

Supplementary Material

Optimisation of a pollen DNA metabarcoding method for diet analysis of flying-foxes (*Pteropus* spp.) in Queensland, Australia

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Supplementary Methods

Optimisation of DNA extraction and sequencing

DNA extraction: Using a preliminary batch of 11 samples from *P. scapulatus* from 4 sites, two kits for DNA extraction were compared: the Nucleospin DNA Food Kit (Macherey-Nagel, Düren, Germany), and the Nucleospin DNA Stool Kit (Macherey-Nagel, Düren, Germany). DNA was extracted with the Stool Kit following the manufacturers protocol. DNA was extracted using the Food Kit following the manufacturers protocol, with the following modifications: samples were homogenised using sterile 3 mm tungsten carbide beads for 1 min in a TissueLyser II (Qiagen, Hilden, Germany). Following homogenisation, we then followed the manufacturer's protocol for extraction of DNA using the NucleoSpin DNA Food Kit except the volume of Elution Buffer CE was decreased to 50 µL to increase the concentration of the final extraction.

DNA sequencing: In the first DNA sequencing run, we analysed 49 samples. We trialled two primer pairs for each marker, giving a short and a long PCR product. If the DNA in faecal samples is degraded, then a shorter PCR product is more likely to amplify. However, if the DNA is of good enough quality to amplify a long PCR product, then this will lead to finer taxonomic resolution. The primer combinations are outlined in Table S4. All 49 samples in the sequencing run were analysed with the primer pairs giving short PCR products, while a subset of 7 samples were also analysed with the primer pairs giving long PCR products. The two DNA barcode markers were amplified in separate reactions for each sample. We used an indexing strategy that gave each sample a unique combination of two indices, that were used for both DNA barcodes. The PCR reactions contained primers at a final concentration of 200nM, 12.5 µL of KAPA HiFi ReadyMix (KAPA Biosystems, Boston, Massachusetts, USA), and 8.5 µL of the template DNA (up to 20 ng/µL) in a 25 µL reaction. To increase the chance of detecting all species in the mixture, each DNA extraction was included in three PCR reactions that were amplified separately, i.e., the reaction contents described above were divided between three PCR tubes. The PCR conditions for long PCR fragments

included an initial period of heat activation for 3 min at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; followed by a final extension of 10 min at 72°C and then held at 10°C. For the short fragments, following a gradient PCR to determine optimal annealing temperature, PCR was conducted with an initial denaturing step of 95 °C for 3 minutes; followed by 35 cycles of: 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min; and a final extension at 72 °C for 10 min.

Supplementary Results

Comparison of DNA extraction methods

Consistently higher DNA concentrations were achieved using the NucleoSpin Food Kit compared to the NucleoSpin Stool Kit (Table S3). While DNA concentration is not the only measure of quality of DNA extraction, we detected no evidence of DNA degradation in any extractions, and the extracted DNA from both methods amplified successfully via PCR, demonstrating that neither method was affected by inhibitors.

Comparison of long and short PCR products

The preliminary run of Illumina MiSeq generated 2,304,061 paired-end 250bp sequencing reads. Of these 449,420 were from the 58 samples sequenced with short PCR products and 1,854,641 were from the 25 samples sequenced with long PCR products. After separating sequences into ITS2 and *rbcL* reads, and analysis via the dada2 bioinformatics pipeline, there remained 47,432 short ITS2 reads, 259,035 short *rbcL* reads, 456,195 long ITS2 reads, 139,295 long *rbcL* forward reads, and 139,152 *rbcL* reverse reads (Table S5). Most ITS2 short reads were lost at the filtering stage, due to poor quality read 2 sequences. Most *rbcL* long reads were lost at the merging step, due to a lack of overlap between the 250bp paired-end reads for the ~550bp PCR product. Based on these results, subsequent analyses used long PCR products of ITS2 and sequenced them with 300bp paired-end reads.

