



**Full Length Article**

## Isolation and Identification of Endophytic Bacteria from the Roots of the Endangered Plant *Ostrya rehderiana* Chun

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### Abstract

*Ostrya rehderiana* Chun, one of the most endangered species in China mainly because of difficult transplantation, is distributed in Western Tianmu Mountain of the Zhejiang Province in China. There are presently only five plants still remaining in the wild. Since plant endophytes affect the growth and development of the host plant either directly or indirectly, in this study, we adopted the tissue block separation method to isolate 22 endophytic bacteria strains from the roots of *O. rehderiana*, with 10 strains from wild *O. rehderiana* and 12 from *O. rehderiana* plantations. From morphological analysis and 16S rDNA sequence analysis, 6 known genera were identified from the 22 strains. Among the isolated strains, eight were *Bacillus* (the dominant endophytic bacteria in the root of *O. rehderiana*), six were *Paenibacillus*, three were *Agrobacterium*, three were *Paraburkholderia*, one was *Rhizobium*, and one was *Pseudomonas*. Both wild and plantation *O. rehderiana* contained *Bacillus*, *Paenibacillus* and *Paraburkholderia*, which presumably could promote the growth of *O. rehderiana*. Besides, the *Rhizobium* strain isolated from wild *O. rehderiana* might be beneficial to the growth of *O. rehderiana*, but the *Agrobacterium* strains from the plantations might impair the growth of *O. rehderiana*. © 2020 Friends Science Publishers

**Keywords:** *Ostrya rehderiana* chun; Root; Endophyte; Phylogenetic tree; Endangered species

### Introduction

Endophytes, including endophytic fungi and endophytic bacteria, are a group of microorganisms that are inseparable from and closely associated with their host plants (Wani *et al.*, 2015). Although previous studies largely focused on endophytic fungi, endophytic bacteria are also receiving rising interests lately in studying the interaction between plants and microorganisms (Pinski *et al.*, 2019). Many endophytic bacteria have been found to possess various beneficial effects for their host, including nitrogen fixation, growth promotion, disease prevention, and stress resistance (Chihaoui *et al.*, 2015; Tétard-Jones and Edwards, 2016; Song *et al.*, 2017; Khare *et al.*, 2018; Enebe and Babalola, 2019). However, some endophytic bacteria (accounting for about 10%–20% in total) also appeared to be detrimental to the growth and development of their hosts (He *et al.*, 2004; Ellouze *et al.*, 2014). It was found by van Peer *et al.* (1990) that a significantly higher percentage of *Pseudomonads* obtained from the endorhizosphere (30%) reduced plant growth than those obtained from the root surface (4%). Jin *et al.* (1996) reported on some endophytic bacteria in wheat seedlings that could inhibit wheat growth to varying extent. Besides, some endophytes demonstrate pathogenic effects in their host plants. Bending *et al.* (2002) reported that most *Bacillus* isolates

stimulated growth of *S. luteus* along the *P. sylvestris* root, whereas the isolates of *Pseudomonas* and *Serratia* inhibited root colonization, and the *Burkholderia* and *Serratia* isolates inhibited the formation of the ectomycorrhiza by 97% and 41% respectively. Newman *et al.* (2003) reported that *Xylella fastidiosa* could cause Pierce's disease as well as several other major agricultural diseases in grapevine. They observed the movement of *X. fastidiosa* through bordered pits to neighboring vessels and proposed that vessel-to-vessel movement was a key colonization strategy whose failure resulted in vessel plugging and disease.

