

Culturable Endophytes Diversity Isolated from *Paeonia ostii* and the Genetic Basis for Their Bioactivity

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Abstract

Paeonia ostii is known for its excellent medicinal values as Chinese traditional plant. To date, the diversity of culturable endophytes associated with *P. ostii* is in its initial phase of exploration. In this study, 56 endophytic bacteria and 51 endophytic fungi were isolated from *P. ostii* roots in China. Subsequent characterization of 56 bacterial strains by 16S rDNA gene sequence analysis revealed that nine families and 13 different genera were represented. All the fungal strains were classed into six families and 12 genera based on ITS gene sequence. The biosynthetic potential of all the endophytes was further investigated by the detection of putative polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes. The PCR screens were successful in targeting thirteen bacterial PKS, five bacterial NRPS, ten fungal PKS and nine fungal NRPS gene fragments. Bioinformatic analysis of these detected endophyte gene fragments facilitated inference of the potential bioactivity of endophyte bioactive products, suggesting that the isolated endophytes are capable of producing a plethora of secondary metabolites. These results suggest that endophytes isolated from *P. ostii* had abundant population diversity and biosynthetic potential, which further proved that endophytes are valuable reservoirs of novel bioactive compounds.

Key words: *Paeonia ostii*, endophytes, diversity, polyketide synthase, nonribosomal peptide synthetase

Introduction

Endophytes are bacterial (including actinomycetes) or fungal microorganisms that spend part of or their entire life cycle inter- and/or intra-cellularly, colonizing healthy tissues of host plants without manifesting apparent symptoms of disease (Wani et al. 2015). These microorganisms produce numerous novel bioactive products, such as antibiotics, anticancer reagents, biological control agents, and other useful bioactive compounds for medical, agricultural, and industrial uses (Nisa et al. 2015; Venugopalan and Srivastava 2015). Thus, researchers have shown interest in bio-prospecting of endophytic microbial communities inhabiting plants from different ecosystems.

Medicinal plants are rich sources of precious bioactive compounds. Given their long-term association with each other, medicinal endophytes participate in metabolic pathways of medicinal plants, thereby producing analogous or novel bioactive compounds (Egamberdi-

eva et al. 2017). Endophytic fungi of medicinal plants provide various bioactive secondary metabolites with unique structures; these metabolites include alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, and xanthenes (Aly et al. 2011; Deshmukh et al. 2015). Meanwhile, endophytic bacteria are presumed capable of producing a wide range of undescribed metabolites. Novel antibiotics produced by endophytic bacteria of medicinal plants include ecomycins, pseudomycins, munumbicins, and kakadumycins. Therefore, endophytes of medicinal plants are considered potential sources of novel bioactive compounds.

Recent studies have demonstrated that active compounds of endophytes can be categorized under two classes, namely, polyketides and nonribosomal peptides, which are biosynthesized by polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) systems (Amoutzias et al. 2016). A typical modular PKS is composed of acyltransferase (AT), ketosynthase

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(KS), and acyl carrier protein domains (Crawford et al. 2009). KS domain condenses an extender unit onto the growing polyketide chain during polyketide biosynthesis and is involved in production of structurally diverse metabolites. Encoded NRPSs are composed of multiple modules. Each module consists of an adenylation (A) domain, which facilitates selection and activation of amino acids; a peptidyl carrier protein domain; and a condensation (C) domain, which catalyzes peptide bond formation between two amino acids (Strieker et al. 2010). Each module spans 1000–1100 amino acids long; according to collinearity rule, the number and order of modules define the number and order of amino acids in NRPs, respectively (Nikolouli and Mossialos 2012).

Recent advances in development of bioinformatics tools have enabled application of functional gene-based molecular screening strategy for rational selection of endophytes; such biosynthetic screening strategy offers promising potential for targeted drug discovery. Genes for PKSs and NRPSs are among the most intensively encoded in endophytic microbial genomes. PKS and NRPS pathways are suitable targets for functional gene-based screening because of their roles in biosynthesis of many bioactive microbial metabolites (Evans et al. 2011). Using a PKS gene-based molecular screening strategy, researchers have discovered a new polyketide from endophytic fungi isolated from *Salicornia herbacea* containing PKS I genes (Wang et al. 2014). Therefore, encoding biosynthesis genes of PKSs and NRPSs in endophytes can be used as indicator in screening different endophytic strains to isolate novel polyketides and nonribosomal peptides.

Moutan cortex, which is the root bark of the medicinal tree peony *Paeonia ostii*, contains numerous pharmacological compounds with anti-microbial, anti-inflammatory, and anti-cancer activities (Lau et al. 2007). Various compounds that have been identified in the Moutan cortex include paeonoside, paeonolide, apiopaeonoside, paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, benzoylpaeoniflorin, paeonol, and sugars (Chen et al. 2006a). Although considerable research effort has been devoted to biodiversity of endophytic microorganisms associated with medicinal plants, limited studies have explored the diversity and biosynthetic potential of endophytes in the medicinal tree peony. Thus, we performed 16S rRNA and ribosomal internal transcribed spacer (ITS) gene sequence analysis to investigate species diversity of culturable endophytic bacteria and fungi of *P. ostii* collected from Henan Province, China. We used degenerate primers of PKS and NRPS genes to screen the potential capacity of endophytes to synthesize secondary metabolites. The objective of this endeavor is to bio-prospect endophyte resources that can be potentially applied in pharmaceutical and agricultural fields.

Experimental

Materials and Methods

Sample collection and endophyte isolation. Samples for endophyte isolation were collected in April 2016 from a *P. ostii* plantation within and surrounding Luoyang City (34°43' N, 112°24' E). The area is characterized by a temperate monsoon climate with a mean annual temperature of 14.9°C and mean annual rainfall of ≈ 530 to 600 mm. The soils of this area are cinnamon soils derived from carbonatite. The fifty individual plants, and each of plants, representing about 10-year growth, were selected randomly. The plant root tissues were dug, transferred into sterile biosafety bags, and transported to the laboratory for further analysis. The samples were washed in running tap water to remove the clays on the surface of root tissue and checked for disease symptoms or superficial damage. Symptom-free root samples were then separated into 10 pieces, and each of pieces, containing 5 g root tissues. Surface sterilization was performed on 2–3 cm tissue samples according to methods described by Guo et al. (2003). The samples were sterilized by washing with 70% ethanol for 1 min, followed by immersion in 3% sodium hypochlorite for 2 min and rinsing with 70% ethanol for 30 s. Disinfected samples were washed with sterilized water for five times and then drained (Potshangbam et al. 2017).

To isolate endophytic bacteria, surface-sterilized root segments were ground by pestle and mortar with 3 ml phosphate-buffered saline buffer (pH 7.0). Then, 100 µl aliquots of suspension were plated on tryptic soy agar (TSA) medium and Luria-Bertani (LB). Plates were incubated at 28°C for 3–5 days. Single colonies displaying different morphological characteristics were selected and stored in 15% glycerol at –80°C for further analyses. To isolate endophytic fungi, root tissues were cut into 0.5 cm pieces and placed on potato dextrose agar (PDA) medium. Five plant fragments were placed on each plate. In total, 280 root segments from 50 individual *P. ostii* plants were investigated. Plates were incubated at 25°C for 5–20 days in the dark and checked regularly. A mycelium from the colony margin was transferred to a new petri dish containing PDA medium to achieve single conidial selection. To determine successful sterilization, sterile distilled water used in the final rinse was plated on fresh TSA, LB and PDA plates, incubated at 28°C for 7 days, and then examined for any remaining epiphytic microorganism.

Identification of endophytic bacteria and fungi. The endophytic bacteria were identified according to the analysis of 16S rDNA. The endophytic fungi were identified according to morphology of the fungal culture, and the internal transcribed spacer sequences of nuclear ribosomal DNA (ITS1-5.8S-ITS2 rDNA

Table I
PCR primers for identification and screening the biosynthetic genes of endophytic isolates.

