Research article

Rhizospheric fungi of *Panax notoginseng*: diversity and antagonism to host phytopathogens

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ABSTRACT

Background: Rhizospheric fungi play an essential role in the plant–soil ecosystem, affecting plant growth and health. In this study, we evaluated the fungal diversity in the rhizosphere soil of 2-yr-old healthy *Panax notoginseng* cultivated in Wenshan, China.
Methods: Culture-independent Illumina MiSeq and culture-dependent techniques, combining molecular and morphological characteristics, were used to analyze the rhizospheric fungal diversity. A diffusion test was used to challenge the phytopathogens of *P. notoginseng*.
Results: A total of 16,130 paired-end reads of the nuclear ribosomal internal transcribed spacer 2 were generated and clustered into 860 operational taxonomic units at 97% sequence similarity. All the operational taxonomic units were assigned to five phyla and 79 genera. Zygomycota (46.2%) and Ascomycota (37.8%) were the dominant taxa; *Mortierella* and unclassified Mortierellales accounted for a large proportion (44.9%) at genus level. The relative abundance of *Fusarium* and *Phoma* sequences was high, accounting for 12.9% and 5.5%, respectively. In total, 113 fungal isolates were isolated from rhizosphere soil. They were assigned to five classes, eight orders (except for an *Incertae sedis*), 26 genera, and 43 species based on morphological characteristics and phylogenetic analysis of the internal transcribed spacer. *Fusarium* was the most isolated genus with six species (24 isolates, 21.2%). The abundance of *Phoma* was also relatively high (8.0%). Thirteen isolates displayed antimicrobial activity against at least one test fungus.
Conclusion: Our results suggest that diverse fungi including potential pathogenic ones exist in the rhizosphere soil of 2-yr-old *P. notoginseng* and that antagonistic isolates may be useful for biological control of pathogens.

1. Introduction

Soil microbial communities display high structural, genetic, and functional diversity, and play extremely complex roles in many aspects of soil ecosystems, such as biotic interactions and nutrient cycling [1]. Microbial diversity in soil is an important factor determining soil health and is considered one of the main drivers in soil suppressiveness [2]. The rhizosphere is one of the most complex environments that are influenced by plant roots and active microhabitat where plant roots and microbes interact [3,4]. Composition of the rhizosphere microbiota can negatively or positively influence plant traits such as stress tolerance, health, development, and productivity [5,6]. Conversely, host plants are able to shape their own rhizosphere microbiome by adjusting environmental factors such as pH, soil nutrients, and root architecture [5], and recent studies have shown that plant

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genotype also has a great influence on the structure of rhizosphere microbial communities [7−9]. Fungi and fungus-like organisms form one of the most diverse groups of the Eukarya, and represent an essential functional component of soil microbial communities [10]. Under unfavorable conditions, some fungi can cause plant diseases and sometimes even total loss of the crop yields. In many instances, these diseases are caused by a complex of fungal species. At the same time, other fungal groups can antagonize phytopathogens, decompose plant residues, supply nutrients for plants, and stimulate plant growth [11].

The culture-dependent approach has been applied to analyze the fungal diversity for several decades [12]. In the past 20 yrs, many molecular methods, such as terminal-restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and real-time quantitative PCR detecting system (Q-PCR), have widely been used to study the diversity and composition of soil microbial communities [13,14]. With methodological advances, high-throughput sequencing techniques, such as 454 pyrosequencing and MiSeq by Illumina (San Diego, CA, USA), have been considered more powerful tools for estimating microbial communities [14,15]. Compared to the 454 pyrosequencing, MiSeq sequencing has shorter time for generating good-quality paired-end reads and a much higher throughput at a low price [15−17]. The length of reads can reach 2 × 300 bp. Due to lower costs and greater throughput, Illumina MiSeq sequencing is increasingly being utilized to estimate the diversity and composition of microbial communities in various ecosystems.

Panax notoginseng (Burk.) F. H. Chen (Araliaceae), a well-known traditional Chinese medicinal plant mainly cultivated in the mountainous areas in Yunnan and Guangxi, is widely used for the treatment of cardiovascular diseases, inflammation, trauma, and internal and external bleeding due to injury, and has also been used as a tonic [18,19]. Grown under shaded conditions with high humidity for at least 3 yrs, cultivation of *P. notoginseng* is easily affected by the soil environment, and *P. notoginseng* is also susceptible to a number of soil-borne diseases [20]. Among the diseases, the root-rot disease complex caused by a single pathogen or a combination of pathogens is most destructive because it results in yield reduction, or no harvest, and a lower content of active ingredients [21]. The reported pathogens include *Alternaria panax*, *Alternaria tenuissima*, *Cylindrocarpon destructans*, *Cylindrocarpon didynum*, *Fusarium oxysporum*, *Fusarium solani*, *Phytophthora cactorum*, *Phoma herbarum*, and *Rhizoctonia solani* [22]. Fungal pathogens, endophytes, and rhizosphere microbes of *Panax ginseng* and *Panax quinquefolius* have been reported [23−27]. Investigation on fungi associated with *P. notoginseng* has been focused on pathogens, and few reports were about the diversity of fungal community in the rhizosphere soil of *P. notoginseng*.

