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Fungal diversity in major oil-shale mines in China

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ABSTRACT

As an insufficiently utilized energy resource, oil shale is conducive to the formation of characteristic microbial communities due to its special geological origins. However, little is known about fungal diversity in oil shale. Polymerase chain reaction cloning was used to construct the fungal ribosomal deoxyribonucleic acid internal transcribed spacer (rDNA ITS) clone libraries of Huadian Mine in Jilin Province, Maoming Mine in Guangdong Province, and Fushun Mine in Liaoning Province. Pure culture and molecular identification were applied for the isolation of cultivable fungi in fresh oil shale of each mine. Results of clone libraries indicated that each mine had over 50% Ascomycota (58.4%–98.9%) and 1.1%–13.5% unidentified fungi. Fushun Mine and Huadian Mine had 5.9% and 28.1% Basidiomycota, respectively. Huadian Mine showed the highest fungal diversity, followed by Fushun Mine and Maoming Mine. Jaccard indexes showed that the similarities between any two of three fungal communities at the genus level were very low, indicating that fungi in each mine developed independently during the long geological adaptation and formed a community composition fitting the environment. In the fresh oil-shale samples of the three mines, cultivable fungal phyla were consistent with the results of clone libraries. Fifteen genera and several unidentified fungi were identified as Ascomycota and Basidiomycota using pure culture. *Penicillium* was the only genus found in all three mines. These findings contributed to gaining a clear understanding of current fungal resources in major oil-shale mines in China and provided useful information for relevant studies on isolation of indigenous fungi carrying functional genes from oil shale.

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Introduction

Oil shale is a type of fine-grained sedimentary rock that contains organic matter. Components of organic matter trapped in oil shale can yield significant amounts of oil and combustible gas when they undergo destructive distillation (Dyni, 2003). With the globally rising demand for energy and the gradual exhaustion of conventional energy resources such as petroleum, natural gas, and coals, oil shale is seen as an important replacement energy in the 21st century, considering its abundant reserves, advantageous features, and feasibility of exploitation (Bunger et al., 2004).

In the past, oil shale was mainly utilized with physical and chemical techniques. However, in recent years, the advantages of microbial metallurgy have been highlighted, such as simple technical processing, low-carbon and environmentally friendly operation, low energy consumption, and low costs (Anjum et al., 2012). In early studies, most inorganic matter in oil shale was directly or indirectly biodegraded by microorganisms under mild conditions, and kerogens remained as energy sources (Findley et al., 1974; Craig Meyer and Yen, 1976; Vrvic et al., 1988, 1990; Cvetković et al., 1993; Beškoski et al., 2008). Until 1997, organic matter in oil shale was found to be the primary energy source for

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indigenous bacteria (Krumholtz et al., 1997). Subsequently, oil shale was confirmed to provide the sole carbon source and energy for the growth of individual or mixed bacterial strains accompanying structural changes of oil shale (Permanyer et al., 2010; Matlakowska and Sklodowska, 2009; Matlakowska et al., 2010). In addition, mixed strains (*Rhodococcus erythropolis* and *Rhodococcus ruber*) that produce biosurfactants were used to degrade the organic matter in oil shale, indicating that the maximum extraction ratio of shale oil was as high as 26% (Haddadin et al., 2009). According to existing literature, bacteria play dominant roles in oil-shale bio-utilization. However, studies on fungi in oil shale are limited. In general, fungi are involved in almost all of the biological and biochemical reactions in the environment. Early studies found that some fungi can live on the surface of oil-shale particles in oil-shale deposits of the eastern US; however, further studies were not carried out (Pfister et al., 1991). In 2006, the known wood-rotting fungus *Schizophyllum commune* was applied for the biotransformation of oil shale, proving that the organic matter in oil shale can be decomposed and released to the surrounding environment as organic heavy metal complexes (Wengel et al., 2006). Although this study showed the potential of fungi for oil-shale bio-utilization, the strain used was not isolated from the habitat of oil shale. Indigenous microorganisms are capable of developing functional genes and special physiological metabolisms fitting the surrounding environment in the long-term adaptation process, which make them exhibit more proficient bio-utilization abilities for materials in their environment of origin (Qi et al., 2011; Matlakowska and Sklodowska, 2011). Therefore, exploring valuable indigenous fungi is an important and feasible way to propel the bio-utilization of oil shale. A survey on fungal diversity in the original environment is a fundamental prerequisite because it can provide useful information for targeted and expanded screening of valuable indigenous fungi.