References

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Table S1: Collection details and sequencing run details of faecal samples analysed in this study

Sample ID	Species	Camp	Sex	Collection date	Sequencing run	Comments
1709001	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709002	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709003	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1709004	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709005	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709006	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709007	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709008	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709009	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709010	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709011	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709012	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	
1709026	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF13218_C2CMY	
1709027	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1709028	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709029	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	<1000 ITS2 reads; <1000 rbcL reads
1709030	<i>Pteropus scapulatus</i>	Charters Towers	female	Sep-17	AGRF_CAGRF20335_CHNRT	
1709032	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1709033	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF13218_C2CMY	
1709034	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1710004	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF13218_C2CMY	
1710006	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1710007	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1710008	<i>Pteropus scapulatus</i>	Charters Towers	male	Oct-17	AGRF_CAGRF20335_CHNRT	
1710009	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads

1710010	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	
1710014	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	
1710015	<i>Pteropus scapulatus</i>	Charters Towers	female	Oct-17	AGRF_CAGRF20335_CHNRT	
1710016	<i>Pteropus scapulatus</i>	Charters Towers	male	Oct-17	AGRF_CAGRF20335_CHNRT	
1711003	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF13218_C2CMY	
1711006	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF13218_C2CMY	
1711007	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	
1711008	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711009	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	
1711010	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1711011	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1711012	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1711013	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	
1711014	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711018	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	ITS2 failed; <1000 rbcL reads
1711021	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711022	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711023	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711024	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711025	<i>Pteropus scapulatus</i>	Charters Towers	female	Nov-17	AGRF_CAGRF20335_CHNRT	
1711026	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	
1711027	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	
1711028	<i>Pteropus scapulatus</i>	Charters Towers	male	Nov-17	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
Bat poo1	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-17	AGRF_CAGRF13218_C2CMY	
Bat poo2	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-17	AGRF_CAGRF20335_CHNRT	
Bat poo3	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-17	AGRF_CAGRF20335_CHNRT	
Bat poo4	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-17	AGRF_CAGRF20335_CHNRT	
Bat poo5	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-17	AGRF_CAGRF20335_CHNRT	
1804002	<i>Pteropus alecto</i>	Coen	female	Apr-18	AGRF_CAGRF20335_CHNRT	
1804003	<i>Pteropus alecto</i>	Coen	unknown sex	Apr-18	AGRF_CAGRF20335_CHNRT	

1804004	<i>Pteropus alecto</i>	Coen	male	Apr-18	AGRF_CAGRF13218_C2CMY	
1804006	<i>Pteropus alecto</i>	Coen	male	Apr-18	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1804007	<i>Pteropus alecto</i>	Coen	male	Apr-18	AGRF_CAGRF20335_CHNRT	
1804008	<i>Pteropus alecto</i>	Coen	male	Apr-18	AGRF_CAGRF20335_CHNRT	
1804009	<i>Pteropus alecto</i>	Coen	male	Apr-18	AGRF_CAGRF20335_CHNRT	
1804010	<i>Pteropus alecto</i>	Coen	female	Apr-18	AGRF_CAGRF20335_CHNRT	ITS2 failed
1806500	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806501	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806502	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806503	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806504	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806505	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806506	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806507	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806508	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	
1806509	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF20335_CHNRT	Control, ITS2 failed
1806510	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806511	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806512	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806513	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806514	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806515	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806516	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806517	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806518	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1806519	<i>Pteropus scapulatus</i>	Mary Valley	ground	Jun-18	AGRF_CAGRF21447_CPBYC	
1808001	<i>Pteropus scapulatus</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808002	<i>Pteropus scapulatus</i>	Charters Towers	female	Aug-18	AGRF_CAGRF20335_CHNRT	
1808003	<i>Pteropus scapulatus</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808005	<i>Pteropus scapulatus</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808006	<i>Pteropus scapulatus</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808007	<i>Pteropus scapulatus</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	

1808009	<i>Pteropus alecto</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808010	<i>Pteropus alecto</i>	Charters Towers	female	Aug-18	AGRF_CAGRF20335_CHNRT	
1808011	<i>Pteropus alecto</i>	Charters Towers	female	Aug-18	AGRF_CAGRF20335_CHNRT	<1000 rbcL reads
1808012	<i>Pteropus alecto</i>	Charters Towers	male	Aug-18	AGRF_CAGRF20335_CHNRT	
1808500	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808501	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808502	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808503	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808504	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808505	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808506	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808507	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808508	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808509	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808510	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808511	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808512	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808513	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808514	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808515	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808516	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808517	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808518	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1808519	<i>Pteropus scapulatus</i>	Charters Towers	ground	Aug-18	AGRF_CAGRF21447_CPBYC	
1809001	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	
1809002	<i>Pteropus alecto</i>	Charters Towers	female	Sep-18	AGRF_CAGRF20335_CHNRT	
1809003	<i>Pteropus alecto</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	<1000 ITS2 reads; <1000 rbcL reads
1809004	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	
1809005	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	
1809006	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	
1809007	<i>Pteropus scapulatus</i>	Charters Towers	male	Sep-18	AGRF_CAGRF20335_CHNRT	
1809009	<i>Pteropus alecto</i>	Charters Towers	female	Sep-18	AGRF_CAGRF20335_CHNRT	