In this study, the diversity of the rhizospheric fungal community of 2-yr-old healthy *P. notoginseng* plants was characterized using a MiSeq sequencing platform (2 × 300 bp). The culturable fungal community was also analyzed by culture-dependent methods, and the antagonistic activities of the fungal isolates were also challenged by the pathogens, *A. panax*, *F. oxysporum*, *F. solani*, and *P. herbarum*, isolated from rotten roots of *P. notoginseng*. The further purposes include the following: (1) finding antagonistic fungi for the development of biocontrol agents for soil-borne diseases of *P. notoginseng*; (2) exploring the active substances from antagonistic fungal strains; and (3) investigating the fungal structure for in-depth studies on microbial ecological functions.

2. Materials and methods

2.1. Site and sampling

Soil samples were collected in a *P. notoginseng* plantation (N 23°35′, E 103°40′) in Wenshan, Yunnan, in early June 2013. Soil adhering around the roots was gently taken off, put into a sterile plastic bag, and transported to the laboratory within 24 h. Soil samples were preserved at −80°C and 4°C before use.

2.2. Soil DNA extraction and MiSeq sequencing

DNA was extracted from 0.5 g frozen soil sample using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer 2 (ITS2) region was amplified according to the newly developed primers fITS9 and ITS4 [28]. Amplifications were carried out in a total volume of 50 μL using 10 ng of DNA, 5 μL 10 × polymerase chain reaction (PCR) buffer, 5 μL deoxy-ribonucleoside triphosphate (dNTP) (10mM each), 2.5 unit Plantium Taq, and 0.5 μL each of Bar-PCR primer (50μM) and Primer R (50μM). PCR cycling conditions were as follows: 5 min at 94°C; 25 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C; and 7 min at 72°C. The PCR products were purified and run using a MiSeq Benchtop for 2 × 300 bp paired-end sequencing (Illumina). The quality check was performed using PRINSEQ-lite 0.19.5. All sequences were aligned using UCLUST v1.1.579 (http://www.drive5.com/uclust/downloads1_1_579.html), and complete linkage clustering was used to define operational taxonomic units (OTUs) with 97% identity as a cutoff. Rarefaction analysis was performed using MOTHUR v.1.22.2 [29]. Diversity in the sample was estimated using the Shannon−Weiner index and the species richness was expressed as the number of OTUs by the nonparametric indices abundance-based coverage estimate (ACE) and Chao1 [30]. In addition, taxonomy assignment was performed using the Ribosomal Database Project (RDP) classifier [31].

2.3. Isolation of fungi

Fungal isolation was carried out using the dilution method. Soil sample (10 g) was mixed with 90 mL sterile water in a conical flask, and the soil solution was diluted from 10^{-1} to 10^{-3} after shaking for 1 h in an oscillator. A volume of 0.1 mL soil solution (10^{-3}) was coated evenly on potato dextrose agar with penicillin sodium salt (100 mg in 1 L medium) and kanamycin sulfate (50 mg in 1 L medium) to suppress bacterial growth. All plates were incubated at room temperature for 1 wk or until fungal growth was observed. Emergent hyphae were transferred and purified on sterile potato dextrose agar plates. Fungal isolates were grouped into morphotypes on the basis of their morphological differences.

2.4. Molecular identification and diversity analysis

Genomic DNA was extracted from pure cultures of a representative isolate of each fungal morphotype using the method of Guo et al [32]. The nuclear ribosomal internal transcribed spacer (ITS) regions including the *5.8S* gene were subsequently amplified using the primer set ITS1 and ITS4 [33]. The amplification was performed in a total volume of 50 μL containing 10−50 ng of genomic DNA, 0.4μM of each primer, 0.2mM dNTP, 2.5 unit *Easytaq* DNA polymerase, and 5 μL 10× *EasyTaq* buffer (TransGen Biotech Co., Ltd, Beijing, China). Conditions for amplification were as follows: an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C, 2 min at 72°C, and a final extension at 72°C for 10 min. Purified PCR products were directly sequenced

with primer pairs as mentioned above in the ABI 3730-XL DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA). DNA sequences were analyzed with DNASTAR version 5.03 software (DNASTAR, Inc., Madison, WI, USA), and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST searches.