Although China has abundant oil-shale resources that rank No. 4 in the world, available research and information on the microbial resources in oil shale are limited. In this study, three main oil-shale production areas in China were selected, including Huadian Mine in Jilin Province, Maoming Mine in Guangdong Province, and Fushun Mine in Liaoning Province. By combining the clone libraries of ribosomal deoxyribonucleic acid internal transcribed spacer (rDNA ITS) with traditional pure culture, the composition and structure, dominant group and diversity of fungus communities in the special habitats were revealed in detail. This study was aimed at providing a useful basis of fungal resources for the bio-utilization of oil shale and bioremediation of the ecological environment in oil-shale areas.

1. Materials and methods

1.1. Study plot setting and sample collection

The sampling was carried out in July 2012. In Maoming Mine in Guangdong Province (21°41'N, 110°58'E), Fushun Mine in Liaoning Province (41°50'N, 123°57'E), and Huadian Mine in Jilin Province (43°00'N, 126°47'E), the most representative fresh oil shale, weathered oil shale, and sandy soil were

collected. The samples from the same mine were equally mixed for microbial total DNA extraction to construct the clone library. Fresh oil-shale samples were used for pure culture.

The samples in one mine were taken as examples. For each sample, three sampling plots with size of 10 m × 10 m and spacing of about 500–1000 m were set in each sampling area. The multi-point sampling method was used in each sampling plot. First, the surface shale or sandy soil was removed to avoid microorganisms in the external environment entering into samples. Next, crushed samples were collected with sterile gloves. After mixing, samples were filtered with a 4 mm sieve. An adequate amount of each sample was placed in 50 mL sterile centrifuge tubes by the quartering approach. Subsequently, the samples were brought back in an icebox and preserved in the laboratory at 4 °C. All sampling tools were autoclaved. The climatic characteristics of each mine are listed in Table 1, and the geological characteristics of each mine are listed in Table 2. The basic features of samples are listed in Tables 3 and 4.

1.2. Extraction of microbial total DNA

The improved sodium dodecyl sulfate SDS-high-salt extraction method was used based on Zhou's method (Zhou et al., 1996). For the specific operation procedures and the productivity and purity of extracted DNA, please refer to our previous publication (Jiang et al., 2014).

1.3. Clone libraries of fungal rDNA ITS

1.3.1. Construction of clone libraries

With ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' as primers (Gardes and Bruns, 1993), 550 bp of rDNA ITS was amplified. The 50 µL PCR system consisted of 25 µL of 2 × MasterMix (Biotek, China), 50 ng of microbial total DNA, 2 µL of each primer (10 µmol/L), and double distilled water. PCR amplification was performed on a C1000™ Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C denaturation for 30 sec, 55 °C annealing for 30 sec, 72 °C elongation for 45 sec, and a final elongation step at 72 °C for 10 min. PCR products were recycled and purified with the MiniBEST DNA Fragment Purification Kit (TaKaRa, China). The PCR product was ligated into the pUM-T vector (Biotek, China), and the ligation reaction was used to transform competent *Escherichia coli* strain DH5α (Biotek, China) to generate rDNA ITS clones. Recombinant clones were selected on Luria-Bertani agar plates containing 20 µg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5 mmol/L IPTG (isopropyl-β-D-thiogalactopyranoside), and 100 µg/mL ampicillin. Plates were incubated overnight at 37 °C. The presence of inserts was determined by PCR with white (positive) bacterial colonies by using primers M13F: 5'-GTAAACGACGGCCAG-3' and M13R: 5'-CAGGAAACAGCTATGACCATG-3'.