1811519	<i>Pteropus scapulatus</i>	Charters Towers	ground	Nov-18	AGRF_CAGRF21447_CPBYC	
1811601	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811602	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811603	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811604	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811605	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811606	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811607	<i>Pteropus scapulatus</i>	Moura	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811610	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811611	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811612	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811613	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811614	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811615	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811616	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1811617	<i>Pteropus scapulatus</i>	Connors River	ground	Nov-18	AGRF_CAGRF20335_CHNRT	
1812001	<i>Pteropus scapulatus</i>	Mt Isa	female	Dec-18	AGRF_CAGRF21447_CPBYC	
1812002	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1812003	<i>Pteropus scapulatus</i>	Mt Isa	female	Dec-18	AGRF_CAGRF21447_CPBYC	
1812004	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1812005	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1812007	<i>Pteropus scapulatus</i>	Mt Isa	female	Dec-18	AGRF_CAGRF21447_CPBYC	
1812008	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1812009	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1812010	<i>Pteropus scapulatus</i>	Mt Isa	male	Dec-18	AGRF_CAGRF21447_CPBYC	
1901001	<i>Pteropus scapulatus</i>	Woodford	unknown sex	Jan-19	AGRF_CAGRF21447_CPBYC	
1901002	<i>Pteropus scapulatus</i>	Woodford	unknown sex	Jan-19	AGRF_CAGRF21447_CPBYC	
1901003	<i>Pteropus scapulatus</i>	Woodford	unknown sex	Jan-19	AGRF_CAGRF21447_CPBYC	
1901004	<i>Pteropus scapulatus</i>	Woodford	unknown sex	Jan-19	AGRF_CAGRF21447_CPBYC	
1901005	<i>Pteropus scapulatus</i>	Woodford	unknown sex	Jan-19	AGRF_CAGRF21447_CPBYC	
1901300	<i>Pteropus scapulatus</i>	40 mile scrub	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901301	<i>Pteropus scapulatus</i>	40 mile scrub	ground	Jan-19	AGRF_CAGRF21447_CPBYC	

1901514	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901515	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901516	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901517	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901518	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1901519	Negative control	Charters Towers	ground	Jan-19	AGRF_CAGRF21447_CPBYC	
1903001	<i>Pteropus conspicillatus</i>	Gordonvale	female	Mar-19	AGRF_CAGRF21447_CPBYC	
1903002	<i>Pteropus conspicillatus</i>	Gordonvale	female	Mar-19	AGRF_CAGRF21447_CPBYC	
1903003	<i>Pteropus conspicillatus</i>	Gordonvale	female	Mar-19	AGRF_CAGRF21447_CPBYC	
1903004	<i>Pteropus conspicillatus</i>	Gordonvale	male	Mar-19	AGRF_CAGRF21447_CPBYC	
1904001	<i>Pteropus conspicillatus</i>	Gordonvale	unknown sex	Apr-19	AGRF_CAGRF21447_CPBYC	
1904002	<i>Pteropus conspicillatus</i>	Gordonvale	unknown sex	Apr-19	AGRF_CAGRF21447_CPBYC	
1904003	<i>Pteropus conspicillatus</i>	Gordonvale	unknown sex	Apr-19	AGRF_CAGRF21447_CPBYC	
1905320	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905321	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905322	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905323	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905324	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905325	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905326	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905327	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905329	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905330	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905331	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	