2.5. Determination of antagonistic fungi against pathogens of root-rot disease

Antimicrobial activities of isolated fungi were determined by the agar diffusion method. The root-rot pathogens *A. panax*, *F. oxysporum*, *F. solani*, and *P. herbarum* were used as the targets for antagonistic determination. Selected isolates were inoculated into 250 mL conical flasks, each with 50 mL of potato dextrose broth, and incubated at 28°C on a shaker at 170 rpm for 7 d. Then, 200 μL of culture suspension was added into a well (diameter: 5 mm) of the potato dextrose agar plate with pathogens. After incubation at 28°C for 4 d, the activity was expressed as the diameter of the growth inhibition zone (in mm). Each treatment was performed in triplicate.

3. Results

3.1. Fungal diversity and relative abundance in the rhizosphere soil of 2-yr-old P. notoginseng

After quality filtering, a total of 16,130 paired-end reads were generated from 2-yr-old *P. notoginseng* rhizosphere soil sample using an Illumina MiSeq platform, and the sequencing coverage reached 0.98. The average length of these reads was 346 bp. All reads were clustered into 860 OTUs containing 564 non-singletons and 296 singletons at 97% sequence similarity. Rarefaction curves showed that the number of OTUs observed increased with the number of sequences sampled, and reached the near plateau region at 97% similarity level (Fig. 1A). The number of OTUs observed was still lower than the number of OTUs estimated with the nonparametric Chao1 (Fig. 1B) and ACE (Fig. 1C) indices (1,243 and 1,199,

respectively) at 97% similarity. The Shannon diversity index (H' = 4.44, Fig. 1D) showed that the fungi were highly diverse in 2-yr-old *P. notoginseng* rhizosphere soil.

We assessed the distribution of fungal taxa at phylum and genus levels using the RDP classifier. They were assigned to five phyla and 79 genera. At phylum level, most fungal sequences were distributed in five phyla (Fig. 2A), Zygomycota (46.2%), Ascomycota (37.8%), Basidiomycota (5.9%), Chytridiomycota (1.6%), and Glomeromycota (1.2%), and others belonged to unclassified fungi. At genus level (Fig. 2B), *Mortierella* and unclassified Mortierellales accounted for a large proportion (44.9%). It is noteworthy that the relative abundance of *Fusarium* and *Phoma* sequences was high, accounting for 12.9% and 5.5%, respectively. Nearly all the *Fusarium* sequences were identified to be *F. oxysporum*, which was considered the major pathogen of root rot in *P. notoginseng* [22]. The taxa below 0.7% were grouped together and represented as "other" in Fig. 2B.

3.2. Identification and diversity of isolated fungi

A total of 113 fungal isolates were obtained from the rhizosphere soil of *P. notoginseng*; among them 43 morphotypes were recognizable based on their morphological characteristics. One isolate of each morphotype was used for phylogenetic analysis. ITS sequences of the 43 isolates were sequenced and blasted in GenBank, and the closest matches, the accession number in GenBank (Nos. KP230805−KP230847), are listed in Table 1. The phylogenetic analysis was performed with MEGA 5 program (http://www.megasoftware.net/); the resulting phylogenetic tree of all sequences obtained in this study, together with selected GenBank sequences of close relatives, is shown in Fig. 3.

