DNA metabarcoding of pollen transported by moths Version 2

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Abstract

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Protocol

Extract DNA

Step 1.

DNA extractions are carried out using a HotSHOT approach, as follows (see Truett *et al.,* (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*, **29**, 52-54.

1. Excise moth proboscis at the base using a sterile scalpel.
2. Place proboscis in 30 µl HotSHOT alkaline lysis reagent (Truett *et al.,* 2000) in individual well of 200 µl strip tube
3. Shake for 10 minutes at 2000 rpm on Variomag Teleshake plate shaker
4. Remove proboscis using sterile forceps
5. Seal remaining liquid (rinse) with a drop of mineral oil
6. Heat to 95 °C for 1 hour
7. Neutralize rinse with 30 µl HotSHOT neutralizing reagent (Truett *et al.,* 2000)

PCR1

Step 2.

Extracted DNA is amplified at the *rbcL* region using a custom universal primer pair, rbcL-3C (rbcL-3CF: 5’-CTGGAGTTCCGCCTGAAGAAG-3’; rbcL-3CR: 5’-AGGGGACGACCATACTTGTTCA-3’).

1. Set up a PCR reaction containing:

- 8 µl of undiluted DNA extract
- 10 µl MyFi mix
- 0.5 µl each of forward and reverse primers in 10µM solution (final concentration: 0.25µM per primer)
- 1 µl of 1x TE buffer solution
- Drop of mineral oil to seal well

2. Carry out PCR reaction with the following program:

1. Initial denaturation at 98 °C for 5 minutes
2. 45 cycles of: 98 °C for 15 seconds; 56 °C for 15 seconds; 72 °C for 45 seconds
3. Incubation at 72 °C for 10 minutes, then 4 °C for 10 minutes

PCR2

Step 3.

Amplified DNA from PCR1 is further amplified and tags added to identify the source well of each amplicon within its plate. Each well is treated with a unique pair of primers, sourced from 16 tag variants of the forward primer and 12 tag variants of the reverse primer.

1. Set up a PCR reaction containing:

- 2 µl of undiluted PCR1 product
- 10 µl MyFi mix
- 6 µl of molecular biology-grade H_2O
- 0.5 µl each of forward and reverse primers in 10µM solution (final concentration: 0.25µM per primer)
- 1 µl of 1x TE buffer solution
- Drop of mineral oil to seal well

2. Carry out PCR reaction with the following program:

1. Initial denaturation at 98 °C for 5 minutes
2. 25 cycles of: 98 °C for 30 seconds; 30% speed ramp to 58 °C for 1 minute; 72 °C for 2 minutes
3. Incubation at 72 °C for 10 minutes, then 4 °C for 10 minutes

Plate pooling and cleaning

Step 4.

Wells are pooled to create a single library per plate, and each library cleaned to remove leftover primers.

1. Pool 10 µl of PCR2 product from each well within a plate to create a single library for each plate.
2. Clean each library using Mag-Bind RXN PurePlus magnetic beads, following the protocol of Kitson (2016).

PCR3

Step 5.

Illumina MiSeq adapter tags are added to pooled, cleaned PCR2 product to identify the source plate of an amplicon and permit sequencing on the Illumina MiSeq. A unique pair of tagged adapter primers was used for each plate.

1. Set up a PCR reaction containing:

- 5 µl of undiluted, cleaned library from step 4
- 10.5 µl MyFi mix
- 3.5 µl of molecular biology-grade H_2O
- 1.05 µl each of forward and reverse primers in 10µM solution
- Drop of mineral oil to seal well

2. Carry out PCR reaction with the following program:

    1. Initial denaturation at 95 °C for 3 minutes
    2. 12 cycles of: 98 °C for 20 seconds; 72 °C for 1 minute
    3. Incubation at 72 °C for 5 minutes, then 4 °C for 10 minutes

Library pooling and cleaning

Step 6.

Plate libraries are pooled to create a single library for sequencing, and cleaned to remove leftover primers.

1. Measure DNA concentration of the product from each plate using a Qubit Fluorometer.
2. Pool 10 µl of PCR3 product from the plate with the weakest concentration with the same quantity of DNA (and therefore lower volumes) from each other plate.
3. Clean each library using Mag-Bind RXN PurePlus magnetic beads, following the protocol of Kitson (2016).

Sequencing

Step 7.

Final library is sequenced on an Illumina MiSeq following standard protocols.