Primer name	Primer Sequence (5'-3')	Target gene	Product size (bp)	References
24F	AGAGTTTGATC(A/C)TGGCTCAG	16S rDNA	1400–1500	Heuer et al. 1997
1492R	TACGG(C/T)TACCTTGTACGACTT			
ITS1	TCCGTAGGTGAACCTGCGG	ITS	500	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC			
KSaF	TSGCSTGCTTGGAYGCSATC	Bacterial PKS	600–700	Metsä-Ketalä et al. 1999
KSaR	TGGAANCCGCCGAABCCGCT			
A3F	GCSTACSYSATSTACACSTCSGG	Bacterial NRPS	700	Ayuso-Sacido and Genilloud 2005
A7R	SASGTCVCCSGTSGCG TAS			
KAF1	GARKSICAYGGIACIGGIAC	Fungal PKS	700–800	Amnuaykanjanasin et al. 2005
KAR1	CCAYTGIGCICCRTGICCIGARAA			
AUG003	CCGGCACCACCGNAARCCHAA	Fungal NRPS	600–700	Slightom et al. 2009
AUG007	CCGGACCATGTCGCCNGTBYKRTA			

sequence). Genomic DNA of endophytic isolates was extracted using Genomic DNA Kits (TaKaRa, China) in accordance with manufacturer's protocol. Bacterial and fungi isolates were identified by partial sequencing of 16S rDNA and ITS, respectively. Table I provides 16S rDNA and ITS gene primers used in this study. The 50 µl polymerase chain reaction (PCR) reaction mixture contained 100 ng of DNA extract, 1 × Taq reaction buffer, 20 pmol of each primer, 200 µM of each deoxynucleotide triphosphates, and 1.5 U of Taq DNA polymerase (Promega, USA). After initial denaturation at 94°C for 5 min, thermal cycling proceeded as follows: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. After 35 cycles, the final extension step proceeded at 72°C for 10 min. PCR products were purified using TIANquick Midi Purification Kits (Tiangen, China) and sequenced at Sangon Biotech Co. Ltd (Shanghai, China). All obtained sequences were compared with those in the GenBank database by using the BLASTN search program. Similar sequences were further aligned by CLUSTALX (version 1.81). A phylogenetic tree was constructed based on evolutionary distance data by using MEGA software (version 5.0) (Tamura et al. 2011). Phylogenetic analysis was conducted by neighbor-joining method. Bootstrap analysis was performed with 1000 replications to determine the support for each clade.

Detection and analysis of biosynthetic genes PKS and NRPS. Four sets of degenerate primers targeting genes encoding PKS and NRPS were used to screen biosynthetic potential of bacterial and fungal isolates. Utilized primers are listed in Table I. PCR reaction mixture was the same as that described in the previous section. Genes were amplified using the following protocol: a denaturation step at 94°C for 5 min; 35 amplification cycles at 94°C for 1 min; 57°C (for A3F-A7R), 58°C (for KSaF-KSaR), or 60°C (for KAF1-KAR1 and

AUG003-AUG007) for 1 min; 72°C for 2 min; and a final extension at 72°C for 5 min. Degenerate PCR products containing multiple sequences were cloned using pGEM[®]-T Easy cloning kits for sequencing (Promega, USA) and transformed into *Escherichia coli* DH5α. A clone library was established in accordance with manufacturer's instructions. Transformants were screened under standard PCR conditions and sequenced at Sangon Biotech Co. Ltd (Shanghai, China). To perform PKS and NRPS gene fragment analysis, an ExPASy translation tool was employed to identify amino acid sequences from DNA sequences (Gasteiger 2003). Amino acid substrates, which were recognized by the A domain binding pockets, were predicted using the NRPSpredictor analysis tool available at NRPSpredictor2 (<http://nrps.informatik.uni-tuebingen.de/Controller?cmd=SubmitJob>) (Rottig et al. 2011). All the obtained amino acid sequences were compared with those in the GenBank database by using the BLASTP search program. Similar sequences were further aligned by CLUSTALX, and a phylogenetic tree was constructed based on evolutionary distance data by using MEGA software (version 5.0).

Results

Isolation of endophytic bacteria and fungi from *P. ostii* root tissues. Healthy *P. ostii* root tissues were used for endophyte isolation. No colonies emerged after the final rinsing in sterilization, suggesting that surface sterilization was effective, and that subsequent isolates were endophytes. Colony morphology of endophytic isolates on TSA, LB and PDA media indicated that 56 bacteria and 51 fungi isolates were obtained, implying that a highly diverse range of bacterial and fungi endophytes was derived from root tissues of *P. ostii*.

Table II
The distribution of endophytic bacteria and fungi within *Paeonia ostii*.

Genera	No. of isolates	Relative abundance (%)
Endophytic bacteria		
<i>Streptomyces</i>	Md1-1, Md1-2, Md1-3, Md1-18	7.1
<i>Promicromonosporaceae</i>	Md1-20	1.8
<i>Microbacterium</i>	Md1-5, Md1-29	3.6
<i>Citricoccus</i>	Md1-48	1.8
<i>Bacillus</i>	Md1-4, Md1-6, Md1-8, Md1-9, Md1-10, Md1-11, Md1-12, Md1-13, Md1-15, Md1-16, Md1-17, Md1-19, Md1-21, Md1-22, Md1-24, Md1-25, Md1-26, Md1-31, Md1-33, Md1-35, Md1-36, Md1-39, Md1-37, Md1-41, Md1-42, Md1-43, Md1-44, Md1-45, Md1-46, Md1-50, Md1-51	55.4
<i>Psychrobacillus</i>	Md1-27	1.8
<i>Lysinibacillus</i>	Md1-14, Md1-30	3.6
<i>Planococcus</i>	Md1-49	1.8
<i>Xanthomonas</i>	Md1-23, Md1-28	3.6
<i>Pseudomonas</i>	Md1-34	1.8
<i>Serratia</i>	Md1-32, Md1-47	3.6
<i>Enterobacter</i>	Md1-38, Md1-40, Md1-52, Md1-53, Md1-56	8.9
<i>Lelliottia</i>	Md1-7, Md1-54, Md1-55	5.4
Endophytic fungi		
<i>Cylindrocarpon</i>	Mdf-3, Mdf-10, Mdf-13, Mdf-15, Mdf-36, Mdf-38, Mdf-43, Mdf-47	15.7
<i>Fusarium</i>	Mdf-5, Mdf-6, Mdf-7, Mdf-8, Mdf-11, Mdf-18, Mdf-22, Mdf-23, Mdf-25, Mdf-28	19.6
unclassified <i>Nectriaceae</i>	Mdf-26	2.0
<i>Thelonectria</i>	Mdf-1, Mdf-17	4.0
<i>Cephalosporium</i>	Mdf-4, Mdf-48	4.0
<i>Leptosphaeria</i>	Mdf-2, Mdf-9, Mdf-12, Mdf-16, Mdf-20, Mdf-29, Mdf-33, Mdf-34, Mdf-37, Mdf-40, Mdf-46	21.6
<i>Alternaria</i>	Mdf-27, Mdf-30, Mdf-31, Mdf-32, Mdf-39, Mdf-41, Mdf-42, Mdf-44, Mdf-45, Mdf-49, Mdf-50, Mdf-51	23.5
<i>Acrocalymma</i>	Mdf-35	2.0
<i>Cladosporium</i>	Mdf-14	2.0
<i>Macrophomina</i>	Mdf-19	2.0
<i>Phomopsis</i>	Mdf-24	2.0
<i>Mucor</i>	Mdf-21	2.0

Identification and phylogenetic analysis of endophytic bacteria and fungi. Isolates were classified by performing DNA sequencing of 16S rDNA genes of bacteria. PCR products of all 56 bacteria isolates were obtained from 16S rDNA, generating approximately 1400–1500 bp gene fragments. Similarly, PCR products of all 51 fungi isolates were acquired from ITS, generating approximately 400–500 bp gene fragments. PCR products were sequenced and then analyzed by BLASTN. All isolates exhibited 96–100% similarity to sequences in the National Center for Biotechnology Information (NCBI) database. Sequences with the highest similarities were further aligned by CLUSTALX, and phylogenetic trees were constructed. The 16s rDNA and ITS gene sequences were deposited in the GenBank

with accession numbers MF581407 – MF581462 and MF574221 – MF574271, respectively.

