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Preparation of long rDNA amplicons of arbuscular mycorrhizal fungi (Glomeromycota) from soil and root DNA for high-fidelity long-read sequencing

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We use this protocol and it's working

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Abstract

This protocol describes the preparation of long rDNA amplicons (~2.7 kb) of arbuscular mycorrhizal (AM) fungi (phylum Glomeromycota) from environmental DNA for high-fidelity long-read sequencing. The workflow targets AMF communities in both soil and fine roots, using DNA extracted with common commercial kits. The method employs a nested PCR strategy adapted from Kolaříková et al. (2021, New Phytologist), which amplifies a contiguous fragment spanning most of the SSU rRNA gene, the complete ITS region, and the D1–D2 domains of the LSU rRNA gene. In the first round, broad AMF primer combinations NS31/LSUmAr13+24 and AML1/LSUmAr13+24 are used, following the LSUmAr13 + LSUmAr24 LSU primer design of Tedersoo et al. (2018, New Phytologist) rather than the original LSUmAr1–4 mix. This primer scheme provided robust amplification from diverse soil and root DNA extracts in test runs. The pooled first-round products are then subjected to a Glomeromycota-specific second PCR with NS31_Glo3/wLSUmBr, yielding ~2.7 kb amplicons that cover SSU–ITS–LSU for AMF lineages. The resulting libraries are suitable for downstream long-read sequencing on PacBio Revio/Sequel IIe or Oxford Nanopore platforms. Because the primer scheme is AMF-focused but not site-specific, the protocol can be applied to a wide range of soil- or root-associated AMF communities and integrated with existing SSU, ITS, and LSU datasets for phylogenetic and ecological analyses.

Guidelines

This protocol describes a nested PCR workflow for amplifying near-full-length SSU–ITS–LSU rDNA of arbuscular mycorrhizal (AM) fungi (Glomeromycota) from soil and root DNA. It is optimized for environmental samples with low DNA yield or humic contamination and can be applied prior to long-read sequencing (e.g., PacBio or Nanopore). All steps should be carried out using sterile, DNA-free consumables to minimize contamination.

Materials

Materials and Reagents

- 0.2 mL PCR tubes
- Nuclease-free water
- DNA extraction kit for soil: NucleoSpin Soil DNA Kit (Macherey-Nagel, Germany) or equivalent
- PCR enzyme: KOD One PCR Master Mix (TOYOBO, Japan)
- Magnetic bead purification: AMPure XP (Beckman Coulter, USA)
- Primers (PAGE/HPLC purified):
  1. NS31 (5′-TTGGAGGGCAAGTCTGGTGCC-3′; Simon et al. 1992)
  2. AML1 (5′-ATCAACTTTCGATGGTAGGATAGA-3′; Lee et al. 2008)
  3. LSUmAr13 (5′-GCTCDYACTCAAATCTATCAAA-3′; Krüger et al. 2009)
  4. LSUmAr24 (5′-GCTCTDACTCAAAYCTATCGAT-3′; Tedersoo et al. 2017)
  5. NS31_Glo3 (5′-AminoC6-TTGYTGCRGTTAAAAAGCTCG-3′; Kolaříková et al. 2021)
  6. wLSUmBr (5′-AminoC6-AACACTCGCAYAYATGYTAGA-3′; Schlaeppi et al. 2016)
- 70% ethanol (molecular grade)
- Low-TE buffer for elution
- Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, United States)
- Qubit Assay Tubes (Thermo Fisher Scientific)
- DNA ladder (1 kb–3 kb range)

Equipment

- Thermal cycler (e.g., TaKaRa PCR Thermal Cycler Dice Touch, Takara Bio Inc.)
- Micropipette (P1000, P200, P100, P20, P10, and P2)
- Vortex mixer
- Magnetic stand (e.g., NGS MagnaStand (YS-Model) 8Ch × 0.2 mL PCR tube, Nippon Genetics Co., Ltd., Tokyo, Japan)
- Microcentrifuge
- Qubit fluorometer (Qubit 4 fluorometer, Thermo Fisher Scientific)
- Gel electrophoresis system
- PacBio Revio sequencer (outsourced service)

Safety warnings

- Avoid cross-contamination between PCR rounds; use separate work areas for template preparation and post-PCR handling.
- Over-cycling (>35 cycles) may produce chimeric amplicons.
- AMPure beads are sensitive to overdrying—dry only until the surface just turns matte.
- The long rDNA amplicons (~2.7 kb) are prone to shearing; minimize pipetting and vortexing.
- For root DNA, ensure that only fine roots (not woody tissue) are used; otherwise PCR inhibition may occur.

Ethics statement

No animal or human subjects were involved in this study. Soil and root samples were collected from managed research sites with permission from the responsible authorities.

Before start

- Prepare all reagents, primers, and consumables in a clean workspace dedicated to pre-PCR operations.
- Prepare 70% ethanol and magnetic beads (e.g., AMPure XP) for purification steps in advance.
- Ensure that the thermocycler and magnetic stand are clean and functional before starting.

DNA extraction

4h

1 **For soil samples:**
Extract total genomic DNA from approximately 500 mg of soil using the NucleoSpin Soil DNA Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Perform all steps at room temperature unless otherwise noted, and elute DNA with 50 µL of SE buffer.

2h

2 **For root samples:**
Extract total gDNA from approximately 20 mg of fine roots using the ISOPLANT II Kit (Nippon Gene Corporation, Tokyo, Japan) following the manufacturer's protocol. Elute DNA with 50 µL of the supplied buffer.