1905332	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905333	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905335	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905336	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905337	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905338	<i>Pteropus scapulatus</i>	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1905339	Negative control	Townsville, Calcium	ground	May-19	AGRF_CAGRF21447_CPBYC	
1906001	<i>Pteropus scapulatus</i>	Mary Valley	female	Jun-19	AGRF_CAGRF21447_CPBYC	
1906002	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906003	<i>Pteropus scapulatus</i>	Mary Valley	female	Jun-19	AGRF_CAGRF21447_CPBYC	
1906004	<i>Pteropus scapulatus</i>	Mary Valley	female	Jun-19	AGRF_CAGRF21447_CPBYC	
1906005	<i>Pteropus scapulatus</i>	Mary Valley	female	Jun-19	AGRF_CAGRF21447_CPBYC	
1906006	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906007	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906008	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906009	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906010	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906011	<i>Pteropus scapulatus</i>	Mary Valley	male	Jun-19	AGRF_CAGRF21447_CPBYC	
1906012	<i>Pteropus scapulatus</i>	Mary Valley	female	Jun-19	AGRF_CAGRF21447_CPBYC	
1807501	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807502	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807503	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807504	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807505	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807506	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807507	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807508	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807509	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807510	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	

1807511	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807512	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807513	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807514	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807515	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807516	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807517	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	
1807518	<i>Pteropus scapulatus</i>	Charters Towers	ground	Jul-19	AGRF_CAGRF21447_CPBYC	

Table S2: Collection details and accession numbers of plant specimens sequenced for the DNA barcode reference databases

Species	Identified by	Locality name	Geographic coordinates	Collection date	<i>RbcL</i> sequence genbank accession(s)	ITS2 sequence genbank accession(s)
<i>Eucalyptus shirleyi</i>	Andrew Ford	Fletchervale station	19.8576°S, 146.0739°E	01-December-2017	NA	MW139077, MW139078, MW139079
<i>Terminalia platyphylla</i>	Andrew Ford	Serenity Road	20.0401°S, 144.3233°E	01-December-2017	MW178157	MW139082, MW139083, MW139084
<i>Eucalyptus xanthoclada</i>	Andrew Ford	Serenity Road	20.0401°S, 144.3233°E	01-December-2017	NA	MW139080, MW139081
<i>Corymbia dallachiana</i>	Andrew Ford	Southwick Station	19.8200°S, 144.6583°E	01-December-2017	NA	MW139072
<i>Corymbia nesophila</i>	Matt Bradford	Laura	15.5044°S, 144.4818°E	08-May-2018	NA	MW139073, MW139074
<i>Corymbia stockeri</i>	Matt Bradford	Lakefield	15.4320°S, 144.4240°E	08-May-2018	NA	MW139092, MW139093
<i>Corymbia clarksoniana</i>	Matt Bradford	Lakefield	15.2383°S, 144.44038°E	08-May-2018	NA	MW178183, MW139071
<i>Eucalyptus megasepala</i>	Matt Bradford	Laura	15.5044°S, 144.4818°E	08-May-2018	MW178139	MW139074, MW139076
<i>Grevillea pteridifolia</i>	Matt Bradford	Laura	15.5044°S, 144.4818°E	08-May-2018	MW178145	NA
<i>Petalostigma banksii</i>	Matt Bradford	Morehead River	15.029°S, 143.6758°E	08-May-2018	MW178154	MW139131, MW139132
<i>Brachychiton bidwillii</i>	Matt Bradford	Raglan	23.7263°S, 150.8171°E	11-September-2018	MW178133	MW139088, MW139089, MW139090
<i>Ficus racemosa</i>	Matt Bradford	Connors River	22.1459°S, 149.0788°E	31-October-2018	MW178144	NA
<i>Alyxia ruscifolia</i>	Matt Bradford	Raglan	23.7263°S, 150.8171°E	11-September-2018	MW178132	MW139085, MW139086, MW139087