The resulting phylogenetic tree of bacterial isolates (Fig. 1) revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support. All bacterial isolates from *P. ostii* roots belonged to three phyla (i.e., Actinobacteria, Firmicutes, and Proteobacteria), clustered into groups corresponding to six taxonomic orders (i.e., Streptomycetales, Micrococcales, Bacillales, Xanthomonadales, Pseudomonadales, and Enterobacterales), and further classified into nine families and 13 genera. Majority of the isolates were categorized under *Bacillus* (55.36%), followed by *Enterobacter* (8.93%) and *Streptomyces* (7.14%) (Table II). As the

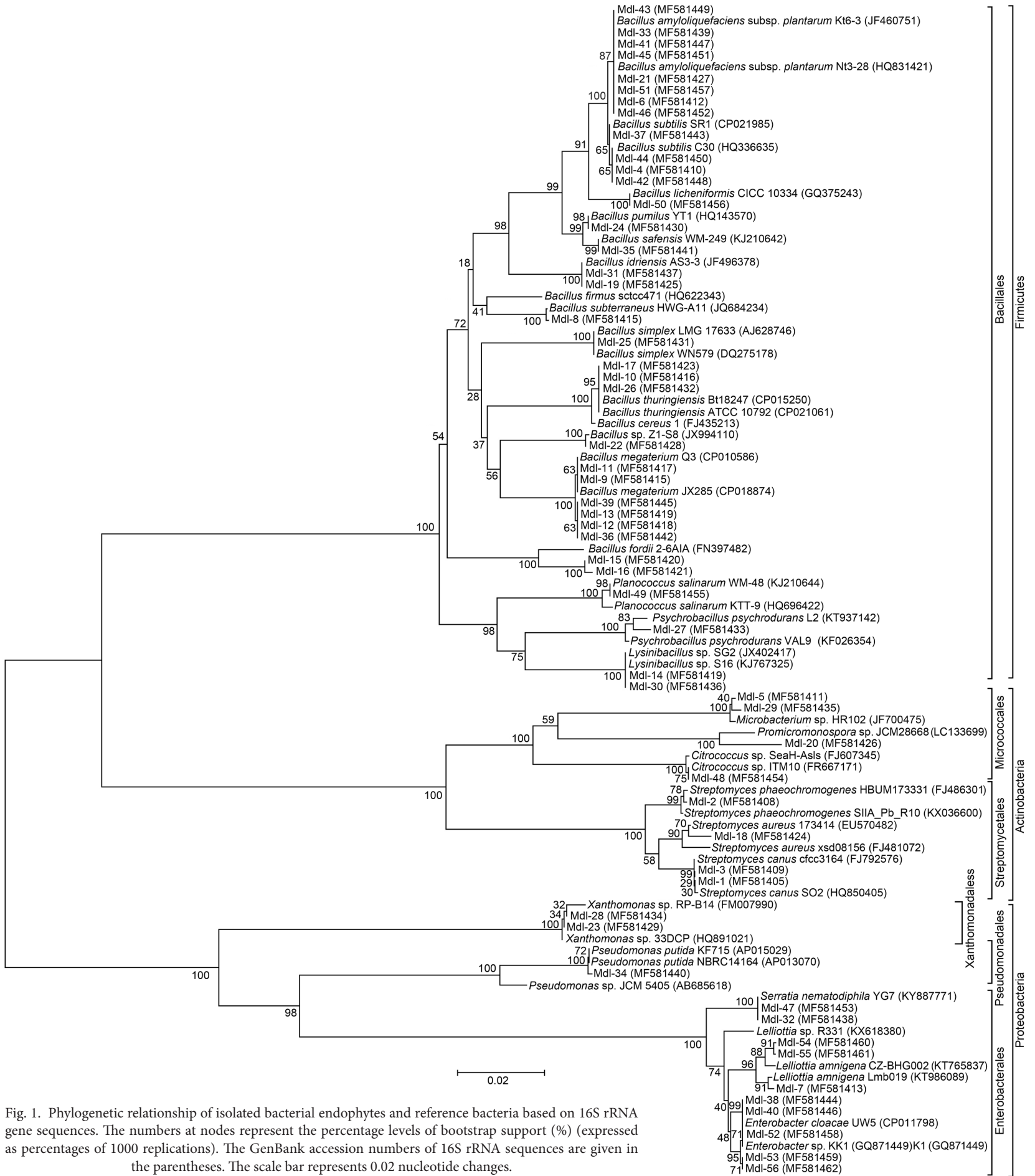


Fig. 1. Phylogenetic relationship of isolated bacterial endophytes and reference bacteria based on 16S rRNA gene sequences. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The GenBank accession numbers of 16S rRNA sequences are given in the parentheses. The scale bar represents 0.02 nucleotide changes.

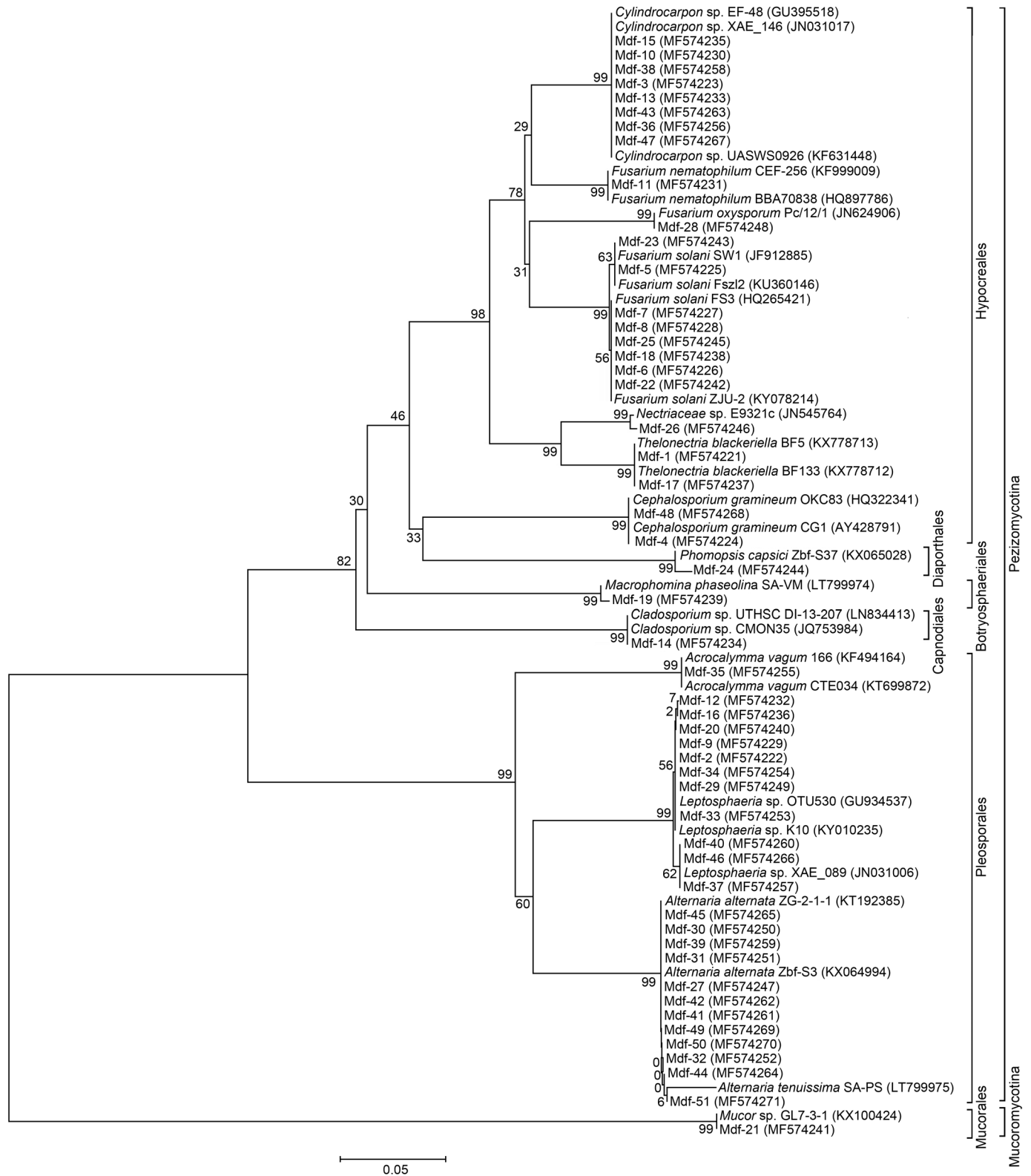


Fig. 2. Phylogenetic relationship of isolated fungal endophytes and reference fungal based on ITS gene sequences. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The GenBank accession numbers of ITS sequences are given in the parentheses. The scale bar represents 0.05 nucleotide changes.

most abundant genus of isolates, *Bacillus* displayed high diversity of phylogenetically related species. Specifically for *Bacillus*, eight isolates showed an identical sequence to that of *B. amyloliquefaciens*, six isolates presented an identical sequence to that of *B. megaterium*, four isolates displayed an identical sequence to that of *B. subtilis*, and the other isolates exhibited identical sequences to those of *B. cereus*, *B. fordii*, *B. idriensis*, *B. subterraneus*, *B. licheniformis*, *B. pumilus*, *B. safensis*, *B. simplex*, and *Bacillus* sp. (Fig. 1).