Based on the phylogenetic analysis of the 99 sequences (43 isolates and 56 sequences from GenBank), the isolates were distributed into two major groups. Forty-two isolates belonging to ascomycetes were grouped together in the first group, and the second group consisted of one zygomycetesisolate (PH30042). Five different fungal subgroups were identified, with four corresponding to Ascomycota − Sordariomycetes, Eurotiomycetes, Chaetothyriomycetes, and Dothideomycetes; and one subgroup belonging
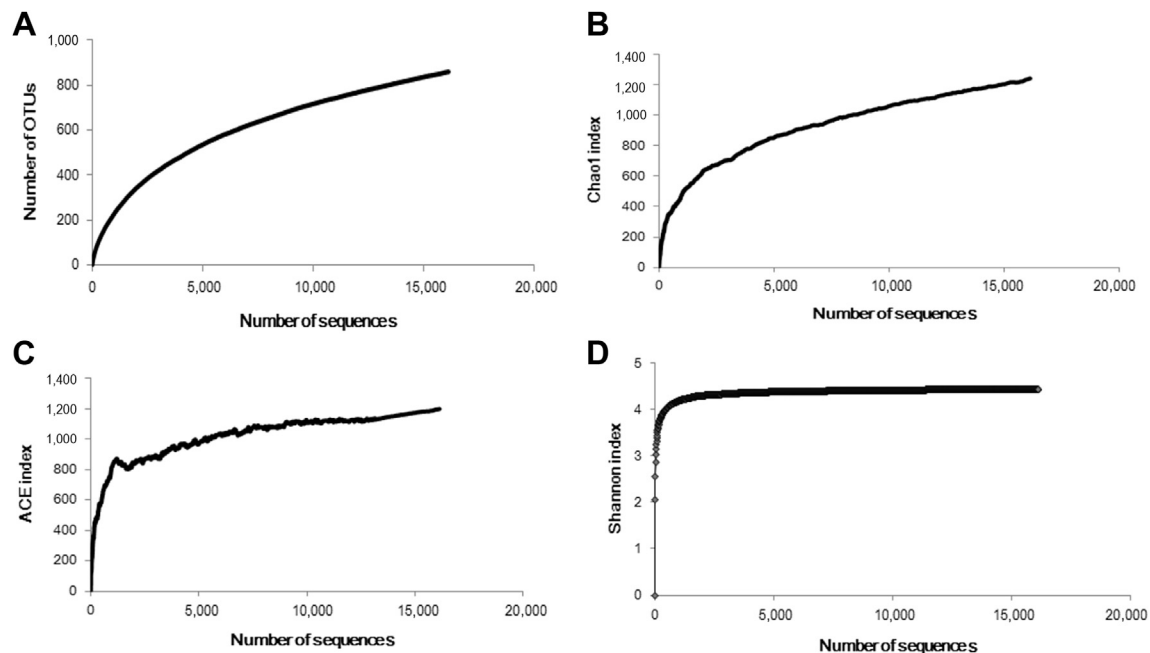
Fig. 1. Rarefaction curves showing the number of similarity-based OTUs at a cluster distance value of 0.03. Rarefaction analysis of (A) species richness, (B) Chao1 index, (C) ACE index, and (D) Shannon index. Four rarefaction curves reached the near plateau phase, representing good sampling depth.
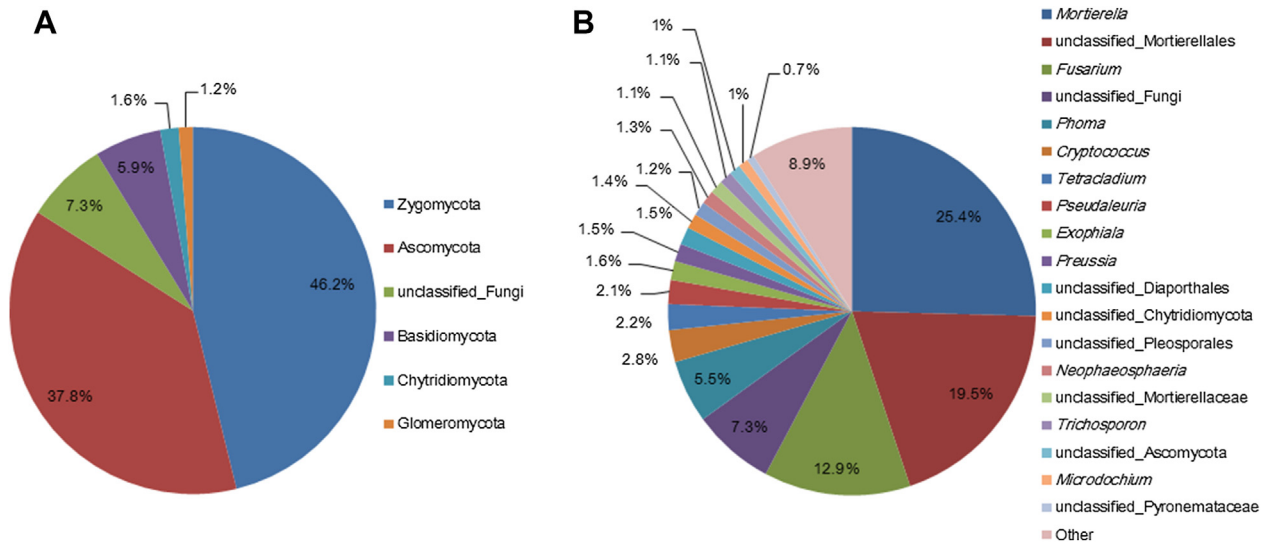
Fig. 2. Proportional distribution of fungal taxa. Distribution of fungal (A) phyla and (B) genera present in rhizosphere soil of 2-year-old healthy *Panax notoginseng*, as revealed by MiSeq sequencing. "Other" in Fig. 2B represents all the taxa below 0.7%.

to Zygomycota, Zygomycetes. Sordariomycetes was the most dominant class (59.3%) comprising three orders (Hypocreales, Sordariales, and Xylariales) and a fungal strain of *Incertae sedis* (PH30006), and 23 species. Dothideomycetes (24.76%) included two orders, Pleosporales and Capnodiales, and 14 species.