Primer synthesis and sequencing of positive clones were completed by Sangon Biotech (Shanghai, China) Co., Ltd. Chimeras were detected online by the Bellerophon system. DNAMAN software was used to remove vector sequences, and the VecScreen system was used to examine whether the

Table 1 – Climatic characteristics of the three mines.

Area	Climate	Seasonal features	Annual average temperature (°C)	Annual average precipitation (mm)
Maoming Mine	Subtropical monsoon climate	Relatively long summer, warm winter, and equable climate	16.5–22.8	1500–1800
Huadian Mine	Temperate continental monsoon climate	Long and cold winter and remarkable temperature difference among four seasons	3.9–4.5	760–790
Fushun Mine	Temperate continental monsoon climate	Long and cold winter and remarkable temperature difference among four seasons	6.0–7.1	740–760

removal was complete. All sequences were aligned with known sequences in the GenBank (NCBI) database. Sequences with similarity over 94% belonged to the same genus, and those with similarity over 97% belonged to the same species. The library coverage (C) was calculated by Eq. (1):

$$C = 1 - (n_1/N) \times 100\% \quad (1)$$

where n_1 is the number of clones that appeared only once in the library; N is the number of positive clones. The library coverage indicated the ratio of genera covered in the library in all genera of the community. The higher it was, the closer to saturation the storage capacity was (Wagner and Loy, 2002).

1.3.2. Analysis of community diversity

Four α diversity indexes used in ecology were analyzed (Simpson, 1949; Lloyd et al., 1968). Richness (R), Shannon's diversity index (H'), Simpson's diversity index (D), and Pielou's evenness index (E) reflected the community diversity from different perspectives, and were calculated by Eqs. (2)–(5):

$$R = S \quad (2)$$

$$H' = -\sum P_i \ln P_i \quad (3)$$

$$D = 1/\lambda \quad (4)$$

$$E = H'/H'_{\max} \quad (5)$$

where S is the number of operational taxonomic units (OTUs) in the library; P_i is the ratio of i individual numbers in the overall individual numbers; $\lambda = \sum P_i^2$; $H'_{\max} = \ln S$.

The Jaccard index (EJ) was used to compare the similarity between communities, which was calculated by Eq. (6)

$$EJ = a/(b + c - a) \quad (6)$$

where a is the number of fungal genera shared by the two libraries to be tested; b and c are the numbers of fungal genera in

the two libraries, respectively. According to the principle of the Jaccard index, the more fungal genera are shared by two communities, the greater the similarity or the closer their relationship is, and vice versa. When EJ falls in the 0–0.25 range, it indicates an extreme dissimilarity. When EJ is in the 0.25–0.50 range, it indicates medium dissimilarity. When EJ falls in the 0.50–0.75 range, it indicates medium similarity. When EJ is in the 0.75–1.00 range, it indicates an extremely high similarity.

1.4. Pure culture and molecular identification of cultivable fungi

Samples were smeared on potato dextrose agar (PDA) medium (Tianhe, China) and then cultured at 28 °C for 96 hr. Sterilized samples were taken as controls. For each sample, the treatment was repeated three times, and three parallels were set for each dilution degree (10^{-1} – 10^{-4}) to eliminate accidental factors. Each colony was purified for over five generations until the mycelia in the plate were completely identical by microscopic examination. The Ezup column fungal genome DNA extraction kit (Sangon, China) was used to extract the total DNA of fungal colonies, and then Primers ITS1 and ITS4 were used to amplify rDNA ITS. Bioedit and MEGA 4 were employed to construct the phylogenetic tree with a neighbor-joining algorithm and Kimura 2 parameter model (Tamura et al., 2007).

All sequences were deposited in the NCBI GenBank under accession numbers KJ626303–KJ626307 and KJ653454–KJ653470.

2. Results and discussion

2.1. Fungal rDNA ITS clone libraries

2.1.1. Diversity at the phylum level

The coverage of the three clone libraries all exceeded 85% (Table 5), which was high enough to represent and characterize the diversity of fungus communities in the oil-shale mines. At

Table 2 – Geologic features of the three mines.