The resulting phylogenetic tree of fungal isolates (Fig. 2) revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support. According to phylogenetic tree, endophytic fungi obtained from *P. ostii* roots can be assigned to two different phyla (i.e., Pezizomycotina and Mucoromycotina). Pezizomycotina accounted for 98% of collected isolates, including Pleosporales (47.0% of total isolates), Hypocreales (45.0%), Capnodiales (2.0%), Botryosphaerales (2.0%), and Diaporthales (2.0%). Majority of the Pleosporales were represented by genera of *Leptosphaeria* (21.6%) and *Alternaria* (23.5%). The second most abundant order was Hypocreales, which was dominated by *Fusarium* (19.6%) and *Cylindrocarpon* (15.7%) species.

Mucoromycotina (*Mucor*) accounted for only 2.0% of total isolates (Table II).

Screening of endophytic bacterial and fungal PKS and NRPS gene fragments. Bacterial and fungal endophytes were surveyed for the presence of PKS and NRPS genes. Partial sequences of these complex biosynthesis genes were obtained from 15 out of 56 (27%) bacterial isolates, and positive results were achieved in 13 out of 56 (23%) and 5 out of 56 (9%) of the genetic screens for KS (PKS) and A (NRPS) domains, respectively. Three bacterial isolates, namely, Md1-2, Md1-24, and Md1-50, contained both putative PKS and NRPS genes (Table III). PCR screens of fungal endophytes revealed that 18 out of 51 (35%) fungal isolates contained either PKS (10 out of 51, 20%) or NRPS (9 out of 51, 18%). Only the fungal isolate Mdf-41 contained both PKS and NRPS genes (Table IV). Sequences of bacterial PKS and NRPS genes and fungal PKS and NRPS genes were deposited in the GenBank with accession numbers MF589505-MF589517, MF589518-MF589522, MF680559-MF68568, and MF680550-MF680558, respectively (Table IV).

Phylogenetic analysis of bacterial PKS and NRPS. Using BLASTP query, nucleotide sequences of KS and A domain gene fragments were translated and

Table III
PKS and NRPS genes in endophytic bacteria isolated from *Paeonia ostii*.

Gene	No. of isolates	Amino acid residues	Accession number	Top BLASTP match (GenBank accession No.)	Identity (%)	Predicted binding pocket (amino acid substrate)
PKS	Md1-2	226	MF589505	polyketide synthase, <i>Nostoc</i> sp. (AGJ72843)	144/226(64%)	Not done
PKS	Md1-4	227	MF589506	polyketide synthase, <i>Bacillus</i> sp. (ACG70843)	226/227(99%)	Not done
PKS	Md1-6	227	MF589507	type I ketosynthase, <i>Bacillus</i> sp. (AIO09656)	220/224(98%)	Not done
PKS	Md1-9	224	MF589508	type I ketosynthase, <i>Bacillus</i> sp. (AIO09656)	220/222(99%)	Not done
PKS	Md1-21	223	MF589509	type I ketosynthase, <i>Bacillus</i> sp. (AIO09652)	222/222(100%)	Not done
PKS	Md1-24	227	MF589510	polyketide synthase, <i>Bacillus</i> sp. (ACG70842)	224/227(99%)	Not done
PKS	Md1-37	227	MF589511	polyketide synthase, <i>Bacillus</i> sp. (ACG70841)	226/227(99%)	Not done
PKS	Md1-41	227	MF589512	polyketide synthase, <i>Bacillus</i> sp. (ACG70841)	226/227(99%)	Not done
PKS	Md1-43	226	MF589513	type I ketosynthase, <i>Bacillus</i> sp. (AIO09656)	219/224(98%)	Not done
PKS	Md1-44	224	MF589514	type I ketosynthase, <i>Bacillus</i> sp. (AIO09656)	218/222(98%)	Not done
PKS	Md1-45	229	MF589515	polyketide synthase, <i>Bacillus</i> sp. (ACG70842)	226/229(99%)	Not done
PKS	Md1-50	227	MF589516	polyketide synthase, <i>Bacillus</i> sp. (ACG70843)	227/227(100%)	Not done
PKS	Md1-51	223	MF589517	type I ketosynthase, <i>Bacillus</i> sp. (AIO09656)	221/222(99%)	Not done
NRPS	Md1-2	232	MF589518	non-ribosomal peptide synthetase, <i>Streptomyces hiroshimensis</i> (BAH68742)	158/217(73%)	DFECLSVVT-(Val)
NRPS	Md1-18	232	MF589519	non-ribosomal peptide synthetase, <i>Streptomyces hiroshimensis</i> (BAH68742)	158/217(73%)	DFECLSVVT-(Val)
NRPS	Md1-24	252	MF589520	nonribosomal peptide synthase, <i>Bacillus</i> sp. (KIA75709)	249/252(99%)	DAKDLGVVD-(Glu)
NRPS	Md1-47	233	MF589521	non-ribosomal peptide synthetase, <i>Pseudomonas</i> sp. (WP_085703687)	225/233(97%)	DAWVFGVVI-(Glu)
NRPS	Md1-50	245	MF589522	non-ribosomal peptide synthetase, <i>Bacillus velezensis</i> (WP_069007535)	243/245(99%)	DFWNIGMVH-(Thr)

Table IV
PKS and NRPS genes in endophytic fungi isolated from *Paeonia ostii*.

Gene	No. of isolates	Amino acid residues	Accession number	Top BLASTP match (GenBank accession No.)	Identity (%)	Predicted binding pocket (amino acid substrate)
PKS	Mdf-4	250	MF680559	related to fusarin C cluster-polyketide synthase/NRPS, <i>Rhynchosporium agropyri</i> (CZS94917)	222/250(89%)	Not done
PKS	Mdf-15	238	MF680560	ketoacyl-synt-domain-containing protein, <i>Coniochaeta ligniaria</i> (OIW26903)	201/240(84%)	Not done
PKS	Mdf-17	211	MF680561	beta-ketoacyl synthase domain-containing protein, <i>Metarhizium album</i> (KHO00577)	201/240(84%)	Not done
PKS	Mdf-26	231	MF680562	PKS protein, <i>Trichoderma parareesei</i> (OTA00034)	175/231(76%)	Not done
PKS	Mdf-41	234	MF680563	polyketide synthase PksF, <i>Alternaria alternata</i> (XP_018382155)	233/234(90%)	Not done
PKS	Mdf-43	238	MF680564	ketoacyl-synt-domain-containing protein, <i>Coniochaeta ligniaria</i> (OIW26903)	227/234(97%)	Not done
PKS	Mdf-44	234	MF680565	polyketide synthase PksF, <i>Alternaria alternata</i> (XP_018382155)	131/217(60%)	Not done
PKS	Mdf-47	250	MF680566	related to fusarin C cluster-polyketide synthase/NRPS, <i>Rhynchosporium agropyri</i> (CZS94917)	233/234(99%)	Not done
PKS	Mdf-49	234	MF680567	polyketide synthase PksF, <i>Alternaria alternata</i> (XP_018382155)	233/234(99%)	Not done
PKS	Mdf-51	234	MF680568	polyketide synthase PksF, <i>Alternaria alternata</i> (AFN68297)	233/234(99%)	Not done
NRPS	Mdf-2	244	MF680550	acetyl-CoA synthetase-like protein, <i>Stagonospora</i> sp. (OAK98265)	218/244(89%)	No prediction
NRPS	Mdf-6	234	MF680551	nonribosomal peptide synthetase 1, <i>Neonectria ditissima</i> (KPM37793)	195/235(83%)	DIGFVGGIF-(Ile)
NRPS	Mdf-8	231	MF680552	nonribosomal peptide synthetase 1, <i>Neonectria ditissima</i> (KPM37793)	208/231(90%)	DVTLVGCVV-(Cys)
NRPS	Mdf-9	222	MF680553	nonribosomal peptide synthetase, <i>Cenococcum geophilum</i> (OCK98900)	195/222(88%)	DVAFIGSIH-(Phe)
NRPS	Mdf-18	228	MF680554	nonribosomal peptide synthetase, <i>Cenococcum geophilum</i> (OCK98900)	198/228(87%)	DVAFIGSIH-(Phe)
NRPS	Mdf-20	246	MF680555	acetyl-CoA synthetase-like protein, <i>Stagonospora</i> sp. (OAK98265)	223/246(91%)	No prediction
NRPS	Mdf-22	238	MF680556	nonribosomal peptide synthetase 1, <i>Neonectria ditissima</i> (KPM37793)	220/237(93%)	DAMLVGAVI-(Gln)
NRPS	Mdf-41	240	MF680557	nonribosomal peptide synthase, <i>Alternaria alternata</i> (XP_018382376)	201/240(84%)	DAILVGAVV-(Gln)
NRPS	Mdf-50	238	MF680558	nonribosomal peptide synthetase 1, <i>Neonectria ditissima</i> (KPM37793)	217/237(92%)	DAMLVGAVI-(Gln)