<i>Bauhinia hookeri</i>	Matt Bradford	Moura	24.5031°S, 145.410°E	10-December-2018	MW178147	MW139113, MW139114, MW139115
<i>Syncarpia glomulifera</i> ssp. <i>glomulifera</i>	Matt Bradford	Wongabel	17.3655°S, 145.4881°E	23-October-2019	MW178155	MW139133
<i>Lophostemon suaveolens</i>	Matt Bradford	Wongabel	17.3655°S, 145.4906°E	23-October-2019	MW178146	MW139112
<i>Syzygium suborbiculare</i>	Matt Bradford	Coen	13.7728°S, 143.2526°E	05-October-2019	MW178156	MW139134, MW139135, MW139136
<i>Eucalyptus tereticornis</i>	Matt Bradford	Rossmoya	23.0377°S, 150.4072°E	02-November-2018	MW178143	MW139108, MW139109, MW139110, MW139111
<i>Eucalyptus tereticornis</i>	Matt Bradford	Herberton	17.355°S, 145.408°E	10-August-2018	MW178142	MW139106, MW139107
<i>Eucalyptus populnea</i>	Andrew Ford	The Overflow	22.2553°S, 149.0011°E	17-October-2017	MW178141	MW139104, MW139105
<i>Eucalyptus crebra</i>	Matt Bradford	The Overflow	22.2553°S, 149.0011°E	17-July-2017	MW178138	MW139101, MW139102
<i>Eucalyptus platyphylla</i>	Matt Bradford	Carrington	17.3022°S, 144.4689°E	10-October-2018	MW178140	MW139103
<i>Eucalyptus camaldulensis</i>	Eric Vanderduys	Mount Isa	20.655°S, 139.519°E	09-January-2019	MW178170	MW139170
<i>Corymbia stockeri</i>	Matt Bradford	Chillagoe	17.1046°S, 144.4035°E	10-June-2018	MW178135	MW139094, MW139095, MW139096
<i>Corymbia torelliana</i>	Matt Bradford	Atherton	17.2607°S, 145.4842°E	10-October-2018	MW178136	MW139097, MW139098
<i>Corymbia clarksoniana</i>	Matt Bradford	Alice river	15.7129°S, 142.6583°E	10/16/18	MW178134	MW139091
<i>Melaleuca bracteata</i>	Matt Bradford	Many Peaks	24.6312°S, 151.281°E	02-November-2018	MW178149	MW139119, MW139120, MW139121, MW139122
<i>Melaleuca bracteata</i>	Matt Bradford	Minamoolka Station	17.5701°S, 145.3249°E	23-January-2019	MW178148	MW139116, MW139117, MW139118

<i>Melaleuca viridiflora</i>	Matt Bradford	Walkamin	17.1040°S, 145.4367°E	05-May-2018	MW178153	MW139128, MW139129, MW139130
<i>Meleleuca nervosa</i>	Matt Bradford	Walkamin	17.1588°S, 145.4308°E	05-May-2018	MW178151	MW139125, MW139126
<i>Meleleuca viminalis</i>	Matt Bradford	Calliope River	24.071°S, 150.8787°E	12-September-2018	MW178152	MW139127
<i>Melaleuca leucadendra</i>	Matt Bradford	Charters Towers	19.8848°S, 146.2282°E	16-November-2017	MW178150	MW139123, MW139124
<i>Corymbia confertiflora</i>	Eric Vanderduys	Georgetown	18.247°S, 143.067°E	22-January-2019	NA	MW139148, MW139149
<i>Corymbia dallachiana</i>	Matt Bradford	Bowen	20.1309°S, 148.0451°E	29-November-2018	MW178162	MW139150, MW139151
<i>Corymbia dallachiana</i>	Matt Bradford	Croydon	17.86282°S, 141.50491°E	22-January-2019	NA	MW139152, MW139153
<i>Corymbia intermedia</i>	Matt Bradford	Yeppoon	23.138°S, 150.749°E	17-January-2019	MW178164	MW139156, MW139157, MW139158
<i>Corymbia polycarpa</i>	Matt Bradford	Goldsborough Station	20.3158°S, 144.9896°E	03-April-2019	MW178165	MW139159, MW139160, MW139161
<i>Corymbia terminalis</i>	Eric Vanderduys	Cloncurry	20.7175°S, 140.3477°E	09-January-2019	MW178166	MW139162, MW139163, MW139164
<i>Corymbia tesellaris</i>	Matt Bradford	Rockhampton	23.306°S, 150.382°E	17-January-2019	MW178167	MW139165
<i>Cryptostegia grandiflora</i>	Matt Bradford	Goldsborough Station	20.298°S, 147.010°E	02-April-2019	MW178168	MW139166, MW139167
<i>Eucalyptus camaldulensis</i>	Matt Bradford	Mount Isa	20.655°S, 139.519°E	07-January-2019	MW178137	MW139099, MW139100
<i>Eucalyptus coolabah</i>	Matt Bradford	Rockhampton	23.225°S, 150.345°E	17-January-2019	MW178171	MW139171, MW139172
<i>Eucalyptus microtheca</i>	Matt Bradford	Blackbull	17.825°S, 141.495°E	22-January-2019	MW178175	MW139178, MW139179
<i>Eucalyptus raveretiana</i>	Matt Bradford	Rockhampton	23.312°S, 150.816°E	17-January-2019	MW178169	MW139168, MW139169
<i>Ficus opposita</i>	Matt Bradford	Goldsborough Station	20.298°S, 147.010°E	02-April-2019	MW178179	MW139186