Among the 43 identified fungal isolates, *Mortierella alpina*, *F. oxysporum*, and *F. solani* were the dominant species, which accounted for 6.19%, 5.31%, and 5.31% of the total isolates, respectively. *Fusarium* was the most isolated genus with 24 isolates (21.24%) and also the most diverse genus with six species, followed by *Phoma* and *Pestalotiopsis*, representing 7.96% and 5.3% of the total isolates (each with 4 species), respectively. Although *Mortierella* accounted for 6.19% of the total isolates, the genus contained only one species. Species richness ($S = 43$) and Shannon diversity index ($H' = 3.62$) showed that the fungal species diversity was high in the rhizosphere soil of 2-yr-old *P. notoginseng* (Table 1).

3.3. Distribution of fungi with antimicrobial activity

Thirty-five of the 43 fungal isolates were challenged by four pathogenic fungi causing root rot of *P. notoginseng*, excepting eight potential pathogenic fungi, PH30010, PH30011, PH30018, PH30026, PH30033, PH30038, PH30043, and PH30045. Thirteen isolates (37.1%) displayed antimicrobial activity against at least one pathogenic fungus (Table 2). The antagonists were distributed in nine genera, mainly in *Pestalotiopsis* (3 species), *Cladosporium* (2 species), *Trichoderma* (2 species), and *Chaetomium* (2 species) (Table 2). Of the 13 isolates, eight were active against *A. panax*, 10 demonstrated activity against *F. oxysporum*, five displayed activity against *F. solani*, and only one showed weak activity against *P. herbarum*. Isolate PH30019 exhibited antimicrobial activity against all pathogen fungi tested. Four isolates (PH30007, PH30016, PH30019, and PH30035) exhibited moderate or strong antimicrobial activity against *F. oxysporum* and *F. solani*.

4. Discussion

Fungal diversity analysis using high-throughput sequencing techniques is becoming more common [34]. Compared to 454 pyrosequencing, Illumina sequencing has more advantages under the same effect [15,35]. Furthermore, the MiSeq platform can

generate longer paired-end reads (2×300 bp). To our knowledge, a few studies have used MiSeq as a sequencing platform to study diversity and community composition of microbes [36−38]. Therefore, in this study, this new sequencing technique was employed to analyze the fungal diversity in the rhizosphere soil of *P. notoginseng* for the first time. A total of 16,978 raw reads were generated from the sequencer, and 16,130 sequences (95%) passed the quality filtering. A total of 860 OTUs were identified using a 97% cutoff during sequence clustering. The number of OTUs observed was lower than that of OTUs estimated with the nonparametric ACE and Chao1 indices at 97% similarity. Zygomycota was the dominant fungal phylum and represented 46.2% of all fungal DNA sequences, in which *Mortierella* accounted for a high relative abundance. *Mortierella* is ubiquitous in the bulk and rhizosphere soil from a range of temperate and tropical forests and agricultural habitats. A large number of *Mortierella* species exist in the rhizosphere soil of *P. notoginseng*, inferring that they play a potential role in keeping microecological balance as protective microbes to suppress soilborne pathogens by competing for nutrition, or assisting the host plants with the uptake of phosphorus and nitrogen. Curlevski et al [39] suggested that *Mortierella* spp. might be an important component of phosphorus cycling.

DNA barcoding is increasingly applied to catalogue and classify biodiversity [40]. Generally, in culture-independent researches, fungal diversity is studied based on the analysis of either the small subunit or the ITS region of the nuclear ribosomal RNA gene. Owing to its higher rate of evolution than the small subunit fragment, the ITS region has been selected as the universal barcode for fungal identification [41]. Due to length constraints of high-throughput metabarcoding, early metabarcoding studies were restricted to the use of either ITS1 or ITS2 [42,43]. However, because of the difference between the rates of evolution of ITS1 and ITS2, the composition of fungal communities characterized by ITS1 or ITS2 may be different. Bazzicalupo et al [40] suggested that ITS2 may be more variable and recovers higher operational taxonomic riches than ITS1, although both fragments performed equally well when evaluating community structures. Monard et al [34] proposed that ITS1 appeared to be a better choice for sequencing, and analysis of both ITS regions were complementary. In addition, selection of the primer is very important to reduce the quantity of the out-targeted sequences and chimeras. Ihrmark et al [28] evaluated the new

Table 1
The analysis of rhizospheric fungi associated with *Panax notoginseng* with the related species based on BLASTN search and their relative abundance

