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SHORT COMMUNICATION

Method for RNA extraction and cDNA library construction from microbes in crop rhizosphere soil

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Abstract Techniques to analyze the transcriptome of the soil rhizosphere are essential to reveal the interactions and communications between plants and microorganisms in the soil ecosystem. In this study, different volumes of $Al_2(SO_4)_3$ were added to rhizosphere soil samples to precipitate humic substances, which interfere with most procedures of RNA and DNA analyses. After humic substances were precipitated, cells of soil microorganisms were broken by vortexing with glass beads, and then DNA and RNA were recovered using Tris–HCl buffer with LiCl, SDS, and EDTA. The crude extract was precipitated and dissolved in RNAse-free water, and then separated by agarose gel electrophoresis. We determined the optimum volume of $Al_2(SO_4)_3$ for treating rhizosphere soil of rice, tobacco, sugarcane, *Rehmannia glutinosa*, and *Pseudostellaria heterophylla*. The crude nucleic acids extract from rice soil was treated with DNase I and then RNA was purified using a gel filtration column. The purified RNA was reverse-transcribed into single-strand cDNA and then ligated with an adaptor at each end before amplifying ds cDNA. The ds cDNA was sub-cloned for subsequent gene sequence analysis. We conducted qPCR to amplify 16S ribosomal DNA and observed highly efficient amplification. These results show that the extraction method can be optimized to isolate and obtain high-quality nucleic acids from microbes in different rhizosphere soils, suitable for genomic and post-genomic analyses.

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Introduction

Soil is a complex body that contains a high diversity of microbes, mainly bacteria, fungi, and protozoans, which function as the chemical catalysts in soil. In recent years, much research has been conducted on soil microbes, and molecular approaches to explore the biological functions of soil microorganisms have been developed and used extensively. Such approaches include metagenomic (Handelsman et al. 1998; Rondon et al. 1999) and metaproteomic techniques (Wilmes and Bond 2004). There have been many reports on soil metagenomics (Wilmes and Bond 2008; Ying et al. 2008; Schmalenberger et al. 2008). However, these DNA-based studies cannot reveal gene or protein expression in the soil, and thus, are not indicative of soil processes. There have also been several reports on soil metaproteomics (Murase et al. 2003; Singleton et al. 2003; Chen et al. 2009). Recently, Wang et al. (2011) developed a soil protein extraction method suitable for different soils that is a powerful scientific tool for soil rhizospheric metaproteomics. Such techniques allow accurate analyses of the composition of complex microbial communities, and can be used to reveal the interactions between plants and microorganisms in the soil ecosystem (Wu et al. 2011).

To complement these genomic and proteomic methods, techniques to obtain high-quality nucleic acids in sufficient quantities for post-genomic analyses are also required. The first step for such analyses is to isolate total mRNA from the cell type or material of interest. The level of target mRNA is an indicator of the transcription activity in the soil. High-quality mRNA can be used to construct cDNA

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libraries, which are useful for studying the gene functions of soil microbes. However, the activity of nucleases and the fast turnover rate of prokaryotic mRNAs are major problems in extracting soil RNA from environmental samples (Bakken and Frostegård 2006; Costa et al. 2004). Methods for the successful extraction of mRNA from soil and its characterization have lagged behind those for extracting and characterizing DNA. Recently, several methods to characterize mRNA, and thus, to measure gene expression in soil, have been reported (Metcalfe et al. 2002; Krsek et al. 2006; Jacobsen and Holben 2007; Urich et al. 2008; Wang et al. 2009). Peršoh et al. (2008) described a universally adaptable protocol to extract high-purity nucleic acids from soil. The method was suitable to extract nucleic acids from different soil types. The construction of cDNA libraries from soil samples is another vital step in screening for target gene expression in soil samples.

In this study, we optimized the method to extract RNA from soil and used it to extract nucleic acids from the rhizosphere soil of several different crops. The total RNA was then purified and reverse-transcribed into cDNA, which was used to construct a cDNA library.