<i>Nauclea orientalis</i>	Matt Bradford	Georgetown	18.193°S, 142.898°E	22-January-2019	MW178183	MW139192, MW139193
<i>Melaleuca nervosa</i>	Matt Bradford	Valley of Lagoons	18.701°S, 145.080°E	01-April-2019	MW178182	MW139189, MW139190
<i>Angophora costata</i>	Matt Bradford	Kilcoy	26.878°S, 152.584°E	13-February-2019	MW178158	MW139137, MW139138, MW139139
<i>Angophora floribunda</i>	Matt Bradford	Tumoulin	17.5654°S, 145.4540°E	30-November-2018	MW178159	MW139140, MW139141
<i>Banksia integrifolia</i>	Matt Bradford	Coolum	26.473°S, 153.098°E	14-February-2019	MW178160	MW139142, MW139143, MW139144, MW139145
<i>Banksia robur</i>	Matt Bradford	Coolum	26.713°S, 153.075°E	14-February-2019	MW178161	MW139146, MW139147
<i>Corymbia erythrophloia</i>	Matt Bradford	The Caves	23.1726°S, 151.4587°E	29-Feb-2019	MW178163	MW139154, MW139155
<i>Eucalyptus leucophylla</i>	Eric Vanderduys	Mount Isa	20.6716°S, 139.5090°E	07-January-2019	MW178172	MW139173
<i>Eucalyptus major</i>	Matt Bradford	Pomona	26.393°S, 152.883°E	14-February-2019	MW178173	MW139174, MW139175
<i>Eucalyptus melanophloia</i>	Matt Bradford	Yaamba	23.1197°S, 150.0199°E	28-February-2019	MW178174	MW139176, MW139177
<i>Eucalyptus pilularis</i>	Matt Bradford	Woodford	26.947°S, 152.858°E	13-February-2019	MW178176	MW139180, MW139181
<i>Eucalyptus pruinosa</i>	Matt Bradford	Normanton	17.968°S, 141.484°E	22-January-2019	MW178177	MW139182, MW139183
<i>Eucalyptus racemosa</i>	Matt Bradford	Connors River	28.017°S, 153.206°E	14-February-2019	MW178178	MW139184, MW139185
<i>Lophostemon grandiflorus</i>	Eric Vanderduys	Mount Isa	20.655°S, 139.519°E	07-January-2019	MW178180	MW139187
<i>Lophostemon suaveolens</i>	Matt Bradford	Rossmoya	23.054°S, 150.463°E	17-January-2019	MW178181	MW139188
<i>Melaleuca quinquenervia</i>	Matt Bradford	Noosa	26.409°S, 153.072°E	13-February-2019	NA	MW139191
<i>Banksia aquilonia</i>	Matt Bradford	Wongabel	17.3612°S, 145.4802°E	24-October-2019	NA	MW139197
<i>Castanospermum australe</i>	Matt Bradford	Tolga	17.2327°S, 145.4791°E	24-November-2019	NA	MW139198, MW139199