Isolate	Closest species in GenBank (accession No.)	Sequence coverage (%)	Max. identity (%)	Taxon (accession no.)	Relative abundance (%)
	Sordariomycetes				
PH30033	*Plectosphaerella* sp. FJ430715	100	99	*Plectosphaerella* sp. KP230834	3.54
	Plectosphaerella cucumerina JQ796755	99	99		
PH30017	*Gibellulopsis nigrescens* KC156644	99	100	*Gibellulopsis nigrescens* KP230818	1.77
PH30029	*Gliomastix masseei* AB540554	100	99	*Gliomastix masseei* KP230830	2.66
PH30019	*Trichoderma koningiopsis* KF494183	100	99	*Trichoderma* sp.1 KP230820	2.66
	Trichoderma gamsii KJ680371	100	99		
PH30004	*Trichoderma harzianum* FJ442660	100	100	*Trichoderma* sp.2 KP230805	1.77
	Trichoderma aureoviride AF359400	100	100		
PH30008	*Paecilomyces marquandii* KM203617	100	99	*Paecilomyces marquandii* KP230809	4.43
PH30022	*Purpureocillium lilacinum* FJ765024	100	99	*Purpureocillium lilacinum* KP230823	0.88
PH30018	*Cylindrocarpon* sp. HQ637289	100	99	*Cylindrocarpon* sp.1 KP230819	1.77
PH30026	*Cylindrocarpon* sp. AB725901	100	97	*Cylindrocarpon* sp.2 KP230827	2.66
PH30045	*Fusarium solani* HE974455	100	99	*Fusarium solani* KP230846	5.31
PH30025	*Fusarium chlamydosporum* KF494035	100	100	*Fusarium chlamydosporum* KP230826	3.54
PH30010	*Fusarium oxysporum* JX573316	100	100	*Fusarium oxysporum* KP230811	5.31
PH30012	*Fusarium tricinctum* KJ598867	100	100	*Fusarium* sp. KP230813	3.54
	Fusarium acuminatum KF887097	100	100		
PH30034	*Fusarium merismoides* AB586998	100	99	*Fusarium merismoides* KP230835	0.88
PH30043	*Fusarium flocciferum* JN676123	100	100	*Fusarium flocciferum* KP230844	2.66
PH30011	*Volutella ciliata* HQ703419	100	99	*Volutella ciliata* KP230812	3.54
PH30007	*Chaetomium megalocarpum* KC109743	100	99	*Chaetomium* sp. KP230808	2.66
	Chaetomium globosum JN209870	100	99		
PH30021	*Chaetomium globosum* KF053680	100	99	*Chaetomium globosum* KP230822	1.77
PH30006	*Arthrinium arundinis* KF144888	100	99	*Arthrinium* sp. KP230807	0.88
	Arthrinium sacchari AB693901	100	99		
PH30020	*Pestalotiopsis cocculi* EF055191	99	99	*Pestalotiopsis* sp.1 KP230821	0.88
	Pestalotiopsis caudata EF055188	99	99		
PH30024	*Pestalotiopsis disseminata* HQ607992	100	99	*Pestalotiopsis* sp.2 KP230825	0.88
	Pestalotiopsis vismiae EF055218	100	99		
	Pestalotiopsis neglecta EF055222	100	99		
PH30031	*Pestalotiopsis lespedezae* GQ869901	100	100	*Pestalotiopsis lespedezae* KP230832	1.77
PH30044	*Pestalotiopsis neglecta* EF055213	99	100	*Pestalotiopsis* sp.3 KP230845	1.77
	Pestalotiopsis lespedezae EF055206	99	100		
PH30046	*Sarocladium strictum* GQ376096	100	100	*Sarocladium strictum* KP230847	1.77
	Eurotiomycetes				
PH30035	*Penicillium chrysogenum* AY373902	100	100	*Penicillium* sp. KP230836	2.66
	Penicillium allii-sativi KJ775600	100	100		
PH30009	*Penicillium vancouverense* JN617675	100	99	*Penicillium vancouverense* KP230810	1.77
PH30005	*Aspergillus nidulans* AY373888	100	100	*Aspergillus nidulans* KP230806	2.66
	Chaetothyriomycetes				
PH30028	*Exophiala pisciphila* NR121269	100	99	*Exophiala pisciphila* KP230829	2.66
	Dothideomycetes				
PH30027	*Microsphaeropsis arundinis* JQ344344	100	99	*Microsphaeropsis arundinis* KP230828	1.77
PH30041	*Paraconiothyrium brasiliense* KJ767099	92	98	*Paraconiothyrium* sp. KP230842	0.88
PH30037	*Paraconiothyrium cyclothyrioides* KC215138	100	100	*Paraconiothyrium cyclothyrioides* KP230838	1.77
PH30036	*Peyronellaea pinodella* KM030324	100	100	*Peyronellaea pinodella* KP230837	0.88
PH30030	*Phoma* sp. JN207319	100	99	*Phoma* sp.1 KP230831	1.77
PH30038	*Phoma herbarum* KJ767079	100	99	*Phoma herbarum* KP230839	3.54
PH30032	*Tetraplosphaeria sasicola* NR119404	99	97	*Tetraplosphaeria* sp. KP230833	0.88
PH30039	*Phaeosphaeriopsis* sp. JQ936185	100	99	*Phaeosphaeriopsis* sp. KP230840	1.77
PH30040	*Phoma* sp. JQ238617	94	95	*Phoma* sp.2 KP230841	0.88
PH30013	*Phoma medicaginis* KF850377	99	98	*Phoma* sp.3 KP230814	1.77
PH30023	*Pyrenochaeta* sp. HQ914829	100	99	*Pyrenochaeta* sp. KP230824	0.88
PH30016	*Alternaria tenuissima* KM921667	100	100	*Alternaria* sp.2 KP230817	3.54
	Alternaria alternata KM371730	100	100		
PH30015	*Cladosporium cladosporioides* HQ832794	100	100	*Cladosporium cladosporioides* KP230816	2.66
PH30014	*Cladosporium cladosporioides* KJ589542	100	100	*Cladosporium* sp. KP230815	1.77
	Cladosporium tenuissimum KJ152168	100	100		
	Zygomycetes				
PH30042	*Mortierella alpina* FJ025167	100	100	*Mortierella alpina* KP230843	6.19
Shannon diversity index (*H'*)					3.62
Species richness (*S*)					43