Mine	Basin type	Sediment environment	Water properties	Source of organic matters	Paleoclimate	Lithology
Maoming Mine	Cenozoic faulted basin	Semi-deep lacustrine	Fresh water	Aquatic organism and terrestrial advanced plants	Stable and consistent warm and moist climate	Lignite and carbonaceous shale interlayer with gray oil shales
Huadian Mine	Cenozoic faulted basin	Semi-deep and deep lacustrine	Salt water	Dominant source of aquatic organism, less terrestrial supply	Half moist and half arid, cold and dry at the late paleoclimate	Interbed of sandstones, mudstones, and oil shales
Fushun Mine	Cenozoic faulted basin	Semi-deep and deep lacustrine	Salt water	Dominant source of aquatic organism, less terrestrial supply	Turning from warm and moist to hot and arid gradually	Interbed of marlstone, mudstone, and oil shales

Table 3 – Major elemental composition of oil shale in the three mines using X-ray fluorescence spectrometry method (Rigaku, Japan).

Oil-shale mine	Main components and contents (wt.%)									
	SiO ₂	CO ₂	Al ₂ O ₃	Fe ₂ O ₃	K ₂ O	CaO	SiO ₃	MgO	Na ₂ O	TiO ₂
Maoming Mine	30.32	41.43	18.33	4.09	1.39	0.41	2.83	0.61	0.03	0.40
Fushun Mine	43.90	28.41	17.08	5.36	1.26	0.70	0.71	0.91	0.61	0.74
Huadian Mine	40.61	33.66	12.82	3.48	1.47	3.07	1.33	0.89	0.99	0.62

the phylum level, although Ascomycota and (or) Basidiomycota were the main phyla of each library, they showed a distinct difference of proportions in different libraries. Ascomycota accounted for 98.9% and 87.1% respectively in the libraries of Maoming Mine and Fushun Mine, both of which were greatly larger than that in Huadian Mine (58.4%). Basidiomycota were not discovered in the library of Maoming Mine, and accounted for a mere 5.9% in the library of Fushun Mine, but reached 28.1% in the library of Huadian Mine (Fig. 1).

It is known that the type and content of organic matter in a biotope are crucial for maintaining the growth and diversity of microbes, and the availability and composition of organic matter are key factors for microbial biomass and community (Tiquia et al., 2002). Microorganisms in sedimentary rocks sought nutrients and energy from ancient organic matter trapped in these rocks (Krumholz et al., 1997; Petsch et al., 2005). Although the three mines presented apparent differences in many aspects (Tables 1–4), the selected mines were all located in a Cenozoic faulted basin, and organic matter in the oil shale in each mine existed mainly in the same form of kerogen, which is a high-molecular weight polymer and insoluble in ordinary organic solvents (Liu et al., 2009; Dyni, 2003). Generally, resin and asphaltene are very difficultly biodegraded, followed by aromatic hydrocarbons and saturated hydrocarbons (Chaineau et al., 1995). But the biodegradation of kerogen is much more difficult due to its higher complexity and stability in contrast to resin and asphaltene. The bioavailability of kerogen may vary with its types, which can usually be divided into sapropelic, humic-sapropelic and humic types. The sapropelic type consists mainly of aliphatic hydrocarbons and has few cyclic or aromatic structures. The humic-sapropelic type chiefly contains cycloalkanes and straight chain alkanes as well as small amounts of polycyclic aromatic hydrocarbons and heteroatom groups. Relatively speaking, the sapropelic type is more easily bio-utilized in comparison with the two other types. In this study, except for the humic-sapropelic type of Maoming kerogen, the type of the other two mines was predominantly sapropelic due to

different depositional environments, and the organic carbon content of Fushun, Huadian and Maoming mines was 11.72%, 33.31% and 15.88%, respectively (Liu et al., 2009). Based on the above information, the preference for fungal phyla in each mine was determined mainly by the essence of the kerogen in the oil shale, whereas the proportional difference of fungal phyla was subject to the combined impact of various factors, such as kerogen types, organic carbon content, climatic and geological conditions, pH, and water content.