compared with protein sequences in the NCBI database. As shown in Table III, bacterial KS domain fragments exhibited 64–100% similarity to database sequences. Phylogenetic analysis of putative PKS fragments validated BLASTP results and indicated that the majority of KS domain fragments are evolutionarily related to previously identified sequences. As illustrated in Fig. 3, the 12 bacterial PKS fragments amplified from *Bacillus* isolates were detected on a single clade. KS domain sequence of four isolates, namely, Mdf-4 (*B. subtilis*), Mdf-50 (*B. licheniformis*), Mdf-24 (*B. pumilus*), and Mdf-45 (*B. amyloliquefaciens*) was grouped with *B. sub-*

tilis PksN and *B. velezensis* PksJ, which are involved in biosynthesis pathway of antibiotic bacillaene. KS domain fragments of five isolates (i.e., Mdf-6, Mdf-9, Mdf-43, Mdf-44, and Mdf-51) were closely related and were clustered with the BaeN fragments involved in biosynthesis of bacillaene. PKS fragments Mdf-37 and Mdf-41 clustered within a clade containing *Bacillus* KS domains, which included a still uncharacterized KS domain from *Bacillus* sp. and *B. amyloliquefaciens*. KS domain sequence of Mdf-2 was related to *Bacillus* sp. type I KS fragments. The remaining fragment, Mdf-2, was amplified from a strain putatively identified as

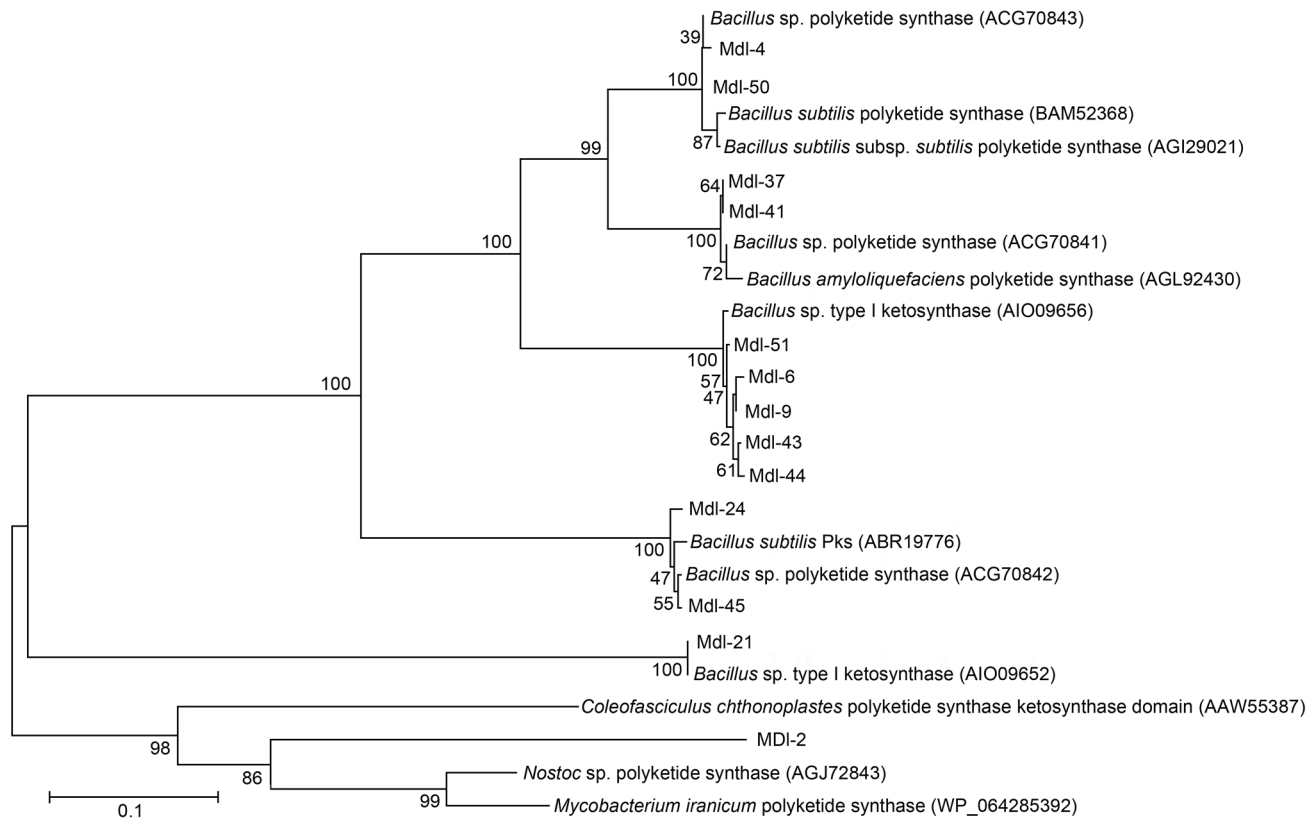


Fig. 3. The phylogenetic relationship of endophytic bacteria based on PKSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.1 amino acid changes.

Streptomyces phaeochromogenes. KS domain sequence of this strain was grouped with putative PKSs from *Nostoc* sp., which are cyanobacterial strains isolated from the Portuguese coast, and a PKS sequence amplified from a symbiont of nontuberculous mycobacterium species.

A domain fragments of bacterial endophytes displayed 73–99% similarity to A domain sequences in the database (Table III). The phylogenetic tree indicated that endophyte-derived fragments were similar to NRPS sequences from *Bacillus*, *Streptomyces*, and *Pseudomonas* in the database (Fig. 4). Sequences amplified from putative *Bacillus* isolates were phylogenetically related to NRPSs of *Bacillus* sp. (Mdl-24 and Mdl-50); these findings were consistent with those of BLASTP analysis. A domain sequence of Mdl-24 was involved in biosynthesis of surfactin, which is a bacterial cyclic lipopeptide. The isolate Mdl-50 was identified as *B. licheniformis* and clustered with the NRPS involved in producing siderophore bacillibactin. The A domain sequences of two isolates, namely, Mdl-2 (*S. phaeochromogenes*) and Mdl-18 (*Streptomyces aureus*), were grouped with NRPS fragments of *Streptomyces hirosheimensis*. *Streptomyces*-related NRPSs were involved in synthesis of numerous bioactive compounds. Another fragment of interest, Mdl-47, was related to NRPS sequences from *Pseudomonas*.