primers—fITS7, gITS7, and fITS9—together with the ITS4 primer to amplify the ITS2 region by 454 sequencing. Primers fITS7 and fITS9 yielded a lower proportion of nonfungal sequences than gITS7. However, the fITS7 primer mismatched against most sequences of Mucorales. Mucoralean fungi are widely dispersed and occur in various habitats as saprobes in most ecosystems, such as soil, dung, dead plant material, and water [44]. Therefore, in this study, we

analyzed the fungal diversity in the rhizosphere soil of *P. notoginseng* by amplifying the fungal ITS2 region using the fITS9f/ ITS4 primer combination. The dataset obtained from the Illumina MiSeq platform was basically satisfactory and also supported the conclusion proposed by Ihrmark et al [28]

In pure-culture experiments, we isolated 113 fungal strains from the rhizosphere soil of 2-yr-old *P. notoginseng*. Based on the
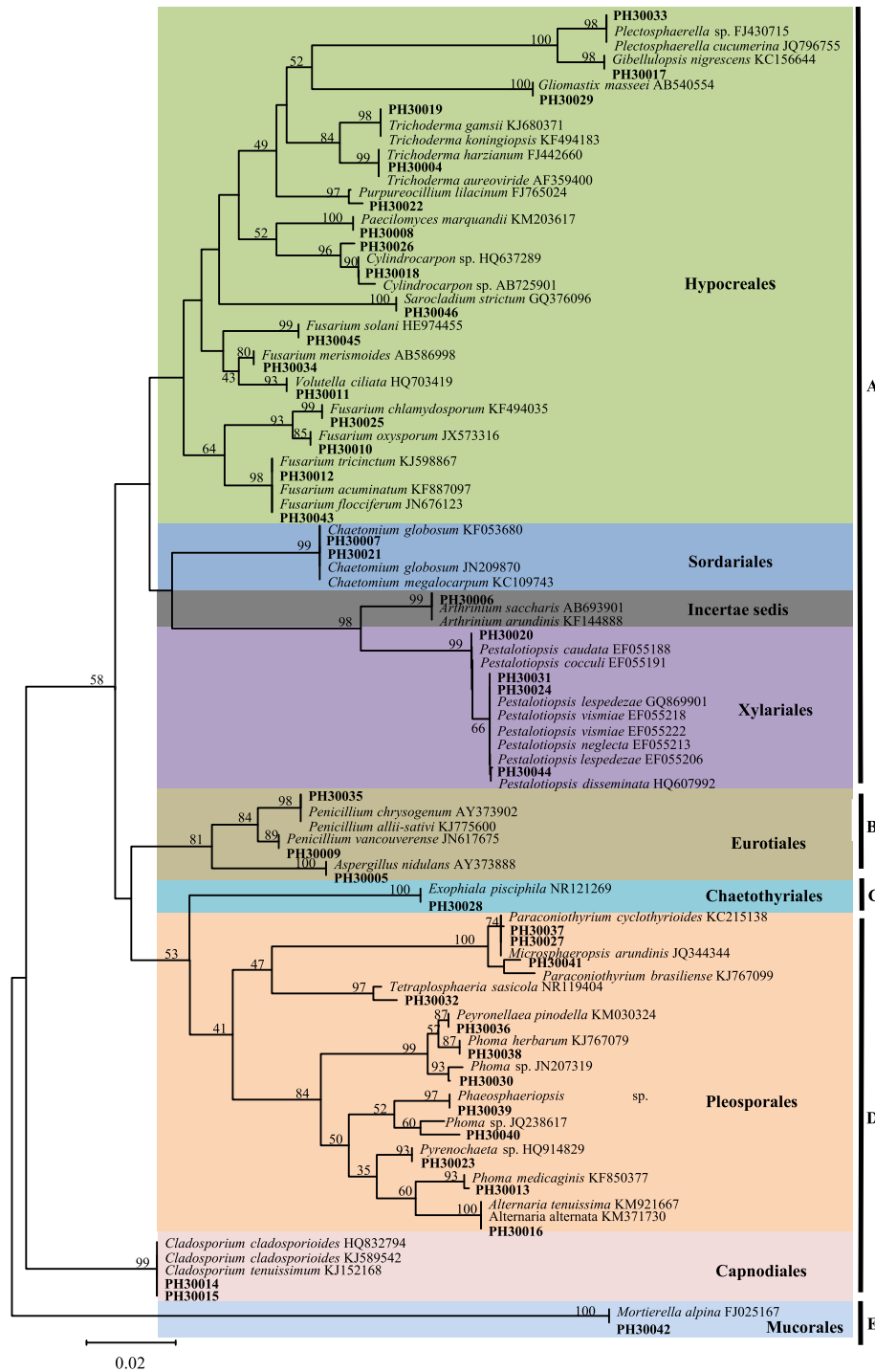
Fig. 3. Neighbor-joining analysis of ITS sequences of 43 taxa from *Panax notoginseng* showing the relationships of culturable fungi with closest relatives. Bootstrap values are shown at the branches (1,000 replicates). The name of some groups is shown in bold letters: A, Sordariomycetes; B, Eurotiomycetes; C, Chaetothyriomycetes; D, Dothideomycetes; and E, Zygomycetes.

