for the majority of nitrogen fixation at the site covered with moss. It was reported that the biological soil crusts widely distributed on the surface of wastelands of copper mine tailings studied and can significantly improve nitrogen level of the upper layer wastelands (Sun et al., 2004). Many studies have confirmed that the biological soil crusts contain cyanobacteria and bacteria with nitrogen fixation (Russow et al., 2005; Yeager et al., 2007). This study proved the cyanobacteria with nitrogen fixation living not only in biological soil crusts, but also in the upper layer of wastelands of copper mine tailings.

Previous investigation found that there was a distinct succession series of plant communities in Yangshanchong wasteland of copper mine tailings (Sun et al., 2004), and microbial biomass and DNA contents from microorganisms increased with succession of plant communities (Shang et al., 2008). In this study, the wasteland samples under vascular plants did not present high diversity of nitrogen-fixers compared to bare wastelands and that under cryptogamic crusts, and cyanobacteria were shown to be critical in early successional stage of the natural ecological restoration (YBL1) (Fig. 1, bands labeled as 7, 10, 15, 16, 17, etc.) of where the efficiency of nutrient content is low (Table 2). Similar results were reported by Kahindi et al. (1997) who also confirmed that, in many mid-successional stages where legumes are absent, the accumulation of organic carbon is normally insufficient to maintain the high C:N ratios required to favor the growth of free-living nitrogen-fixers in the soil, while at later stages of succession there is an abundance of carbon which can act as a substrate for nitrogen-fixation by free-living heterotrophs.

3.2 Main factors causing diversity changes of free-living nitrogen-fixing microorganism community in wastelands of copper mine tailings

In 25 bands excised and sequenced, only three bands (band 12, 16 and 21) existed in all wastelands samples (Fig. 1), and the diversity analysis also showed changes in free-living nitrogen-fixers in different samples. From Figs. 1 and 2, it can be found that the diversity of *nifH* genes in Yangshanchong wasteland of copper mine tailings did not display a consistent successional tendency with development of plant communities. The bare wastelands with algal crust (YBL1 and YBL2) in humid seasons presented a higher diversity of *nifH* genes than cryptogamic crusts (YBA and YBC) and vascular communities (YIA, YIC, YHA, YHC, YZA and YZC). Comparing TIA, TIC1 and TIC2 to YIA and YIC with the same type of plant community, the former showed a higher diversity of *nifH* genes than the later. These results indicated that both the types of plant communities and physico-chemical properties could affect the diversity and structure of free-living nitrogen-fixers in wastelands of copper mine tailings.

Total soil metal contents are considered to be poor indicators of the actual contents in the soil solution to which soil microorganisms are exposed (Giller et al., 2009). Previous study suggests that NH_4OAc-extractable heavy metal is highly correlated with soil microbial metabolism (Jiang et al., 2003). In this study, the available metal contents were used in CCA to identify the effect of heavy metals on the diversity of free-living nitrogen-fixers. The effects of available Cd, As, Cr and Pb were not put into consideration in CCA in view of the low contents. CCA showed that diversity of *nifH* genes in different wasteland

samples was significantly affected by LOI, water content pH and available Zn contents of the samples ($p < 0.05$).

The previous studies indicated that pH is an important factor for the structure of bacterial community (Noll and Wellinger, 2008; Wakelin et al., 2008). And Gros et al. (2004) thought that most nitrogen-fixing microorganisms have an optimum soil pH close to seven. In this study, the samples from the Tongguanshan wasteland with about neutral pH had higher diversity of free-living nitrogen-fixing microorganisms than that from Yangshanchong wasteland with higher pH values.

Water content is very important for all bacteria living in soil, including free-living nitrogen-fixing microorganisms. A positive effect of water content on bacterial community structure was observed in other environments (Gros et al., 2004; Noll and Wellinger, 2008). In our study, the TIA and TIC1 from Tongguanshan wasteland with higher water contents displayed a higher diversity of free-living nitrogen-fixers than the YIA, YIC, YHA, YHC, YZA and YZC from Yangshanchong wasteland with lower water contents. Higher water content was beneficial to the growth and propagation of free-living nitrogen-fixing microorganisms in the wastelands. LOI was predicted to be a remarkable parameter affecting nitrogen-fixing microorganisms (Gros et al., 2004). The increase of organic carbon content may stimulate the growth of heterotrophic free-living nitrogen-fixers belonging to Proteobacteria detected in the copper mine tailings.