Translated A domain sequences were further analyzed to determine whether they contained an eight-residue binding pocket and identify the amino acid that possibly binds to the pocket (Table III). NRPS sequences were successfully predicted for bacterial isolates, including Mdl-2 and Mdl-18, which possessed the same amino acid-binding pocket and can bind the Val residue. *Bacillus* isolates Mdl-24 and Mdl-50 possessed different binding pockets and were thus responsible for adding different amino acids to the growing peptide chain. Analysis of fragments detected from endophytes of *Pseudomonas* Mdl-47 suggested that the binding pocket may integrate a Glu residue into a nonribosomal peptide.

Phylogenetic analysis of fungal PKS and NRPS.

Fungal KS domain fragments exhibited 76–99% similarity to the database sequences (Table IV). Translated PKS fragments were aligned with the reference PKS sequences, and a phylogeny tree was constructed. The resulting phylogram (Fig. 5) revealed that all amplified fungal KS domain sequences belonged to the clade of type I PKSs, which synthesize reduced PKs. Four fungal PKS fragments amplified from *Alternaria* isolates (Mdf-41, Mdf-44, Mdf-49, and Mdf-51) were clustered within a single clade and highly homologous with *Alternaria* PksF, which is involved in biosynthesis pathways

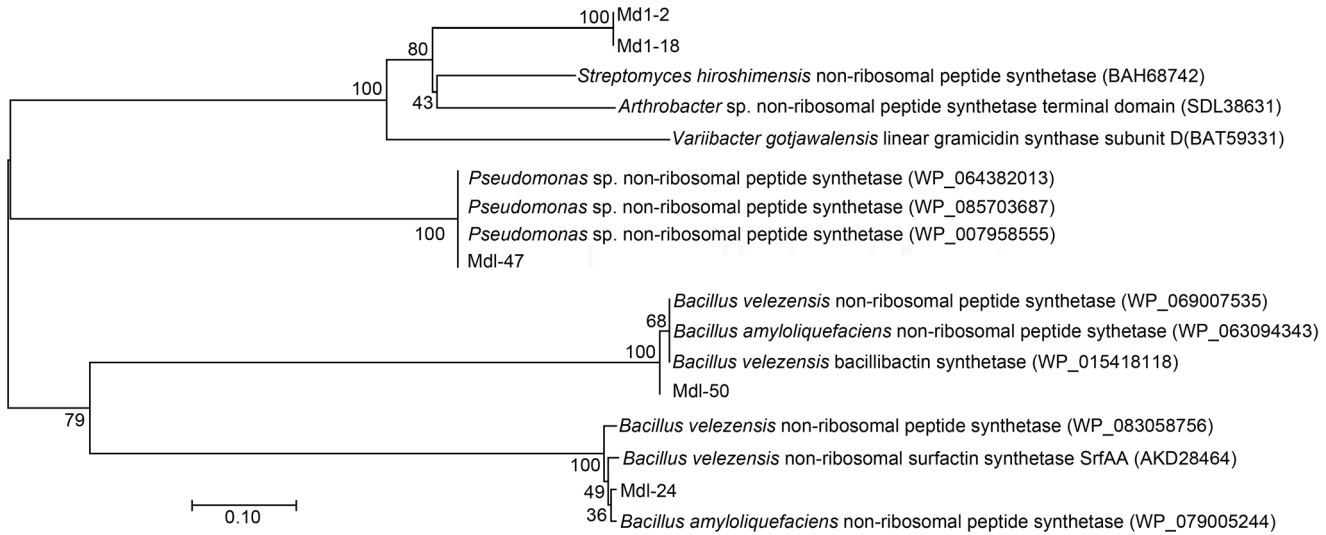


Fig. 4. The phylogenetic relationship of endophytic bacteria based on NRPSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.10 amino acid changes.

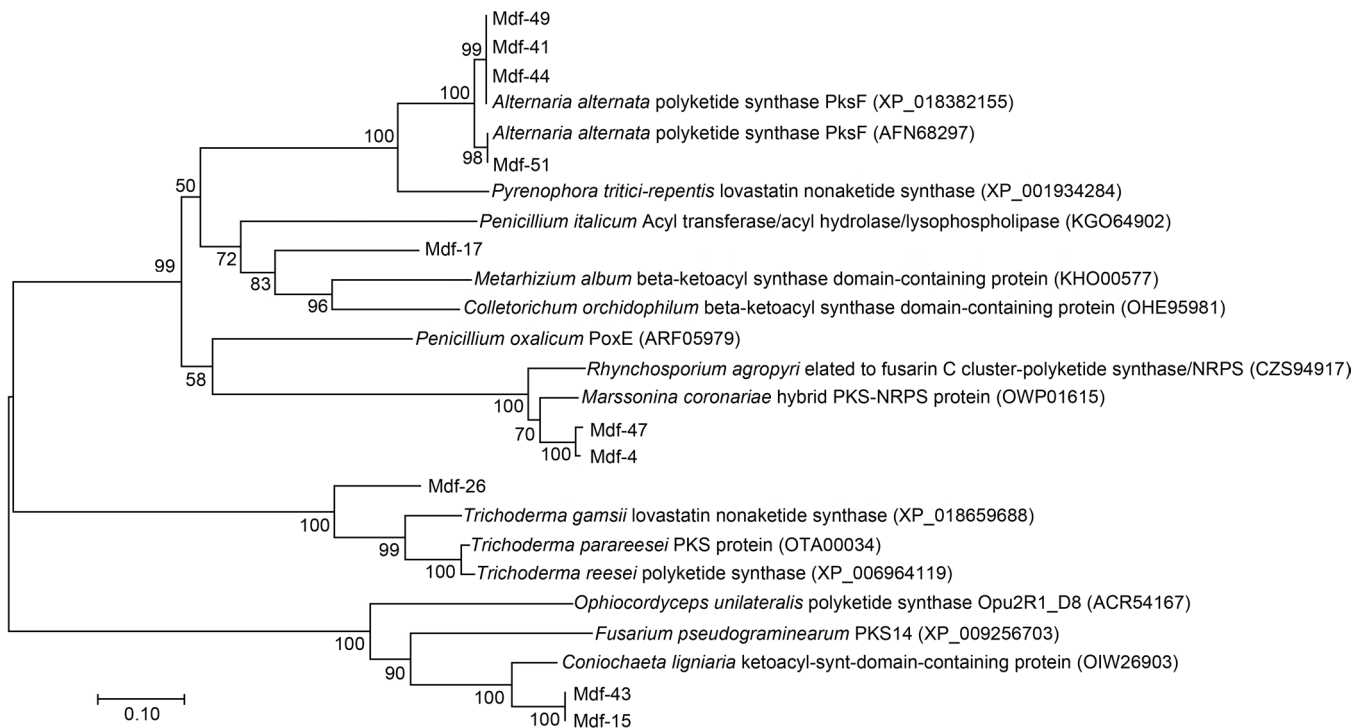


Fig. 5. The phylogenetic relationship of endophytic fungi based on type I PKSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.10 amino acid changes.

of yellow pigment and two new polyene compounds. Isolates Mdf-4 and Mdf-47, which were closely related to each other, were clustered with hybrid PKS-NRPS fragments and showed slight similarity to PoxE fragments of *Penicillium oxalicum*. PKS fragments from endophytes of *Nectriaceae*, Mdf-26, were grouped with the lovastatin nonaketide synthase of *Trichoderma gamsii*. However, three PKS fragments (i.e., Mdf-15, Mdf-17, and Mdf-43)

displayed slight similarity to fungal KS domain proteins, which were uninvolved in synthesis of active substances and therefore perform unknown functions.