Materials and methods

Preparation of soil samples

Different rhizospheric soils were sampled from rice, sugarcane, tobacco, *Rehmannia glutinosa*, and *Pseudostellaria heterophylla* fields as described by Wang et al. (2011). The rice and sugarcane fields were located in Fuzhou and the *P. heterophylla* field in Ningde. Both cities are in Fujian Province, southeast China (26.05N, 119.18E). The *R. glutinosa* field was located in Zhengzhou, Henan Province, central China (35.19N, 113.51E) and the tobacco field in Yuxi, Yunnan Province, southwest China (24.47N, 102.48E). Plant roots, leaves, stones, and insects were removed from the rhizospheric soils. The physical and chemical properties of the soil samples were reported in our previous study (Wang et al. 2011).

Nucleic acid extraction

Nucleic acids were extracted from soil samples using the method of Peršoh et al. (2008) with minor modifications (Table 1). For each rhizospheric soil sample, 0.5 g soil was added to a 2 ml tube. Then, 0.2 mol/L $Al_2(SO_4)_3$ and 4 mol/L NaOH were added to allow the flocculation of humic substances with $Al(OH)_3$. To determine the optimal volume of $Al_2(SO_4)_3$ for each soil sample, we tested different volumes ranging from 30 to 300 μL. After precipitation of humic substances, 325 μL extraction buffer (0.4 mol/L LiCl, 100 mmol/L Tris–HCl, 120 mmol/L EDTA, pH = 8) and 325 μL 10 % SDS (pH = 8) were added. Then, the sample was subjected to a glass bead-beating treatment with a vortex mixer, before adding phenol: chloroform: isoamylalcohol (25:24:1) and chloroform: isoamylalcohol (24:1). The nucleic acids were precipitated in cold ethanol with 3 M sodium acetate (pH = 5.2) at −20 °C for 10 min, and then the mixture was centrifuged and the pellet was washed with 75 % ethanol (RNAse-free). Then, the pellet was re-suspended in 10 μL RNAse-free ddH_2O. The extracted nucleic acid solutions were treated with DNase I (Takara Biotechnology, Dalian, China) for 10 min, and then purified using a RNAPure High Purification Total RNA Extraction Kit (BioTeke Corp., Beijing, China) and a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK).

Table 1 Extraction steps and purification procedures to isolate soil RNA and construct a cDNA library

Step	Procedures
Precipitation of soil humic substances	1. Add 0.2 M $Al_2(SO_4)_3$ + 1 M Tris–HCl buffer + sterile dd H2O, vortex.
	2. Add 4 M NaOH + 0.1 M Tris–HCl buffer, vortex.
	3. Centrifuge and discard supernatant.
Cell disruption and lysis	4. Add glass beads + elution Buffer (0.4 M LiCl, 0.1 M Tris–HCl buffer + 0.12 M EDTA) + 10 % SDS, vortex.
	5. Centrifuge and transfer supernatant.
Removal of proteins, recovery of nucleic acids	6. Add phenol:chloroform:isoamylalcohol (25:24:1), vortex, centrifuge, transfer supernatant.
	7. Add chloroform:isoamylalcohol (24:1), vortex, centrifuge, transfer supernatant.
Precipitation of nucleic acids	8. Add cold ethanol + 3 M sodium acetate; incubate at −20 °C, centrifuge.
Preparation of crude nucleic acid solution	9. Add 75 % ethanol, centrifuge, discard supernatant.
	10. Add RNase-free dd H_2O, dissolve pellet.
Degradation of DNA, purification of RNA	11. Add DNase I, incubate at 37 °C
	12. Apply to gel filtration column for purification.
First strand cDNA synthesis	13. Add M-MLV reverse transcriptase.
Addition of adaptor sequences to first-strand cDNA	14. Ligate 3′ and 5′ adaptor sequences.
Construction of rhizosphere soil cDNA library	15. Transform into *E. coli* DH5α competent cells.

Total RNA reverse-transcription and qPCR to detect expression of 16S ribosomal DNA

The total RNA purified from the soil samples was used to synthesize single-strand cDNA (ss cDNA) using an ExScript RT Reagent Kit (Takara). Gene fragments of 16S ribosomal DNA (16S rDNA) were amplified from the ss cDNA using the following primers; 16s-F: 5′-TGGAGCA TGTGGTTTAATTCGA-3′ and 16s-R: 5′-CCATCTCTGG AAAGTTCTCTGCA-3′. The purified fragment was ligated into pMD-18T (Takara) and then transformed into competent *Escherichia coli* cells and plated onto solid LB medium containing 50 μg/ml ampicillin, which had been overlaid with 10 mM X-Gal and 50 mg/ml IPTG. After incubation overnight at 37 °C, white colonies (putative positive clones) were picked and cultured to isolate plasmid DNA. These were used as the template to prepare the standard curve to calculate the copy number of 16S ribosomal DNA in the ss cDNA. qPCR was performed using a Realplex^4 Real-Time PCR System (Eppendorf, Hamburg, Germany) using SYBR Premix Ex Taq (Takara). Then, different volumes of ss cDNA containing 20,000, 10,000, 5,000, 2,500, 1,250 copies of 16S ribosomal DNA were used to evaluate the efficiency, regression coefficient, and slope.