In this study, the community of free-living nitrogen-fixers was significantly affected by the content of available Zn in the wastelands of copper mine tailings (Fig. 3), in despite of the high content of available Cu. It is considered that the nitrogen-fixers in copper mine tailings with high content of Cu which have a strong tolerance to Cu are sensitive to Zn. The Tongguanshan wasteland with lower content of available Zn showed higher diversity of nitrogen-fixers compared to Yangshanchong wasteland. However, the wasteland sample of YBL1 with the available Zn of 12.983 mg/kg showed higher diversity of nitrogen-fixers than other wasteland samples with low contents of available Zn (except TIA), which could be due either to the generation of heavy metal-resistant bacterial species, especially tolerant cyanobacteria (Fig. 1, bands labeled as 7, 8), or to the contents of bioavailable Zn in the sample that may be somewhat stimulatory to the growth of the Zn-resistent nitrogen-fixers in wastelands of copper mine tailings.

The contamination of copper mine tailings have occurred over a long period, and it was considered that the nitrogen-fixing microorganism community would gradually change its metal tolerance characteristics in response to high wasteland metal contents, as a result, heavy-metal-tolerant species survived and increased in abundance.

The effects of various metal levels on soil microbes is found to be greatly affected by the pH of the soil owing to the strong effects of pH on solubility and speciation of metals in soil (Oliveira and Pampulha, 2006). The lowering in pH can result in increases in the contents of available metals, and the limit values of heavy metals to microorganisms may be reduced in soil with pH values lower than six (CEC, 1986). In this study, the wastelands in natural ecological restoration with pH of neutral or alkaline (pH > 7) did not show high available contents of heavy metals (less than 15 mg/kg Zn), although the total contents were detected in a high level (more than 300 mg/kg Zn) that exceeded the limits in the cases of Zn (150–300 mg/kg dry soil) established by the CEC Directive (Commission of the European Communities, 1986).

The effects of heavy metal contamination on free-living nitrogen-fixers have been reported in previous studies. Mårtensson and Witter (1990) found heterotrophic nitrogen fixation to be severely reduced in metal-contaminated soil in Sweden. McGrath et al. (1995) suggested that nitrogen fixation by free-living heterotrophic bacteria was inhibited in metal contaminated soil. It is reported that the population of free-living nitrogen-fixers is significantly affected in contaminated soil with the heavy metal content exceeded the limit established by the CEC Directive (Commission of the European Communities, 1986), undergoing a decrease of about 80% (Oliveira and Pampulha, 2006; Oliveira et al., 2009).

Although CCA analysis in this study and the previous studies (Giller et al., 1989; Riffkin et al., 1999; Héry et al., 2003; Tan et al., 2003; Beneduzi et al., 2008) indicated that the diversity and structure of nitrogen-fixing microbial community in environmental samples were affected by soil physico-chemical properties, we thought that the species of dominant plant and development period of plant communities were the key factors affecting the diversity and structure of free-living nitrogen-fixing microbial community in wastelands of copper mine tailings. Compared with Tongguanshan wasteland of copper mine tailings with a longer discarded period and higher cover of plant community, Yangshanchong wasteland with a short development period of plant community had not only lower nutrient and water content (Table 2), but also lower cover of plant community. Low cover of plant community and short development period of plant community in Yangshanchong wasteland resulted in the stress of nutrient, water, heavy metal and temperature for microbial community in wasteland substrate. Thus, the long-term colonization of plant communities provided a favorable habitat for free-living nitrogen-fixers in the wastelands by ameliorating pH, reducing the toxicity of heavy metals, increasing organic matter and water content. Poly et al. (2001b) did not support the influence of the plant species and reported that soil management seemed to be the major parameter influencing the *nifH* gene pool by controlling inorganic nitrogen content, and suggested that the variation of inorganic nitrogen content and plant effect were negatively correlated to the *nifH* gene pool. However, Diallo et al. (2004) described that the diversity of nitrogen-fixing bacteria was strongly affected by plant species and season. Bardgett et al. (1999) thought that the abundance and activity of soil microorganisms were more regulated by the dominant plant species than by the physical or chemical properties of the soil. Deslippe et al. (2005) reported that increased exudation of labile carbon

compounds by plant roots may cause shifts of diazotroph community structure. Tscherko et al. (2005) suggested that the increase in vegetation cover can decrease heat stress and dryness at the soil surface, favoring microbial growth and activity.

Our data also indicated that the nitrogen content did not affect the diversity of *nifH* genes in wasteland samples investigated, which was different from the reported results (Poly et al., 2001b; Tan et al., 2003; Coelho et al., 2009).

In conclusion, the free-living nitrogen-fixing microorganisms in wastelands of copper mine tailings include Proteobacteria and Cyanobacteria. Most of the free-living nitrogen-fixers in wastelands of copper mine tailings are unique and uncultured. The species of dominant plant and development period of plant community by ameliorating pH, reducing the toxicity of heavy metals, increasing organic matter and water content affected the diversity and structure of the free-living nitrogen-fixing microorganisms in wastelands of copper mine tailings.

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