2h

3 Measure the extracted gDNA concentration using a Qubit fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

1st PCR: NS31 / LSUmAr13 + LSUmAr24

45m

4 Prepare PCR-master mix in a 0.2 mL PCR tube with the following:

5m

	Component	Volume (total 25 µl)
	Nuclease-free water	X µl
	Forward primer (NS31, 10 µM)	1.25 µl
	Reverse primer (LSUmAr13, 10 µM)	0.675 µl
	Reverse primer (LSUmAr24, 10 µM)	0.675 µl
	2× KOD One mix	12.5 µl
	Template DNA	Y µl (20 ng)

5 Run the following PCR program:
[98 °C 00:00:10 → 66 °C 00:00:05 → 68 °C 00:00:20] × 30 → 8 °C ∞

40m

1st PCR: AML1 / LSUmAr13 + LSUmAr24

45m

6 Prepare PCR-master mix in a 0.2 mL PCR tube with the following: 5m

	Component	Volume (total 25 µl)
	Nuclease-free water	X µl
	Forward primer (AML1, 10 µl)	1.25 µl
	Reverse primer (LSUmAR13 10µl)	0.675 µl
	Reverse primer (LSUmAr24, 10µl)	0.675 µl
	2 × KOD One mix	12.5 µl
	Template DNA	Y µl (20 ng)

7 Run the following PCR program: 40m
[98 °C 00:00:10 → 53 °C 00:00:05 → 68 °C 00:00:20] × 30 → 8°C ∞

Clean-up of 1st PCR products

30m

8 Clean up the 1st PCR products using AMPure XP (Beckman Coulter, USA).

8.1 Equilibrate the beads solution at room temperature and allow the beads to resuspend completely.

8.2 Add 12.5 µL beads solution to the 25 µL PCR products (0.5× bead solution/sample ratio) and mix by pipetting up and down multiple (10 to 20) times.

8.3 Incubate the tube at room temperature for 5 min.

8.4 Place the tube on a magnetic rack (NGS MagnaStand (YS-Model) 8Ch × 0.2 mL PCR tube, Nippon Genetics Co., Ltd., Tokyo, Japan) to capture the beads. Incubate until the liquid is clear (~ 00:03:00 min). 3m

8.5 Discard the supernatant.

8.6 Wash beads by adding 200 µL 70% ethanol.

8.7 Incubate the tube on the magnetic rack at room temperature for 00:00:30 . 30s

8.8 Discard the supernatant.

8.9 Repeat the previous washing steps (8.6-8.8).

8.10 Remove the tube from the magnetic rack.

8.11 Elute the purified DNA by adding 25 µL of TE Buffer and mix by pipetting.

8.12 Incubate the tube at room temperature for 00:02:00 . 2m

8.13 Place the tube on the magnetic rack to capture the beads. Incubate until the liquid is clear (00:01:00). 1m

8.14 Transfer the supernatant to a new PCR tube.

2nd PCR: NS31_Glo3 / wLSUmBr 50m

9 Prepare PCR-master mix in a 0.2 mL PCR tube with the following: 10m

	Component	Volume (total 25 µl)
	Forward primer (NS31_Glo3)	1.25 µl
	Reverse primer (wLSUmBr)	1.25 µl

	Component	Volume (total 25 µl)
	2 × KOD One mix	12.5 µl
	Template DNA (Cleaned 1st PCR products) NS31 / LSUmAr13 + LSUmAr24	5 µl
	Template DNA (Cleaned 1st PCR products) AML1 / LSUmAr13 + LSUmAr24	5 µl

10 Run the following PCR program: 40m

[98 °C 00:00:10 → 62 °C 00:00:05 → 68 °C 00:00:20]×30 → 8 °C ∞

Clean-up of 2nd PCR products 20m

11 Clean up the 2nd PCR products using AMPure XP (0.5× bead solution/sample ratio) following the same procedure as in Section 8. 20m

Quality control 10m

12 Measure the DNA concentration of the purified 2nd PCR products using a Qubit fluorometer with the Qubit dsDNA HS assay kit. 10m

13 OPTIONAL: *
Check the amplified fragments by gel electrophoresis.

Protocol references

Kolaříková Z, Slavíková R, Krüger C, Krüger M, Kohout, P (2021) PacBio sequencing of Glomeromycota rDNA: a novel amplicon covering all widely used ribosomal barcoding regions and its applicability in taxonomy and ecology of arbuscular mycorrhizal fungi. New Phytol 231:490–499. https://doi.org/10.1111/nph.17372

Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota : one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223. https://doi.org/10.1111/j.1469-8137.2009.02835.x

Lee J, Lee S, Young JPW (2008) Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi: PCR primers for arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 65:339–349. https://doi.org/10.1111/j.1574-6941.2008.00531.x

Schlaeppi K, Bender SF, Mascher F, Russo G, Patrignani A, Camenzind T, Hempel S, Rillig MC, van der Heijden MGA (2016) High-resolution community profiling of arbuscular mycorrhizal fungi. New Phytol 212:780–791. https://doi.org/10.1111/nph.14070

Simon L, Lalonde M, Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295. https://doi.org/10.1128/aem.58.1.291-295.1992

Tedersoo L, Tooming-Klunderud A, Anslan S (2018) PacBio metabarcoding of Fungi and other eukaryotes: errors, biases and perspectives. New Phytol 217:1370–1385. https://doi.org/10.1111/nph.14776

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