*Ostrya rehderiana* Chun (*O. rehderiana*), a plant of the *Betulaceae* family in the *Ostrya* genus, is distributed in the Western Tianmu Mountain of the Zhejiang Province in China. It is one of the rarest plants in China and currently there are only five plants grown in the wild. Difficulty in transplantation is one of the main causes of endangerment since transplanted individuals do not readily develop new roots and thus survive (Guan, 2008; Shaw *et al.*, 2014). It remains unknown whether the endophytes in the roots are culpable for the difficulty in root development of transplanted individuals. Therefore, this study adopted the root block separation method to isolate and purify endophytic bacteria from the roots of *O. rehderiana*, and combined with morphology studies with molecular biology

for the identification and evolution analysis of the collected bacteria. The results lay further foundation for understanding the endangerment of *O. rehderiana* and evaluating the effects of its endophytic bacteria on its growth.

## Materials and Methods

### Materials

Root-containing soil blocks were collected from wild *O. rehderiana* plants at the Tianmu Mountain of Hangzhou and from the artificial plantations. The collected soil blocks were cleaned by washing once with tap water to remove dirt and debris on the surface, once more with dilute dishwashing detergent, and then finally rinsed thoroughly with tap water again. Then, the cleaned blocks were immersed in dilute to 1 times of NaClO (saturated solution) for 30 min. After washing with sterilized water for three times, cut root blocks into small cubes (3-5 mm in length), and placed in Potato Dextrose Agar (PDA) medium that contained 50 mg/L streptomycin, 200 mg/L peeled, diced, and filtered potato infusion, 20 g/L dextrose, and 14 g/L agar. In each Petri dish were evenly placed 3-5 root blocks of uniform size with their section facing the PDA medium. Two control groups were also tested, one with a medium free of any material but sterilized water to verify proper sterilization for inoculation, and the other with root blocks removed from the Petri dish after rolling quickly in the PDA medium to verify thorough sterilization of the block surface. The test and control groups were both cultured in dark at 28°C until plaque grew visible on the section. The plaques were numbered and selectively transferred into new medium (PDA with 50 mg/L streptomycin) for further purification. Single colonies of endophytic bacteria were obtained after 3 cycles of cultivation. The growth rate, size, color, texture, etc., of the separated colonies were observed for an initial identification with reference to the Chinese Handbook of Fungi (Dong and Cai, 2001) and Bergey's Manual of Systematic Bacteriology (Bergey's Manual Trust, 2001-2012).

The DNA of the endophytes was extracted with the Ezup spin column bacterial genomic DNA miniprep kit (Sangon Biotech, Shanghai, China) according to the user manual. The PCR amplification of the DNA was carried out using the following primers: 16S rDNA of 27f: AGAGTTTGATCMTGGCTCAG and 1492r: TACGGYTACCTTGTTACGACTT; ITS rDNA upstream: TCCGTAGGTGAACCTGCGG and ITS rDNA downstream: GTCCTCCGCTTATT. The PCR amplification was carried out on a PE 9700 synthesizer (Applied Biosystems, CA, USA) in a medium (20 µL) containing 2×Taq Master Mix (10 µL, Sangon Biotech, Shanghai, China), DNA (1 µL, 100 ng/µL), primer (1 µL, 10 pmol/µL), and double distilled water (7 µL) with 30 cycles as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, elongation at 72°C for 1 min. Pre-denaturation was carried out at 94°C for 5 min and final elongation at 72°C

for 5 min. The products were electrophoresed in 1.5% agarose gel for 60 min at 90 V and stained with EB or pictured with a Bio/Rad gel imager. The electrophoresed PCR products were purified and linked to pMD18-T vector (Takara Bio Inc., Japan) and transcribed into competent *E. coli* DH5a. The positive clones were screened in lysogeny broth containing 50 mg/L ampicillin, and the recombinant plasmids were sequenced by Sangon Biotech (Shanghai, China). The species of the isolated endophytes were determined by homology alignment in Blast (<http://www.ncbi.nlm.nih.gov/blast>) with known nucleic acid sequences in GenBank. Phylogenetic trees were constructed with MEGA6 (<http://www.megasoftware.net>) based on the neighbor-joining method using default parameters.