cDNA library construction

The first-strand cDNA was precipitated with 2.5 volumes of ethanol with 0.1 volume of sodium acetate (3 mol/L, pH = 5.2). The precipitated cDNA was redissolved and then dephosphorylated with calf intestine alkaline phosphatase (CIAP, Takara). Then, T4 DNA ligase (Takara) was used to ligate the cDNAs to the oligonucleotide 5′-CTAA-TACGACTCACTATAGGGCAGCGTGGTCGCGGCCGA GGT-3′, which was phosphorylated at the 5′ end (adaptor 1). The ligation products were precipitated and then redissolved in 10 μL ddH_2O before being treated with T4 Polynucleotide kinase (PNK, Takara) to phosphorylate the 5′ end. Then, another oligonucleotide, 5′-CTAATACGACTCACTA-TAGGGCTCGAGCGGCCGCCCGGGCAG GT-3′ (adaptor 2), was ligated to the other end of the cDNAs. The sequences of the two adaptors were those recommended in the manual of the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) with some modifications. PCR amplification was then performed using the primers F: 5′-ACCTGCCC GGGCGGCCGCTCGA-3′ and R: 5′-GCCCTATAGT GAGTCGTATTAG-3′, which had base complementarity with parts of the two adaptors. The PCR products were purified with an E.Z.N.A. Cycle-Pure Kit (Omega, Doraville, GA, USA), inserted into the pEAST-T4 Zero vector (TransGen Biotech Co., Ltd. Beijing, China), and then transformed into Trans1-T1 phage-resistant chemically competent cells (TransGen). The transformed cells were plated onto solid LB medium containing 50 μg/ml ampicillin. After incubation overnight at 37 °C, white colonies (putative positive clones) were picked and transferred into 1.5 ml tubes containing 500 μL LB liquid medium (AMP^+). The cDNA inserts were then amplified by PCR.

DNA sequencing and nucleic acid homologies

Positive clones were sent to the Shanghai Sangon Biological Engineering Technology and Service Co., Ltd for sequencing. DNA sequences were analyzed at the National Center for Biotechnology Information (NCBI, USA; www.ncbi.nlm.nih.gov) using BlastN and BlastX algorithms.

Results

Optimization of the $Al_2(SO_4)_3$ volume to precipitate humic substances

We screened different volumes of $Al_2(SO_4)_3$ to precipitate humic substances from sugarcane soil, and found that addition of 150 μL of $Al_2(SO_4)_3$ to sugarcane soil gave the best results (a large amount of nucleic acids and lower concentrations of humic acid) (Fig. 1). Using the screening protocol, the following volumes were determined as optimal for treating rhizosphere soil from each crop: rice, 150 μL; tobacco, 120 μL; sugarcane, 150 μL; *R. glutinosa,* 75 μL; and *P. heterophylla,* 120 μL. The nucleic acids extracted from the samples were analyzed by agarose gel electrophoresis (Fig. 2). The quality of the extracted nucleic acids was evaluated via spectrophotometry (Varian, USA). The $A_{260/280}$ ratio of the nucleic acids extracted from rice, tobacco, sugarcane, *R. glutinosa,* and *P. heterophylla* soil was 1.71, 1.69, 1.71, 1.72, and 1.69, respectively, and the amount of nucleic acids extracted from the samples was 22.7, 19.0, 14.3, 24.48, and 17.96 μg per gram of soil, respectively.