<i>Syncarpia glomulifera</i> ssp. <i>glomulifera</i>	Matt Bradford	Wongabel	17.361°S, 145.4802°E	24-October-2019	NA	MW139222
<i>Amyena bifurcata</i>	Eric Vanderduys	Mount Isa	20.5344°S, 139.4863°E	25-June-2019	NA	MW139194, MW139195, MW139196
<i>Corymbia confertiflora</i>	Matt Bradford	Yarraden Station	14.3886°S, 143.3607°E	17-October-2019	NA	MW139203, MW139204
<i>Eucalyptus camaldulensis</i> var. <i>obtusa</i>	Matt Bradford	Moondarra dam	20.5477°S, 139.5420°E	25-June-2019	NA	MW139210, MW139211, MW139212, MW139213
<i>Hakea lorea</i>	Eric Vanderduys	Mount Isa	20.2144°S, 139.8059°E	26-June-2019	NA	MW139217, MW139218
<i>Terminalia aridocola</i>	Eric Vanderduys	Mount Isa	20.5485°S, 139.5448°E	25-June-2019	NA	MW139223
<i>Corymbia aspera</i>	Eric Vanderduys	Mount Isa	20.5485°S, 139.5448°E	25-June-2019	NA	MW139200, MW139201
<i>Lophostemon grandiflorus</i>	Matt Bradford	Moondarra dam	20.5450°S, 139.5430°E	25-June-2019	NA	MW139219, MW139220
<i>Corymbia rhodops</i>	Andrew Ford	Mt Nolan	17.4002°S, 145.2749°E	05-February-2019	NA	MW139207, MW139208, MW139209
<i>Melaleuca viridiflora</i>	Andrew Ford	Staaten River	16.5519°S, 142.7948°E	14-March-2019	NA	MW139221
<i>Eucalyptus populnea</i>	Matt Bradford	Monto	24.8706°S, 151.1212°E	20-November-2019	NA	MW139214
<i>Eucalyptus tereticornis</i>	Matt Bradford	Mulgildie	25.0489°S, 151.2333°E	20-November-2019	NA	MW139215, MW139216
<i>Corymbia dallachiana</i>	Matt Bradford	Walkamin	17.1789°S, 145.4522°E	25-November-2019	NA	MW139205, MW139206
<i>Corymbia citriodora</i>	Matt Bradford	Eidsvold	25.1673°S, 151.1820°E	20-November-2019	NA	MW139202

Table S3: Comparison of DNA extraction methods

Concentration of DNA extractions from flying-fox faecal samples using the Macherey-Nagel NucleoSpin DNA Stool Kit and the Macherey-Nagel NucleoSpin DNA Food Kit

Sample ID	Concentration of extraction with stool kit (ng/μl)	Concentration of extraction with food kit (ng/μl)
1709001	2.2	3.42
1709010	1.68	5.9
1808001	2.82	4.84
1808010	<0.5	0.448
1809001	0.47	1.16
1809011	0.78	2.38
1806500	2.5	2.22
1806509	2.2	7.84
1811601	2.06	4.38
1811611	15.3	61.4
1811612	29.8	97.4

Table S4: PCR and sequencing primers used in the optimised protocol. PCR product lengths include the fusion primers. Illumina tag is shown in **bold**, index, pad and linker sequences in plain text, and marker-specific primer in *italics*.

Region	Primer name	Primer type	Primer sequences (index sequence shown as XXXXXXXX)	References
ITS2 long (450-550 bp PCR product)	ITS-S2F-fusion	PCR	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX <i>XCCTGGTGCTGGTATGCGATACTTGGTGTGAAT</i>	(Chen et al. 2010); fusion primer as in Sickel et al. (2015)
	ITS4R-fusion	PCR	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXAGTC <i>AGTCAGCCTCCTCCGCTTATTGATATGC</i>	(White et al. 1990); fusion primer as in Sickel et al. (2015)
	Read 1 ITS	Sequencing	<i>CCTGGTGCTGGTATGCGATACTTGGTGTGAAT</i>	(Sickel et al. 2015)
	Read 2 ITS	Sequencing	<i>AGTCAGTCAGCCTCCTCCGCTTATTGATATGC</i>	(Sickel et al. 2015)
	Index ITS	Sequencing	<i>GCATATCAATAAGCGGAGGAGGCTGACTGACT</i>	(Sickel et al. 2015)
<i>rbcL</i> long (~600 bp PCR product)	<i>rbcL</i> 2-fusion	PCR	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX <i>XCCTGGTGCTGGTTGGCAGCATTYCGAGTAACTC</i>	(Palmieri, Bozza, and Giongo 2009); fusion primer as in Bell, Loeffler, and Brosi (2017)
	<i>rbcLaR</i> -fusion	PCR	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXAGTC <i>AGTCAGCCGTAATAATCAAGTCCACCRCG</i>	(Kress et al. 2009); fusion primer as in Bell, Loeffler, and Brosi (2017)
	Read 1 <i>rbcL</i>	Sequencing	<i>CCTGGTGCTGGTTGGCAGCATTYCGAGTAACTC</i>	(Bell, Loeffler, and Brosi 2017)
	Read 2 <i>rbcL</i>	Sequencing	<i>AGTCAGTCAGCCGTAATAATCAAGTCCACCRCG</i>	(Bell, Loeffler, and Brosi 2017)
	Index <i>rbcL</i>	Sequencing	<i>CGYGGTGGACTTGATTTTACGGCTGACTGACT</i>	(Bell, Loeffler, and Brosi 2017)
ITS2 short (350-450 bp PCR product)	UniPlan tF-fusion	PCR	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX <i>XCCTGGTGCTGGTTGTGAATTGCARRATYCMG</i>	(Moorhouse-Gann et al. 2018); fusion primer, current study
	UniPlan tR-fusion	PCR	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXAATA <i>AGTAAGCTCCCGHYTGAYTGRGGTDC</i>	(Moorhouse-Gann et al. 2018); fusion primer, current study