## Results

### Separation and Preliminary Identifications

After the root blocks of *O. rehderiana*, whose surface had been sterilized, were cultured in PDA for 2-3 days, cells began to grow on the section. No cell was grown in the control group, which confirmed that the surface of the root blocks had been completely sterilized and the observed cells on the sections were endophytes from within the roots. A total of 22 strains of endophytic bacteria were obtained eventually and numbered as follows, with the plant source indicated in the parenthesis: TM (wild), TMR01 (plantation), TMR02 (plantation), TMR03 (plantation), TMR04-1 (plantation), TMR04-2 (plantation), TMR05 (plantation), TMR06-1 (plantation), TMR06-2 (plantation), TMW07-1 (wild), TMW07-2 (wild), TMR08 (plantation), TMR09 (plantation), TMR10 (plantation), TMR13 (plantation), TMW14 (wild), TMW15 (wild), TMW16 (wild), TMW17 (wild), TMW19-1 (wild), TMW19-2 (wild), and TMW20 (wild). Among them, 10 strains were from wild plants and 12 strains were from plantations (Fig. 1). The cultivated colonies were deemed to be endophytic bacteria according to their surface morphology, color, texture, growth rate, edge morphology, etc.

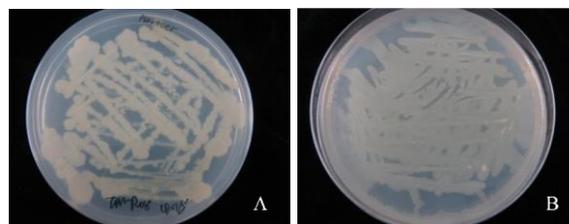
### Molecular Identification and Evolution Analysis

All DNA isolated from 22 strains amplification with 16S rRNA general primers gave a single band with a size of 900-1500 bp (Fig. 2), whereas no band was obtained when the ITS rDNA general primer was used. After the PCR products were cloned, the recombinant plasmids of the positive clones identified by restriction endonuclease were sequenced, and the resulting sequences were found to highly similar to sequences in GenBank database. Since Blast analysis showed that all strains could be traced to some strain with a high level of homology in GenBank database, the 22 strains were classified into 6 known genera, including 8 strains of *Bacillus* (TMR01 (GenBank accession: MK615900), TMR02 (GenBank accession: MK615901),

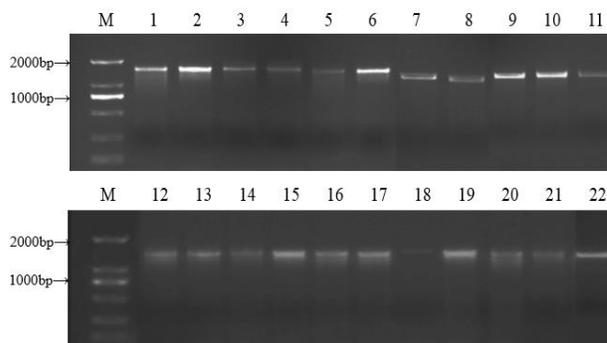
TMR04-1 (GenBank accession: MK615902), TMR06-1 (GenBank accession: MN186891), TMR10 (GenBank accession: MK615909), TMW17 (GenBank accession: MK615914), TMW19-1 (GenBank accession: MK61591), and TMW20 (GenBank accession: MK615916)), 6 strains of *Paenibacillus* (TMR04-2 (GenBank accession: MK615903), TMW07-1 (GenBank accession: MK615905), TMW07-2 (GenBank accession: MK615906), TMW14 (GenBank accession: MK615911), TMW15 (GenBank accession: MK615912), and TMW16 (GenBank accession: MK615913)), 3 strains of *Agrobacterium* (TMR05 (GenBank accession: MK615904), TMR08 (GenBank accession: MK615907), and TMR09 (GenBank accession: MK615908)), 3 strains of *Paraburkholderia* (TMR03 (GenBank accession: MN150518), TMR06-2 (GenBank accession: MN150520), and TMW19-2 (GenBank accession: MN150516)), 1 strain of *Rhizobium* (TM (GenBank accession: MK615899)), 1 strain of *Pseudomonas* (TMR 13 (GenBank accession: MK615910)). Among them, *Bacillus* accounted for 36.36% of the total bacteria and was thus the dominant endophytic bacteria, followed by *Paenibacillus*, which accounted for 27.27% of the total. The 16S rDNA sequence analysis of endophytic bacteria in the same genus showed high similarity among TMR01, TMR02, TMR04-1, and TMW17 in *Bacillus*, high similarity among TMR10, TMW19-1 and TMW20 in *Bacillus*, high similarity between TMR04-2 and TMW07-1 in *Paenibacillus*, high similarity among TMW07-2, TMW14, and TMW15 in *Paenibacillus*, high similarity between TMR05 and TMR08 in *Agrobacterium*, and high similarity between TMR03 and TMR06-2 in *Paraburkholderia*. Phylogenetic trees were then constructed with MEGA6 (Fig. 3).