Oil shale is formed by co-deposition of fine-grained detrital minerals and organic matter from the decomposing remains of decaying plants, algae and prokaryotic microorganisms under reducing conditions. The process was accompanied by the formation of the original microbial community. Indirect evidence indicated that microbes played an important role in deposition and early diagenesis of oil shale (Dyni, 2003). Organic components, which consist of cellulose and lignin of plants and lower organisms, are one of the original ingredients in the formation of kerogen in oil shale (Dyni, 2003). In Ascomycota, many genera can metabolize cellulose through the production of extracellular enzymes (Lyons et al., 2003). They decompose cellulose into cellose first and then into glucose. Afterward, they absorb glucose as nutrition. This characteristic may be the primary reason for Ascomycota to be the absolutely dominant category in the habitat of oil shale. In Basidiomycota, some genera metabolize not only cellulose but also lignin (Johnsen et al., 2001). The result indicated that Ascomycota and Basidiomycota can be considered as potential fungi for biotransformation of oil shale.

2.1.2. Diversity at the genus level

At the genus level, the differences of constituent compositions between libraries were significant (Fig. 2). Besides the content and type of organic matter, the influence of environmental factors (Tables 1–4) and mining practices on the non-uniformity of microorganism distribution should be considered. The ecological niche of different microflora can be differentiated by the selective absorption of nutrition, varying amounts of available nutrients, and different hydrothermal conditions. With

Table 4 – Water content, pH value, and total DNA content of samples.

Samples of origin	Water content (%)	pH value	Total DNA content (ng/g dry sample)
Maoming Mine	8.16 ± 1.66a	3.76 ± 0.26a	650.12 ± 88.17a
Fushun Mine	3.44 ± 0.66b	7.42 ± 0.08b	268.36 ± 55.29b
Huadian Mine	8.74 ± 0.98a	8.40 ± 0.06c	228.60 ± 52.31b
F-value	6.083	228.612	11.977
p-value	0.007	<0.001	<0.001

Data are presented as means ± SD (n = 3).

Different alphabets in the same column represent significant difference ($p < 0.05$).

The water contents were tested by oven drying method. The pH values were determined by potentiometry. Total DNA contents were obtained by improved SDS-high-salt extraction method.

Table 5 – Number of positive clones, coverage, and diversity and evenness indexes of clone libraries of Funshun Mine, Huadian Mine and Maoming Mine.

Clone library	Fushun	Huadian	Maoming
Number of positive clones	85	89	91
Coverage of diversity (C) (%)	95.3	85.4	96.7
Richness (R) (Number of OTUs ^a)	13	26	6
Shannon's diversity index (H')	1.84	2.80	0.47
Simpson's diversity index (D)	0.75	0.92	0.19
Pielou's evenness index (E)	0.72	0.86	0.26

Note: ^a No. of unique sequences showing <94% identity with recovered sequences from this study.

Coverage of diversity (C) refers to the extent or degree to which the species present in the community are analyzed.

Richness (R) takes into account the total number of different species present in the community.

Shannon's diversity Index (H') takes into account both the abundance and evenness of the species present in the community.

Simpson's diversity index (D) is a measure of diversity which takes into account the number of species present, as well as the relative abundance of each species.

Pielou's evenness index (E) refers to how close in numbers each species in the community are.

higher Shannon's diversity index (generally ranging between 1.5 and 3.5, seldom exceeding 4.5) and Simpson's diversity index closer to 1, communities presented more intensified heritable variation, stronger adaptive capacity for environmental change, and tended to expand the distribution range and enter new environments more easily. When the Pielou's evenness index was closer to 1, it indicated that the higher the evenness degree of fungal distribution was, the more stable the community became. According to Table 5, the highest diversity and the most balanced distribution of relative abundance was in Huadian Mine, showing that its community had the most complex structure, strongest resistance, and highest stability. On the contrary, the diversity and evenness of Maoming Mine were much lower than those of the other two mines. The relative abundance of dominant genera (>10% were seen as dominant genera) and common genera (1–10% were seen as common genera) in Maoming Mine differ remarkably from the other mines (Fig. 2). Although the dominant genus in Maoming Mine is *Penidiella* solely, the proportion of *Penidiella* accounted for up to 90.1%. *Penidiella* is acidophilic and plays an important ecological role in many extreme

