DNA Extraction, PCR Amplification, Sequencing and Phylogenetic Analysis

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Abstract

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Protocol

Step 1.
Before the extraction of genomic DNA, fungal isolate was cultured on PDA plates at 28 °C in darkness for 5-7 days.

Step 2.
100 mg Fungal mycelia were picked out to a mortar and 1000 µl of CTAB lysis buffer was added to grind at 65°C.

Step 3.
The mycelial mixture was transferred to a 1.5 ml Eppendorf tube and 10 µL of β-mercaptoethanol was added.

Step 4.
The Eppendorf tube was mixed thoroughly and water-bathed at 65°C for 60 min with continuous shaking.

Step 5.
The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 12,000×g for 8 min at 4 °C.

Step 6.
The supernatant was transferred to a new microtube and extracted with chloroform/isoamyl alcohol (24:1) at 12,000×g for 8 min at 4 °C.

Step 7.
Repeat step 6 once. The supernatant was transferred to a new microtube and precipitated by adding a 2× volume of chilled isopropanol for 6 h at −20°C.

Step 8.
The resulting pellet was collected by centrifugation (12,000×g, 15 min), washed twice with 75% ethanol, air-dried, resuspended in 20 µl of sterile Millipore water and deposited in -20℃ freezer for storage.

The resulting genomic DNA was used as template to amplify the fungal ITS regions with the universal ITS primers, ITS1F (5□ CTTGGTCATTTAGAGGAAGTAA 3□) and ITS4 (5□ TCCTCCGCTTATTGATATGC 3□), using the polymerase chain reaction (PCR).

Step 9.
Prepare the PCR system (50 µl) as follows on ice cDNA mixture 1 µl dNTP 1 µl upstream primer (10

mM) 1 µl downstream primer (10 mM) 1 µl Taq buffer 5µl Taq 1 µl ddH2O add up to 50 µl

Step 10.

Step 11.
Set up the PCR reaction procedure as described below:Step 1 94℃ 5 minutesStep 2 94℃ 40 seconds55℃ 40 seconds72℃ 55 seconds30 Cycles in Step 2Step 3 72℃ 10 minutesStep 4 4℃ hold

Step 12.
The amplified products were submitted for sequencing (Invitrogen, Shanghai)

Step 13.
The ITS-rDNA sequence were obtained, and the cross peaks were removed using BioEdit5.0.6 software.

Step 14.
Aligned with the sequences in the GenBank by Basic Local Alignment Search Tool (BLAST) programs to find out the sequence homology with closely related organisms (similar≥ 98%).

Step 15.
Phylogenetic analyses of the endophytes were carried out using MEGA 5.1 software, the neighbor-joining (NJ) method was used to infer the evolutionary history of the fungal isolates, and the bootstrapping was carried out using 1,000 replications.

Step 16.
Finally, we submitted ITS-rDNA sequences of the representative isolates to GenBank and obtained the accession number.

Step 17.