Fungal endophyte A domain fragments showed 83–93% similarity to A domain sequences in the database (Table IV). The phylogenetic tree indicated similarity endophyte-derived fragments with NRPSs involved in synthesis of many bioactive compounds

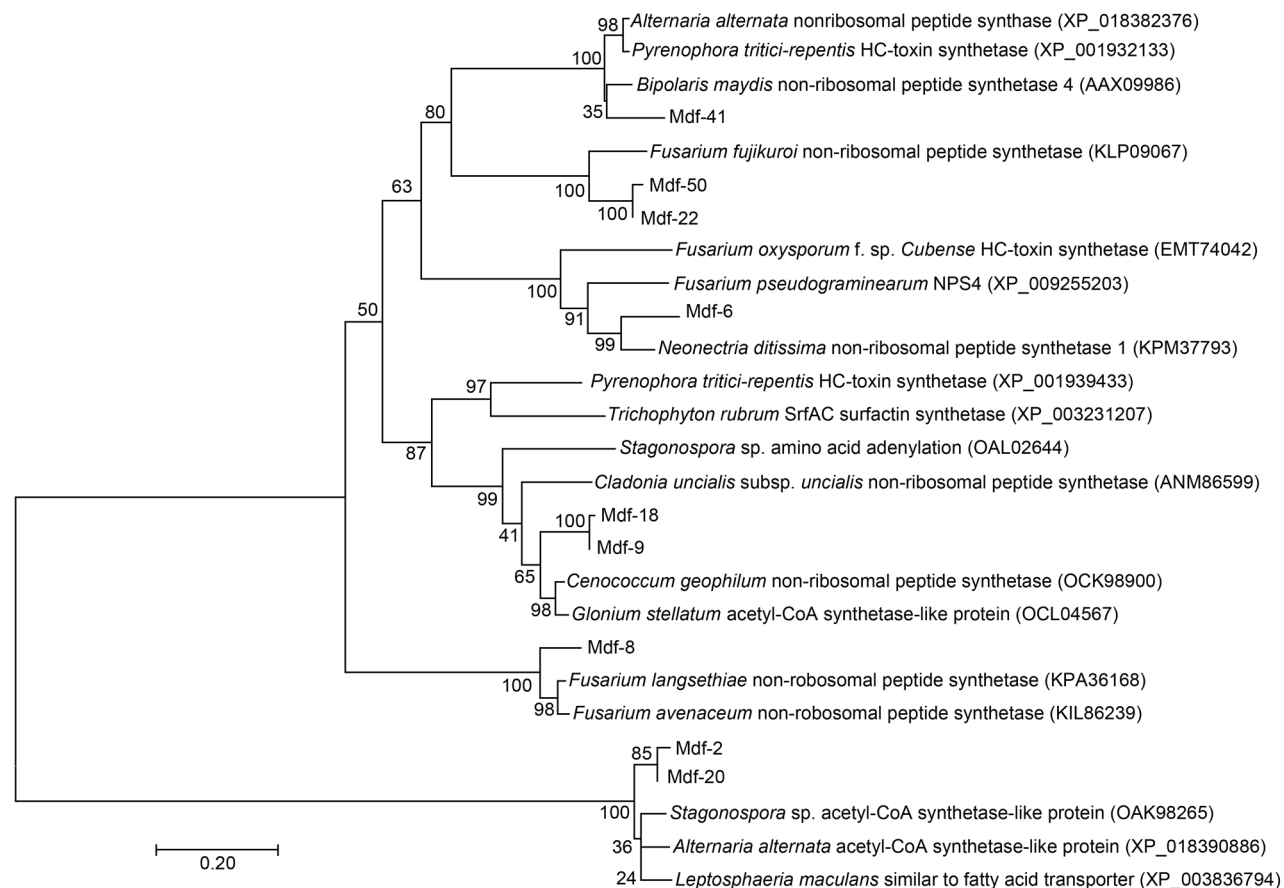


Fig. 6. The phylogenetic relationship of endophytic fungi based on NRPSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.20 amino acid changes.

(Fig. 6). The A domain fragments of isolates Mdf-6, Mdf-22, Mdf-41, and Mdf-50 were clustered with a clade and were involved in biosynthesis pathways of fungal HC-toxin, NPS4, and NPS1. Isolates Mdf-9 and Mdf-18 were closely related to each other and showed slight similarity to the still uncharacterized NRPS fragments of *Cenococcum geophilum*. Gene products of Mdf-9 displayed similarity to putative NRPS fragments of *Fusarium*. The A domain fragments of Mdf-2 and Mdf-20 were clustered with a single clade and were highly homologous with the acetyl-CoA synthetase-like protein, which is involved in fungal biosynthetic and catabolic processes in *Stagonospora*.

The nine translated endophytic fungal NRPS fragments were analyzed to predict active residues of the A domain binding pocket. Seven amino acid binding pockets of fungal NRPSs were predicted successfully (Table IV). However, no NRPS amino acid binding pocket was detected in the other two strains (Mdf-2 and Mdf-20). Mdf-22, Mdf-41, and Mdf-50 showed the same amino acid binding pocket and bound to the Gln residue. Mdf-9 and Mdf-18 presented the same amino acid binding pocket and bound to the Phe residue. The remaining two isolates, Mdf-6 and Mdf-8,

featured different amino acid binding pockets, and they integrated Ile and Cys residues into nonribosomal peptides, respectively.

Discussion

Endophytes participate in long-term symbiotic relationships with their host plants, and many of them produce bioactive substances as consequence of these relationships. Structures of active compounds produced by endophytes are considered superior to those produced by their host plants. As such, endophytes, particularly those from medicinal plants, have become important sources of novel and biologically active secondary metabolites (Wang and Dai 2011). To aid this purpose, a more diverse and comprehensive collection of endophytes must be realized. Screening and isolation of promising strains of endophytes must be conducted to produce novel bioactive compounds for pharmaceutical and agricultural applications.

Tree peony (*P. ostii*) is a Chinese traditional plant considered valuable for its ornamental and medicinal benefits. However, limited information is available

regarding its endophytic community. This study investigated preliminary endophytes diversity from *P. ostii* and their potential ability to synthesize some secondary metabolites. Results showed abundance of a diverse range of endophytes in root tissues *P. ostii*. Endophytes comprised 56 bacterial endophytes classified under 13 different genera and 51 fungal endophytes categorized under 12 fungal genera. A large proportion of the endophytic bacteria and fungi possessed PKS and NRPS genes, suggesting that these endophytes are potential sources of bioactive substances.

Recent studies have explored endophyte diversity in medicinal plants, such as *Aloe vera* (Akinsanya et al. 2015), *Ferula songorica* (Liu et al. 2016), the medicinal cactus *Opuntia humifusa* (Silva-Hughes et al. 2015), and *Rhodiola rosea* (Cui et al. 2015). The most commonly found endophytes belong to the genera *Streptomyces* sp., *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Alternaria* sp., *Leptosphaerulina* sp., *Fusarium* sp., *Colletotrichum* sp., *Phomopsis* sp., *Phyllosticta* sp., and *Cladosporium* sp. (Nair and Padmavathy 2014).

Among the 56 endophytic bacteria obtained from *P. ostii*, *Bacillus* was the most dominant genus (55.4% of all isolates); this finding is consistent with previous reports on different hosts (El-Deeb et al. 2013). In addition to *Bacillus* sp., other genera, such as *Streptomyces* from *Schima wallichii* (Passari et al. 2016) and *Leifsonia* from ginseng roots, have also been reported in medicinal plants (Qiu et al. 2007). Endophytic *Bacillus* spp. isolated from plant tissues are highly abundant and considered microbial factories for numerous biologically active molecules that potentially inhibit phytopathogen growth (Ongena and Jacques 2008). Endophytic *Streptomyces* are considered potential sources of secondary metabolites and various bioactive products that exhibit antimicrobial, antioxidant, and plant-growth-promoting activities (Lam 2006; Nimnoi et al. 2010). These results indicated that tree peony acts as a reservoir of novel endophytic bacteria for isolation of biologically active compounds.

In this study, the dominant endophytic fungal genera in *P. ostii* belong to *Alternaria* (23.5%) and *Fusarium* (19.6%). *Alternaria* has been previously observed as the predominant fungal taxa in 29 traditional Chinese medicinal plants and a well-known medicinal plant in India (Huang et al. 2008; Gond et al. 2012). *Fusarium* was isolated as the dominant endophytic fungal species from five medicinal plant species in the Western Ghats of India (Raviraja 2005). Previous reports have also stated that *Alternaria* and *Fusarium* are common endophytes of tropical, subtropical, and temperate plants (Banerjee 2011; Gong et al. 2015). Colonization and propagation of these endophytes may offer significant benefits to their hosts by producing useful substances. Metabolites with antimicrobial activity have been found

in endophytic fungi isolates from *Fusarium* and *Alternaria* (Brady and Clardy 2000; Raviraja et al. 2006).