environments. The exploitation of Maoming Mine adopted opencast techniques. Meanwhile, Maoming Mine is located in the subtropical monsoon climate with abundant rainfall throughout the year (Table 1). Moreover, the carbon element content in the samples of Maoming Mine was much higher than that in Fushun Mine and Huadian Mine (Table 3). Rains facilitated the carbonatite decomposition into carbonic acid solution, leading to the loss of calcium ions (this finding was reflected in the lower carbon content in Maoming Mine than the other mines, as shown in Table 3). In conclusion, the high proportion of *Penidiella* in the Maoming Mine library was attributed to the pH value in samples of Maoming Mine, which was much lower than that in Fushun Mine and Huadian Mine (Table 4), providing favorable conditions for the growth and metabolism of acidophilic fungi. In addition, *Mrakiella* was found to exist only in the Huadian Mine library, which could be correlated with the crymophilia of *Mrakiella*. Huadian Mine is located in relatively high latitudes, where the solar radiation is less intense, and the climate is relatively cold. The abovementioned details demonstrate the ecological principle that some specific ecological environments determine the corresponding structure of microbial communities. The environmental and climate conditions of each mine generated insignificant impact on the preference for fungal phyla but significantly influenced the composition and structure of fungal genera.

Compared with the other two mines, the largest amount of unidentified fungi was observed in Huadian Mine (Fig. 1), which may be attributed to the exploitation form of underground mining. The subsurface environment is less affected by external conditions and has an anaerobic environment, thus preserving substantial fungi that cannot be cultured by existing technologies or identified by human beings. This finding indicated that substantial amounts of fungi and their genetic resources in oil shale have been wasted and should be further characterized. Similarly, many uncultured microorganisms were discovered in natural asphalt samples with a history of 28,000 years collected in Los Angeles, United States (Kim and Crowley, 2007). Although these uncultured microorganisms in the ancient sediments cannot be isolated and cultured with existing technologies, their functional genes can be inserted into vectors to construct genetically engineered microorganisms. In this way, the functional genes with potential application value can be expressed and utilized for the

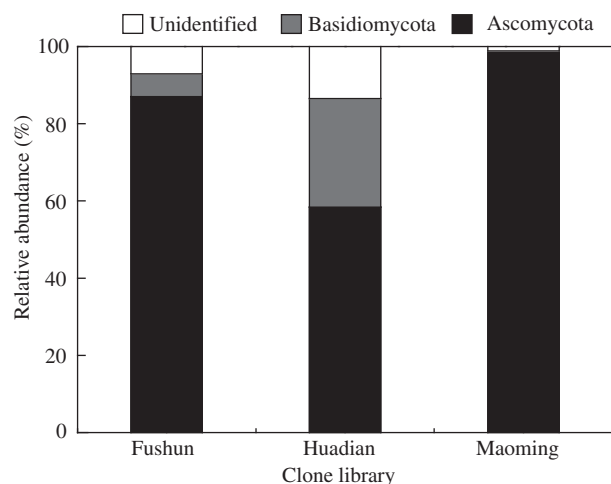


Fig. 1 – Relative abundance of fungal phyla obtained from the clone libraries of the three oil-shale mines (Fushun, Huadian and Maoming) based on rDNA ITS. rDNA ITS: ribosomal deoxyribonucleic acid internal transcribed spacer.