phylogenetic analysis and morphological characteristics, the isolates were assigned to five classes, eight orders (except for an *I. sedis*), 26 genera, and 43 species (Table 1; Fig. 3). Chen et al [27] reported that *Penicillium*, *Trichoderma*, and *Fusarium* were the dominant fungal populations in ginseng rhizosphere soil in northeastern China. The composition, quality, and diversity distribution of soil fungi showed great differences between ginseng and notoginseng, but the proportion of pathogens was high in both plant rhizosphere soils. As evident from the results of culture-

independent experiments, the proportion of *Fusarium* and *Phoma* is high, suggesting that the plant is in danger of pathogen invasion, although it appears to be healthy at the sampling time (early in June). Root-rot diseases of *P. notoginseng* usually occur heavily in July and August, as environmental and weather conditions are optimal for the pathogens. The relatively high frequency of *Fusarium* and *Phoma* may give some interpretation. At the same time, antagonistic isolates were also detected and obtained during this study. The antagonists may be useful in inhibiting or suppressing

Table 2
Antagonistic activities of fungal isolates against pathogenic fungi of *Panax notoginseng*

solate No.	Diameter of the growth inhibition zone (mm)			
	Alternaria panax	*Fusarium oxysporum*	*Fusarium solani*	*Phoma herbarum*
PH30004		10	8	
PH30007		10	11	
PH30014	7	8		
PH30015	8	8		
PH30016		12	14	
PH30017		11		
PH30019	9	13	15	7
PH30020	11			
PH30021	9	9		
PH30024	10			
PH30027		12		
PH30031	9			
PH30035	12	10	10	

the growth of potential root-rot pathogenic fungi in the soil ecological systems. In our previous work, we found a fungus *Aspergillus versicolor* (PH30001) from the rhizosphere soil of 1-yr-old healthy *P. notoginseng*, which produced antagonistic compounds against *F. solani* [45]. Further in-depth studies are needed to investigate the ecological effects of the antagonists. *Cylindrocarpon* species were the major pathogens causing root rot of *P. ginseng* and *P. quinquefolius* [46,47]. Miao et al [22] reported that *C. destructans* and *C. didynum* caused root rot of *P. notoginseng* with weak pathogenicity but wide distribution. Two *Cylindrocarpon* isolates (PH30018 and PH30026) were also obtained during this study. However, *Cylindrocarpon* was not detected in high-throughput sequencing tests. For this case, it may be associated with primer design or various quality-control approaches or database selection. U'Ren et al [48] also confirmed that primer selection influenced pyrosequencing-based inferences of diversity and community composition.

Most environmental microorganisms are unculurable in commonly used media and culture conditions. Rhizosphere is a unique environment, where beneficial microbes and pathogens have an important influence on the growth and health of plants [49]. Root exudates also play an important role in plant–microorganism interactions and functioning of the rhizosphere [50]. Moreover, fertilizers, fungicides, and other agrochemicals applied can also affect the composition and structure of soil microbial communities that may be closely linked with ecosystem functions [51–53]. These findings suggest that more attention should be devoted to changes in the microbial communities. Improving and combining pure-culture and culture-independent techniques can contribute to a better understanding of rhizosphere ecology. This will be helpful in improving the management of notoginseng planting.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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