Molecular methods

DNA extractions

DNA is usually extracted from living or silica dried material, typically ca. 0.05 – 0.1 g. Tissue is washed in water, blotted dry on filter paper and weighed, and ground in an ependorf tube using a plastic pestle and a pinch of sea washed sand (Fisher Scientific cat. no. 525-500). DNA extractions are done using QIAGEN DNeasy® plant mini kits (cat. no. 69104). 100 µl of 65 c buffer is added, and the samples are ground for a few minutes longer. Standard kit protocol is followed, with the first elution in 50 µl buffer AE, and a second elution in 75 µl. Occasionally a modified CTAB procedure is used instead of the QIAGEN kit. Other taxa have been amplified using a modification of Edwards et al., with a final resuspension in 25 µl nanopure water.

PCR

We use two different polymerases. The first is a standard Promega Taq; the second is Clontech's Advantage-GC cDNA Polymerase Mix (BD Bioscience cat. no. 639112).

Recipes:

1.5 units Promega Taq DNA polymerase (Promega, cat. no. M186E, storage buffer A) (0.3 µl at 5 units/µl)
1 x buffer (Promega) (5 µl of 10 X buffer)
2.5 mM MgCl2 (Promega) (5 µl of 25 mM)
200 µM dNTPs (Roche Diagnostics cat. no. 1969 064) (1 µl of each at 10 mM)
300 µM primer (1.5 µl of 10 mM)
DNA
water to 50 µl.

7.5 µl Advantage GC mix
7.5 µl Advantage buffer
1.5 µl of each buffer (at 10 mM)
0.3 µl dNTPs
0.75 µl Advantage polymerase
DNA
water to 35 µl.

Typically 0.3-0.5 µl of DNA extraction is added to each reaction. Reactions are run in a PTC-100 Peltier Thermal Cycler (M.J. Research). For primer sequences, see Table 1.

PCR programme: 1 min @ 94; 35 cycles of 1 min @ 93, 1 min @ 48-50, 3 mins @ 72; 7 mins @ 72; hold @ 4.

PCR products are run out on a 1.6% agarose gel (0.8 g agarose (Sigma cat. no. A-0169) dissolved in 50 ml 1x TBE), typically 5 µl product in 0.5 µl loading solution (10X blue juice, Invitrogen); 1 kb Plus DNA ladder (Invitrogen cat. no. 10787-018) is used as a standard. The gels are stained with ethidium bromide, and visualized under UV.

Successful reactions are cleaned up using QIAquick® PCR purification kits (cat. no. 28106), and eluted in 30 µl buffer EB. The cleaned products were run out on a 1.6% agarose gel, and visualized using UV light.

Sequencing Reactions

1.5 µl BigDye ® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems)
1 µl 5X Sequencing Buffer (Applied Biosystems)
1.6 µl Primer (1 mM)
1.5 – 4.5 µl Template
water to 10 µl.

Sequencing PCR programme: 30 cycles of: 10 sec @ 96, 5 sec @ 50, 4 min @ 60; hold @ 4.

Sequencing reactions are cleaned up using Princeton Separations CentriSep Spin columns (cat. no. CS-901). Old columns are washed and refilled with 85 µl Sephadex (Sephadex ™ G-50 fine DNA grade (Amersham Biosciences cat. no. 17-0573-02) hydrated in nanopure water).

Samples are dried down, then resuspended in ca. 1.25 µl loading solution (30 µl Blue Dextran/EDTA (PE Biosystems part no. 402055) in 120 µl formaline). Sequences are run on an ABI 377 Automated Sequencer, using Long Ranger® XL Singel packs (BioWhittaker Molecular Applications cat. no. 50673) and 64 well sharkstooth paper combs (Applied Biosystems), with c. 1µl resuspended sample loaded in each lane, using a single channel Hamilton syringe.