PCR and Sequencing methods

A modified CTAB method was used to extract DNA which was then suspended in TE buffer and stored at -20°C. PCR amplification was performed using a Corbett PC-960C cooled thermal cycler and negative controls were run for all amplifications. Amplification was performed in a 25μl reaction mix consisting of 2.5μl 10xPCR Buffer, 1.5μl 50mM MgCl2, 1.25μl dNTPs (10mM), 1.0μl of both forward and reverse primers (10pmol), 17.75μl double distilled water, 0.1μl Platinum Taq (5 units/μl) and 1μl of template DNA. The primers used are detailed in Table 2. Amplification of the mitochondrial DNA fragments was conducted with an activation step of 94°C for 4 mins, touchdown from 94°C (30s), 70°C (20s) and 72°C (1m30s) to 94°C, 45°C, 72°C (annealing temperature down 5°C every 2 cycles), followed by 35 cycles of 94°C (30s), 40°C (30s) and 72°C (45s), with a final extension of 72°C for 4 mins. Amplification of nuclear genomic DNA was conducted using an activation step of 95°C for 4mins, touchdown from 95°C for 15s, 55°C for 15s and 72°C for 30s, to 91°C, 51°C and 72°C (annealing temperature down 1°C each 2 cycles), followed by 30 cycles of 90°C (15s), 50°C (15s) and 72°C (30s) with a final extension of 72°C for 4mins. For RPL3int5: activation step of 95°C for 4 mins, touchdown from 95°C for 30s, 55°C for 30s and 72°C for 1min, to 95°C (30s), 45°C (30s) and 72°C (1min) (annealing temperature down 5°C every 2 cycles), followed by 20 cycles of 95°C, 50°C and 72°C, and 30 cycles of 95°C, 47°C and 72°C, with a final extension of 72°C for 6mins. Approximate concentration of amplification products was determined after each extraction and amplification step by electrophoresis in a 1.5% agarose gel stained with 6.0μl ethidium bromide and visualized under ultra-violet light. PCR products were gel purified using the ExoSap cleanup method (nuclear markers), or ammonium acetate precipitation (ND2). Cycle-sequencing reactions were performed in reaction volumes of 20μl, consisting of 0.7μl (nuclear markers) or 1.0μl (ND2) BigDye (Applied Biosystems), 4.5μl 5x Sequencing Buffer, 0.32μl Primer (10 pmol/μl), 13.5μl (nuclear) or 13.2μl (ND2) doubly distilled water, and 1.0μl purified PCR product. PCR sequencing cycles were performed using a Corbett PC-960C cooled thermal cycler, with a denaturation step at 94°C for 5s, annealing at 50°C for 10s, and extension at 60°C for 4 minutes, for 25 cycles. To precipitate sequence products and to remove all unincorporated nucleotides, a mixture of cold 40μl of 95% Ethanol and 3μl Sodium Acetate (3M, ph: 4.6-5.2) was added to each sample and left for 15 minutes at room temperature. Precipitated DNA was pelleted and washed thrice in 150μl of 70% Ethanol. Pellets were dried before being dissolved in 20μl of HiDi formamide and run on an ABI 3100 auto-sequencer.