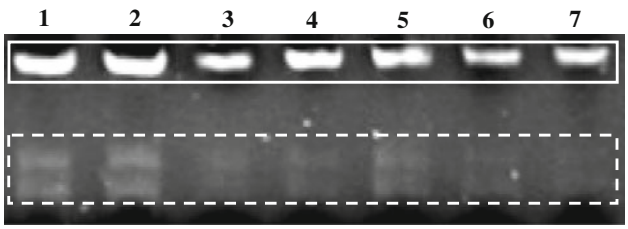

Fig. 1 Optimization of the $Al_2(SO_4)_3$ volume for efficient extraction of DNA and RNA from sugarcane soil samples. *Solid box* shows DNA, *dotted box* shows RNA. In this procedure we used 120, 150, 180, 210, 240, 270 and 300 μL $Al_2(SO_4)_3$ to precipitate humic substances (*lane* 1 to *lane* 7)

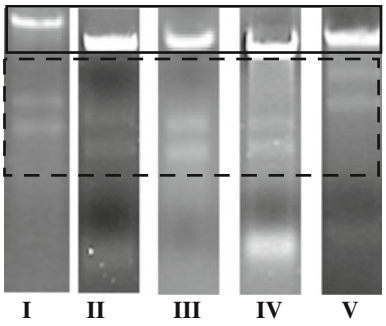

Fig. 2 DNA and RNA extracted from different crop rhizospheric soil separated by gel electrophoresis. **I** tobacco soil; **II** *Pseudostellaria heterophylla* soil; **III** sugarcane soil; **IV** rice soil; **V** *Rehmannia glutinosa* soil. *Solid box* shows DNA, *dotted box* shows RNA. In this procedure, we using the optimal volume of $Al_2(SO_4)_3$ to precipitation of humic substances for different crop rhizospheric soil

Separation and purification of RNA from crude extraction

Total crude extracts from the rice soil sample were selected for isolation of RNA. The DNA was digested by DNase I and then purified by gel filtration. Analyses by gel electrophoresis and UV-spectrophotometric methods showed that the total RNA was successfully purified (Fig. 3I). In addition, the degraded DNA could also be observed as a low molecular weight band (Fig. 3I, lower square), which could have also contained some degraded RNA.

Detection of rice 16S ribosomal DNA from first-strand cDNA

The size of the first-strand cDNA fragments ranged from 0.5 to 3.0 kb (Fig. 3II). Based on the melting curve

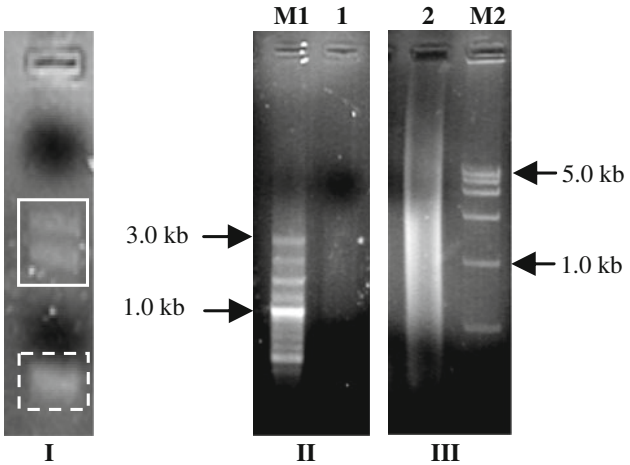

Fig. 3 Purification of total RNA from rice rhizosphere soil (**I**), synthesis of the first-strand cDNA (**II**) and double-stranded cDNA (**III**). *Solid box* shows RNA, *dotted box* shows degraded material. M1 100-bp ladder; 1 first-strand cDNA; M2 15,000 bp DNA marker; 2 double-stranded cDNA

determined from the qPCR, bacterial 16S rDNA was efficiently amplified from the test sample, compared with the no-template control (NTC) (Fig. 4I). Based on the calculated results of 16S rDNA in ss cDNA (data not shown), 16 s rDNA was amplified from different volumes of ss cDNA by qPCR with an efficiency of 0.91, and the regression coefficient or slope was −3.557 (Fig 4II).

Rice soil cDNA library

Analyses of ds cDNA by gel electrophoresis showed that the cDNA fragments ranged from 0.5 to 3.0 kb (Fig. 3III). The rice soil ds cDNA fragments were inserted into the pEAST-T4 Zero vector and transformed into Trans1-T1 phage-resistant chemically competent cells. We constructed a cDNA library to identify expressed genes in rice rhizosphere microorganisms. A total of 328 clones were obtained from the library, and 15 of the cDNA clones were selected for sequencing.

