MOLECULAR IDENTIFICATION OF PHYTOPATHOGENIC FUNGI

Daisy Perez-Brito

Abstract

The importance of fungal species differentiation is critical for plant pathology research and disease control purposes. Traditionally, fungal species have been identified by a range of cultural and morphological characteristics, such as conidial morphology, presence or absence of setae, fungicide sensitivity, and colony color and growth rate. Although valuable, these criteria alone are not always adequate as morphological characteristics may vary under different environmental conditions. Among other techniques, sequence analyses of the internal transcribed spacer (ITS) regions, including the 5.8S rDNA, has been used extensively to identify fungal species. This region is the most widely sequenced DNA region of fungi and has been employed as the universal fungal barcode sequence. It is very useful for molecular systematics at species to genus level and within species.

This protocol is useful for a primary molecular and accurate identification of any fungal species using the sequence analyses of the ITS-5.8S rDNA region

Citation: Daisy Perez-Brito MOLECULAR IDENTIFICATION OF PHYTOPATHOGENIC FUNGI. **protocols.io** dx.doi.org/10.17504/protocols.io.shteb6n
Published: 09 Aug 2018

Protocol

Obtaining mycelial mass for genomic DNA isolation

Step 1.

1. Obtain 10 discs of agar culture (1 x 1 cm) from a 7-day-old colony of target fungus grown on PDA or another culture medium.
2. Transferred them into 250-ml Erlenmeyer flasks containing 50 ml of nutrient broth (NB) medium
3. Incubate at 28°C on an orbital shaker (100 rpm) for three or four days
4. Collect mycelia by filtration
5. Frozen mycelia at -80°C until use, or frozen mycelia at -80°C for 2 hours and lyophilized and store until use (optional).

Fungal genomic DNA isolation:

Step 2.

1. Resuspend fungal mycelia in 1000 µL of sterile distilled water and add two 5-mm sterile porcelain beads, vortex at full speed for 3–5 min.
2. Freeze at –80°C for 10 min. Defrost (3 min, at 65°C in a water bath) and vortex at full speed again for 3 min.
3. Transfer 300 µL of cell suspension to a new 2-mL microcentrifuge tube. Add 800 µL of lysing buffer. Incubate at 65°C for 30 min, with occasional inversion. Cool the mixture to room temperature (RT).

4. Add 500 µL of chloroform and invert gently for 10 min.
5. Centrifuge for 10 min at 15,682g at RT.
6. Transfer top aqueous layer to a new 2-mL microcentrifuge tube
7. Add 700 µL of chilled isopropanol. Mix by very gentle inversion
8. Incubate for 10 min at –80°C
9. Centrifuge for 5 min at 15,682g at RT. Discard the supernatant. Wash pellet with 500 µL of 70% ethanol
10. Dry under vacuum centrifugation (optional).
11. Resuspend the dry pellet in 50 µL of TE buffer

ITS-5.8S rDNA region amplification by PCR

Step 3.

1. Amplified the internal transcribed spacer region, including the 5.8S rDNA, using universal primers ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3')
2. Carried out PCR reaction in 25 µL, consisting of 25 ng of DNA, 1× PCR buffer (Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM of each primer and 1U *Taq* polymerase (Invitrogen).
3. Perform DNA amplification in a thermal cycler as GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer)
4. Amplification program consisted of an initial denaturing step at 95° for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, and a final extension step of 7 min at 72°C.
5. PCR product can be visualized by electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining. The band size obtained will be between 500 and 800 base pairs, depending on the filamentous fungus species studied.
6. The quantity and quality of DNA obtained must be determined. DNA quantified spectrophotometrically and DNA quality by calculating the A260/A280 ratio.

Sequencing

Step 4.

1. PCR products obtained must be purified and sequenced.
2. Note: In our case, we send all our PCR products to Macrogen Inc. Korea for sequencing

Fungal species Identification

Step 5.

1. Fungal sequences should be alignment and edited using the Bio Edit Sequence Alignment program.