Endophytes are presumed ubiquitous in the plant kingdom, and its population depends on host species and location. Previous studies have demonstrated that different host plants feature distinct endophyte community compositions, suggesting that endophytes display host preference (Cohen 2006). Studies have also presented significant differences in both the presence and absence and abundance of endophytes in other medicinal plants. For example, *Brevundimonas* and *Sphingomonas* are the dominant endophytic genera in *Ferula songorica* (Liu et al. 2016), whereas *Aspergillus* is the dominant fungal member in *Aegle marmelos* (Gond et al. 2012). These endophytic genera were not detected in *P. ostii* roots. By contrast, *Enterobacter* (8.93%), *Leptosphaeria* (21.6%), and *Cylindrocarpon* (15.7%) existed in *P. ostii* at high frequencies. Our results further validated specificity and selectivity between endophytes and host plants. Nonetheless, given the inadequate number of our samples, further investigations are needed to prove specificity and selectivity phenomena. Endophyte community is also dynamic, and species composition is affected by various factors (e.g., seasonal changes and host age); however, culture-independent endophytes were not considered in this study.

PKS and NRPS genes serve as appropriate targets for detecting small molecule biosynthesis systems (Moffitt and Neilan 2001; Sauer et al. 2002). Previous PKS and NRPS screening studies have also utilized these biosynthetic pathways to isolate endophytes from other terrestrial and marine environments (Zhou et al. 2011). In the present study, numerous PKS and NRPS genes were detected in endophytes from *P. ostii*. However, type I PKS and NRPS primers were used in this study. These primers cannot amplify all PKS or NRPS genes of endophytes, suggesting that these primers underestimated the diversity of KS and NRPS genes of endophytes from *P. ostii*. Therefore, future works should design new primers that can detect atypical PKS or NRPS genes of endophytes.

Twelve gene sequences detected in bacterial KS domain screen were amplified from isolates, which were identified to belong to the *Bacillus* genus. *Bacillus* spp. are prominent members of endophyte populations (El-Deeb et al. 2013), and they are known for their production of antibiotics, including subtilin, macrolactins, bacillaene, and difficidin (Mongkolthanaruk 2012). These KS domain fragments were grouped with *Bacillus* PksJ, PksN, and BaeN (Fig. 3). Dihydrobacillaene and bacillaene biosynthetic mechanism in *B. subtilis* is split among five large megasynthases (PksJLMNR) and several accessory proteins (Butcher et al. 2007). BaeN, subsequently termed PksN, is a secondary hybrid NRPS-PKS protein involved in biosynthesis of bacil-

laene (Chen et al. 2006b). The PKS gene amplified from *Streptomyces* Md1-2 was similar (64%) to PKS genes of *Nostoc* sp.; these cyanobacteria are prolific sources of bioactive products with interesting biological activities and promising pharmaceutical applications (Burja et al. 2001). PKS of *Nostoc* was mainly involved in biosynthesis of nostophycin and microcystins, and the two cyclic heptapeptides are potent inhibitors of eukaryotic protein phosphatases 1 and 2A (Fewer et al. 2011). Results implied that these putative KS domain genes from tree peony endophytes take part in synthesis of bacillaene and other antibiotics.

NRPS genes of bacterial isolates Md1-24 and Md1-50 were phylogenetically grouped with biosynthesis pathways of diverse *Bacillus* NRPS. The A domain genes of Md1-24 and Md1-50 were grouped with NRPSs of *B. velezensis*, which participate in synthesis of surfactin and bacillibactin synthetase (Fig. 4). Lipopeptides from *Bacillus* are synthesized by NRPS. As lipopeptide-type biosurfactants, surfactins are vital for some bacterial bioformation and root colonization, and they exhibit a wide range of antimicrobial activities (Bais et al. 2004; Aleti et al. 2015). Bacillibactin is catecholate-type siderophore produced by *Bacillus*; it serves as an iron scavenger for overcoming iron limitation. In recent times, endophytic bacterial siderophores have been applied in different fields, such as promoting plant growth of species by enhancing Fe uptake of plants and controlling phytopathogens as potential biocontrol agent (Saha et al. 2016). Other bacterial NRPS genes (Md1-2, Md1-18, and Md1-47) were similar to the still uncharacterized NRPS genes of *Streptomyces* and *Pseudomonas* (Fig. 4). Both *Streptomyces* and *Pseudomonas* are prolific producers of bioactive compounds, including various antibiotics, antitumor, and plant growth hormones (Wu et al. 2011; Passari et al. 2016). Therefore, these NRPS genes may be capable of producing unique bioactive compounds.

The presence of PKS I genes in fungi can increase chances of finding structurally novel polyketides with biological activities. In this study, all 10 KS domain fragments were within clusters of PKSs that synthesize reduced polyketides. Four putative fungal KS domain fragments from *Alternaria* spp. were closely related to *Alternaria alternata* PksF gene involved in biosynthesis of yellow pigment and two new polyene compounds, namely, aslanipyronone and aslaniol (Kasahara et al. 2006). Fungal pigments displayed a wide range of bioactivities; for example, elsinchrome possesses phytotoxic activities, whereas naphthopyrones exhibits antimicrobial activities (Liao and Chung 2008). Detection of pigment and new polyene compounds posits biosynthetic potential of endophytes from *P. ostii*. Numerous polyketide compounds, such as lovastatin and fusarin, were also observed to contain the PKS I

gene (Wang et al. 2014). Lovastatin, which was originally isolated from *Aspergillus terreus*, is widely used as a statin group in cholesterol-lowering agents. Fusarin, which is a broad-spectrum plant toxin, contributes to severity of plant vascular wilt induced by *Fusarium oxysporum*, damping-off, and root rot diseases (Hajjaj et al. 2001; Brown et al. 2012). Therefore, the presence of PKS gene in Mdf-4, Mdf-47, and Mdf-26 isolates implies their genetic potential to produce lovastatin or and fusarin-like metabolites. Other KS domain fragments, such as Mdf-15, Md1f-17, and Mdf-43, were related to the fungal KC domain protein involved in AT biosynthesis in the metabolic process. These amplified KS domains may participate in biosynthetic pathways, but functions relating to polyketide synthesis require further investigation.

Phylogenetic analysis of putative fungal A domains revealed that observed sequences were clustered into two main groups (Fig. 6). One group included seven isolates, namely, Mdf-6, Md1f-8, Mdf-9, Md1f-18, Mdf-22, Mdf-41, and Mdf-50, all of which were similar to fungal HC-toxin, NPS4, and NPS1 genes. NRPS can synthesize nonribosomal peptides, such as mycotoxins and HC-toxin (Bushley and Turgeon 2010). The HC-toxin produced by fungal plant pathogens generally act as effectors that control pathogenicity or virulence in certain plant-pathogen interactions (Tsuge et al. 2013). However, their functions in plants endophytic fungal remain unclear. Thus, further studies are needed to elucidate the potential role of toxin compounds in endophytes-host interactions. Recent studies on NPS genes of *Fusarium graminearum* revealed that NPS1 and NPS4 are related to genes involved in biosynthesis of NPS hydroxamate siderophores (Tobiasen et al. 2006). Therefore, the NRPS gene in endophytic fungi was presumed capable of producing siderophore compounds. The other group included two strains, namely, Mdf-2 and Mdf-20, which are similar to the fungal acetyl-CoA synthetase-like protein, which is utilized for fatty acid biosynthetic processes (Starai and Escalante-Semerena 2004).

In summary, population diversity of endophytes residing in roots of medicinal plant *P. ostii* was studied preliminary. Genetic screening revealed the presence of endophyte-derived PKS and NRPS fragments with putative roles in biosynthesis of secondary metabolites that present a wide array of bioactivities. Findings of this screening encourage future investigations to focus on links between PKS/NRPS genes and bioactivity to clarify full biosynthetic pathways of endophytes. Further studies are also required to determine whether the PKS and NRPS genes identified in this study are functionally dedicated to specialized endophyte-based activities and elucidate chemical components of bioactive metabolites produced by endophytes containing PKS/NRPS genes.

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Conflict of interest

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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