Genetic information for rice soil microbe cDNA

The 15 clones were assigned putative functions based on the homologies of their sequences to those in the nucleotide database (Table 2). Clone R2_14 encoded an epithelial membrane protein; clone R2_22 showed the highest homology to a genomic sequence from *Streptococcus mutans*; clone R2_28 showed homology to the Ebd-P2 pseudogene from *Bos Taurus*; clones R3_11 and R4_4 encoded a beta-1 mRNA OSE-577-637-E3 elongation factor from *Oryza sativa*. Clone R3_32 showed the highest homology to a genomic sequence from *Bordetella parapertussis.* Clone R4_8 showed homology to the gene encoding red fluorescence protein. Clone R5_59 showed the highest homology to microsatellite DNA and clone R5_65 encoded the ATP-binding component of the serine protease from *Shigella flexneri.* The other six clones resembled sequences encoding rRNA from different species.

Discussion

The development of modern molecular biological technologies makes it possible to investigate the communities and functional diversity of soil microorganisms. For example, metagenomics methodologies, that is, methods to isolate and analyze RNA or DNA from soil, are necessary for studies on microbial diversity and functions in soil. Peršoh et al. (2008) described a simple and rapid method for extracting nucleic acids from soil. However, the method involved the use of a specific bead-beating instrument, which many laboratories do not have. In the method described in the present study, a vortex mixer was used for

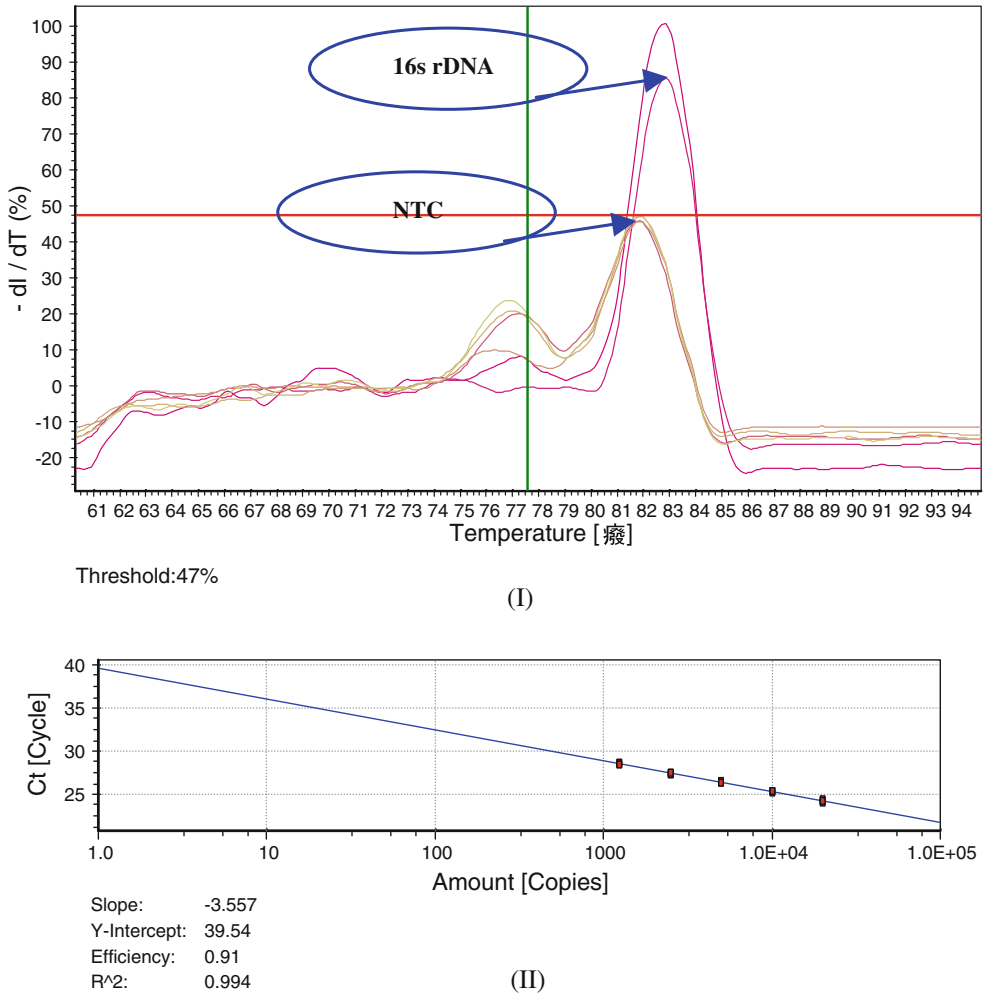

Fig. 4 *Melting curve* analysis of the PCR products of soil rice soil 16S rDNA (**I**) and its standard curve generated by graphing the DNA copies used vs. the CT value