## Discussion

*Bacillus* protects plants from pathogens and pests because it can compete with harmful pathogens for nutrients and space, synthesize antibacterial metabolites or hydrolase to destroy the cell structure of pathogens, and prevent or reduce diseases caused by pathogens (Lopes *et al.*, 2018). Gao *et al.* (2015) reported that the endophytic *Bacillus subtilis* strain E1R-J was a promising biocontrol agent for wheat powdery mildew. *Paraburkholderia* can promote plant growth and may suppress diseases (Donoso *et al.*, 2016; Zúñiga *et al.*, 2017). Rahman *et al.* (2018) reported that *Paraburkholderia* significantly improved both yield and quality of strawberry fruits by spray applications of bacteria in the field on foliage. *Rhizobium* can assist nitrogen fixation to provide abundant nitrogen sources for the growth of plant roots. Zhao *et al.* (2018) colonized the strain *Rhizobium* sp. IRBG74 into the roots of *Arabidopsis thaliana* seedlings and noted enhanced formation and growth of lateral roots. *Agrobacterium* is a Gram-negative bacterium, that has no nitrogen fixation function and mostly causes plant tumors (Zupan and Zambryski, 1995; Barton *et al.*, 2018).



**Fig. 1:** Endophytic bacteria from the root of *Ostrya rehderiana*. A: TMR08 strain; B: TMW15 strain



**Fig. 2:** PCR results for 16S rDNA amplifying sequencing of the 22 endophytic bacteria strains. M: DL 2000 DNA marker; the numbers 1-22 stand for the bacteria strains of TM, TMR01, TMR02, TMR03, TMR04-1, TMR04-2, TMR05, TMR06-1, TMR06-2, TMW07-1, TMW07-2, TMR08, TMR09, TMR10, TMR13, TMW14, TMW15, TMW16, TMW17, TMW19-1, TMW19-2, and TMW20, respectively

Brunner and Pootjes (1969) reported that *Agrobacterium tumefaciens* V-1 could cause tumors in plants. *Pseudomonas* is often beneficial to plant growth. Zhou *et al.* (2016) reported that *Pseudomonas fluorescens* ALEB7B, an endophytic bacterium isolated from the endangered Chinese medicinal plant *Atractylodes lancea*, could produce nitrogen-containing volatile substances ingested by the host plant. The volatile substances not only promoted plant growth significantly but also activated the plant defense response and significantly promoted the accumulation of active ingredients in the plant. In this work, 22 strains of endophytic bacteria were isolated from the roots of *O. rehderiana* grown both in the wild and in plantations. *Bacillus*, *Paenibacillus*, *Paraburkholderia*, *Rhizobium* were isolated from the roots of wild *O. rehderiana*, whereas *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Paraburkholderia*, and *Agrobacterium* were isolated from the roots of the plantation. The *Rhizobium* isolated from the roots of the wild plants was beneficial to plant growth. Both *Pseudomonas* and *Agrobacterium* were isolated from the plantation. Although *Pseudomonas* was beneficial to the colonization and growth of the roots of the artificially transplanted *O. rehderiana*, *Agrobacterium* could cause plant tumors and might not be beneficial to plant growth.