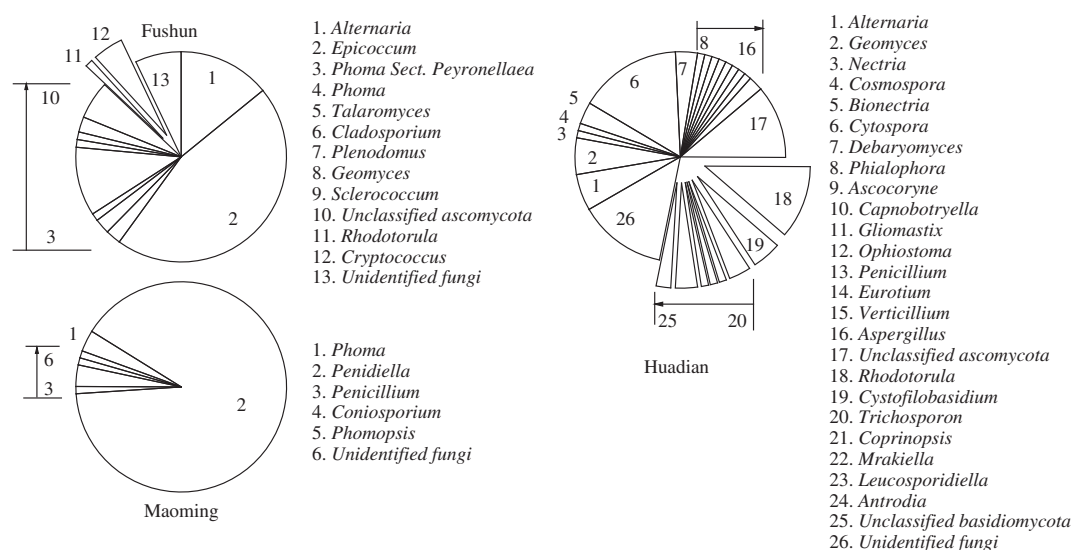


Fig. 2 – Relative abundance of fungal genus obtained from the clone libraries of the three oil-shale mines (Fushun, Huadian and Maoming) based on rDNA ITS. The out-of-position genera in the pies belong to Basidiomycota, whereas the others belong to Ascomycota, excluding unidentified fungi. rDNA ITS: ribosomal deoxyribonucleic acid internal transcribed spacer.

biotransformation of the deposits (Petsch et al., 2005; Voordouw et al., 1996).

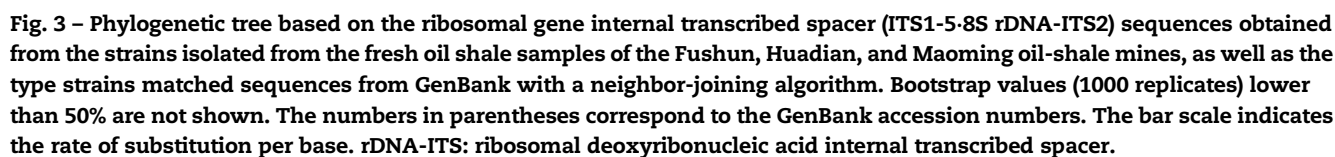
According to the results of clone libraries, we calculated the Jaccard indexes (EJ) among the three mines, and the findings showed that Fushun Mine and Huadian Mine, with relatively similar local ecological conditions, had the highest similarity (EJ = 0.10). The similarity between Fushun Mine and Maoming Mine was medium (EJ = 0.07), whereas Huadian Mine and Maoming Mine, with relatively dissimilar local ecological conditions, showed the lowest similarity (EJ = 0.04). Meanwhile, the EJ among the three communities were all far below 0.25, belonging to the range of extreme dissimilarity. This result indicated that relatively independent adaptations of fungus communities in different mines occurred during the long geological formation and development process driven by phylogenetic or ecological pressures.