the bead-beating step because most laboratories have this piece of equipment. Different volumes of $Al_2(SO_4)_3$ were added to the soil sample during the vortexing step, to determine which volume gave the best results (high levels of nucleic acids and low levels of humic acid). Our results showed that the method could be optimized to extract total RNA from soil microbes in different types of crop soil. We also found that this method is suitable to extract nucleic acids from marine silt (data not shown). We converted the total RNA into cDNA using oligo dT and 6-base random primers, and then the 5′ and 3′ ends of the first-strand cDNA were ligated to two adaptors. A pair of primers with base complementarity to the adaptors was used to amplify single-strand cDNA. The PCR products obtained from these amplifications represented genetic information for soil microbes. Subcloning of the cDNA enabled us to obtain individual clones for sequence analysis. We used RNA isolated from rice soil to construct cDNA libraries, and partial cDNA clones were sequenced and aligned with sequences in the GenBank. Analyses of nucleic acid homologies showed that the cDNA clones represented functional gene and rRNA gene sequences. These sequences were derived from bacteria, fungi, plants, and animals, indicating the diversity of genes in the soil environment. In this study, we could not obtain detailed genetic information about the soil microbes in the samples, since GenBank contains only incomplete data for soil microbes. Additionally, some partial gene sequences were aligned with plant and animal gene sequences. Many of the rRNA gene sequences were from uncultured microbe species, and little information about other functional genes was present in the database. Further studies to obtain genomic information, for example, using the transcriptome approach described in this study, can clarify aspects of the movement of microbes in soil and interactions between microbes and plants.

Table 2 Sequence comparisons for clones from the cDNA library of rice rhizosphere soil microbes

Clones	Gene accession no.	Best homolog in database	Score	%ID/E value	Source
R2_14	NM_007929	Epithelial membrane protein 2	501	99 %/0.0	*Mus musculus*
R2_22	DQ072007	Clone D7, genomic sequence	50	98 %/2e−15	*Streptococcus mutans*
R2_28	AJ620296	Ebd-P2 pseudogene	139	99 %/9e−65	*Bos taurus*
R3_11	EF576070	OSE-577-637-E3 elongation factor beta-1 mRNA, complete cds	669	99 %/0.0	*Oryza sativa*
R3_14	AB546581	Gene for 28S rRNA, partial sequence, clone: LAN35	299	99 %/1e−153	*Uncultured Sordariomycetidae*
R3_22	FN870270	Partial 16S rRNA gene, clone ArcIV_cloneE08	556	99 %/0.0	*Uncultured archaeon*
R3_28	HQ183857	Clone De3134 16S ribosomal RNA gene, partial sequence	975	99 %/0.0	*Uncultured Corallococcus sp.*
R3_32	DQ518948	Bpp5 subtractive hybridization product 6,908 genomic sequence	61	97 %/2e−21	*Bordetella parapertussis strain*
R4_4	EF576070	Clone OSE-577-637-E3 elongation factor beta-1 mRNA	669	99 %/0.0	*Oryza sativa*
R4_6	FR687033	Partial 16S rRNA gene, clone M8C_clone196	342	99 %/2e−177	*Uncultured Beggiatoa sp.*
R4_8	EU016077	Red fluorescence protein DsRed2 gene, complete cds	313	99 %/2e−161	*Expression vector pDsRed2_ER*
R5_56	AB546581	Gene for 28S rRNA, partial sequence, clone: LAN35	349	99 %/0.0	*Uncultured Sordariomycetidae*
R5_59	AM422187	Microsatellite DNA, clone ZcwE05	350	99 %/0.0	*Zalophus wollebaeki*
R5_65	CP001383	ATP-binding component of serine protease	222	99 %/1e−111	*Shigella flexneri*
R6_32	HQ183857	Clone De3134 16S ribosomal RNA gene, partial sequence	978	99 %/0.0	*Uncultured Corallococcus sp.*

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