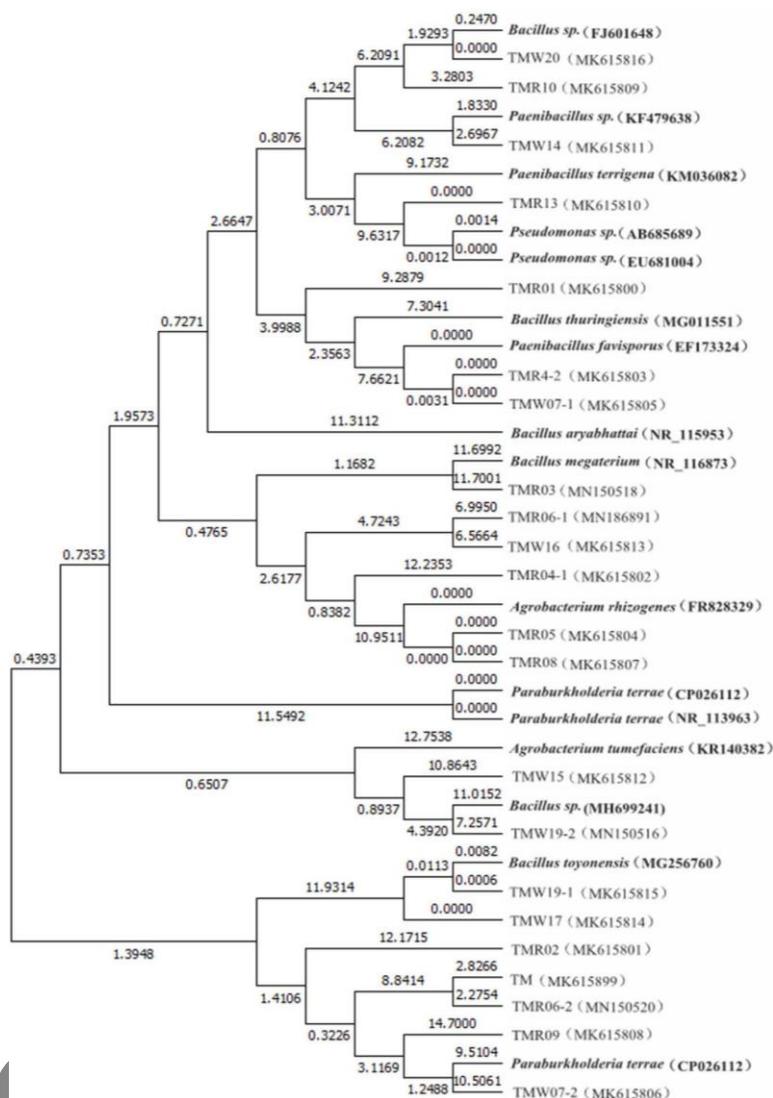


Fig. 3: Phylogenetic trees of the endophytic bacteria based on their 16S rDNA sequences

Since *Bacillus*, *Paenibacillus*, and *Paraburkholderia* were all found in the wild plants and the plantations, they probably could facilitate the growth of *O. rehderiana*.

### Conclusion

Out of 22 endophytic bacterial strains isolated from the roots of *O. rehderiana*, eight were *Bacillus*, six were *Paenibacillus*, three were *Paraburkholderia*, three were *Agrobacterium*, one was *Rhizobium*, and one was *Pseudomonas*. The results give a clue that some isolated endophytic bacteria strains may be beneficial to plant growth and help to plantation of *O. rehderiana*.

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