2.2. Pure culture and molecular identification of cultivable fungi

Pure culture of fresh oil-shale samples of three mines was performed to understand the cultivable fungi species in the oil-shale sediments. Through phenotype identification and rDNA ITS sequence alignment (sequences with similarity higher than 97% were considered as belonging to the same OTU) of 150 isolated strains (50 strains per mine), 15 identified genera and 3 unique rDNA ITS sequences (gz1, jzk1-7 and fz27 in Fig. 3) belonging to unidentified fungi were obtained. Similar to the results of clone libraries, all isolated strains belonged to Ascomycota and Basidiomycota, and the amount of different genera was much more than owned-in-common genera among the three mines. Among the isolated strains, 11 OTUs in Huadian Mine had more than 98% similarity with *Debaryomyces*, *Coprinopsis*, *Rhodotorula*, *Eucasphaeria*, *Aspergillus*, *Sporidiobolus*, *Nectria*, *Bionectria*, *Candida*, and *Penicillium*. One OTU in Huadian Mine could not be identified, but is highly homologous with Ascomycota. Six OTUs in Maoming Mine had more than

96% similarity with *Eupenicillium*, *Penicillium*, *Penidiella*, and an unidentified fungus of Basidiomycota. Six OTUs in Fushun Mine had more than 96% similarity with *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Cryptococcus*, and an unidentified fungus of Ascomycota (Fig. 3). *Penicillium*, which shows strong adaptive capacity and is extensively distributed in various environments, was the only genus found in all 3 mines. Moreover, it was able to secrete relatively complete dextranase with high enzyme activity for decomposing natural wood fibers. Therefore, this genus has potential for the biotransformation of oil shale.

Generally, when the sequence of an isolated strain had more than 97% similarity with the sequence of a relevant type strain in GenBank, they were considered the same species. However, this study adopted the phenotype observation of colonial morphologies and rDNA ITS sequence alignment methods without the in-depth physiological metabolism and biochemical function detection for identification of isolated strains. Moreover, opinions are divided on species taxonomy (Samson and Frisvad, 2004). Hence, genetic diversity was evaluated at the genus level instead of the species level in this study. The findings showed little taxonomical significance for the various species in the same genus, showing certain limitations. However, the results outlined fungal distribution characteristics of major oil-shale mines in China and may be useful for the further survey of biodiversity at the species level. In addition, the method of constructing the clone library of rDNA ITS can bypass the step of strain isolation and cultivation and directly study the composition and structure of microbial populations at the genetic level, which is convenient and efficient and is more suitable for fungal diversity analysis due to the higher richness compared with traditional pure culture. Besides, most fungi do not produce fruiting structures or sporocarps via plate cultivation or lose the spore-bearing function after subculture and purification. Thus, fungi are difficultly separated and cultured through



pure culture. Therefore, the use of culture-independent molecular techniques is an acknowledged effective approach to analyze microbial diversity. Nevertheless, three fungal genera (*Eucasphaeria*, *Sporidiobolus*, and *Candida*) that are excluded in the three clone libraries were identified by pure-cultivation for the following reasons: First, the total DNA of the spore-forming fungi was hard to extract, whereas the dilution-plate method was effective for a group that is apt to produce spores (e.g., Ascomycetes); second, extraction and purification of microbial total DNA may lead to the loss of some DNA; and third, the coverage of clone libraries did not reach 100%, leading to genetic omissions. Overall, the results were consistent for phyla but were slightly inconsistent for genera using the methods of clone library and pure culture. The two methods were mutually complementary for better understanding of the diversity of fungal communities in oil shale.

3. Conclusions

Ascomycota and (or) Basidiomycota were the dominant categories in the habitats of 3 oil-shale mines. The clone libraries presented significant differences at the genus level. Huadian Mine had the greatest amount of unidentified fungi with potential for application and the most abundant diversity and highest evenness, followed by Fushun Mine and Maoming Mine. Jaccard indexes indicated that fungus communities between any 2 of the 3 mines were extremely dissimilar, proving that fungi in different mines evolved separately and speciated independently. By pure culture, 15 identified genera and 3 unidentified rDNA ITS sequences were obtained in the 3 mines. Similar to the results of clone libraries, all of the isolated strains belonged to Ascomycota or Basidiomycota, in which differential genera were more than the owned-in-common genera, and *Penicillium* was the only cultivable owned-in-common genus among the three mines. The combination of traditional and molecular approaches described a comprehensive picture of fungal diversity in oil-shale habitats. These findings can provide beneficial information to further develop and utilize the fungal resources in oil shale in China.

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