	Read 1 ITS short	Sequencing	<i>CCTGGTGCTGGTGTGAATTGCARRATYCMG</i>	current study
	Read 2 ITS short	Sequencing	<i>AATAAGTAAGCTCCCGHYTGAYYTGRRGGTDC</i>	current study
	Index ITS short	Sequencing	<i>GHGACCYCARRTCARDCGGGAGCTTACTTATT</i>	current study
<i>rbcL</i> short (~320 bp PCR product)	rbcLaF- fusion	PCR	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXX XCCTGGTGCTGGTATGTCACCACAAACAGAGACTAAAGC	(Kress and Erickson 2007); fusion primer, current study
	MrbcL1 63-R1- fusion	PCR	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXAGTC AGTCAGCCCGGTCCAYACAGYBGTC CAKGTACC	(Ivanova et al. 2016); fusion primer, current study
	Read 1 rbcL short	Sequencing	<i>CCTGGTGCTGGTATGTCACCACAAACAGAGACTAAAGC</i>	current study
	Read 2 rbcL short	Sequencing	<i>AGTCAGTCAGCCCGGTCCAYACAGYBGTC CAKGTACC</i>	current study
	Index rbcL short	Sequencing	<i>GGTACMTGGACVRCTGTRTGGACCGGGCTGACTGACT</i>	current study

Table S5: Number of sequencing reads remaining in each sequencing run, following each step in the bioinformatics pipeline.

Function “filtered” discards low quality reads and trims low quality sequence from the ends of reads; “denoised” identifies sequencing reads that are likely to contain sequencing errors; “merged” merges the forward and reverse sequencing reads; “nonchim” removes sequences that are likely to be chimeric; and “decontam” removes sequences that are likely to be contaminants.

	input	filtered	denoisedf	denoisedr	merged	nonchim	decontam
Sequencing run 1 ITS2 short	311590	124531	124156	119752	68172	47432	NA ¹
Sequencing run 1 <i>rbcL</i> short	311754	304797	304168	304612	300999	259035	NA
Sequencing run 1 ITS2 long	1199614	525566	524209	524134	470702	456195	452128
Sequencing run 1 <i>rbcL</i> long forward reads	146399	139389	139309	NA	NA ²	139295	138335
Sequencing run 1 <i>rbcL</i> long reverse reads	146399	139389	NA	139302	NA	139152	138192
Sequencing run 2 ITS2	7760594	3652757	3643972	3642588	3273833	3237449	3129419
Sequencing run 2 <i>rbcL</i>	3362556	2093838	2091057	2092200	2073928	2034417	1973615
Sequencing run 3 ITS2	4806873	3758276	3749813	3747863	3403067	3339911	3261146
Sequencing run 3 <i>rbcL</i>	4157706	3787126	3783886	3784987	3767349	3629924	3579131

¹ The “decontam” function was not run for the short sequencing reads, as these were not included in the final analysis.

² The 250bp forward and reverse reads from run 1 were too short to allow merging for the ~550bp long *rbcL* amplicon, and were analysed as single-end reads

Table S5: Taxonomic identifications based on ITS2 long sequences, using the AssignTaxonomy and AssignSpecies functions in dada2. Sequences with fewer than 10,000 reads across the study removed. Samples with fewer than 1000 reads removed. Taxa not identified to species can be placed to species level using local knowledge of species distribution and flowering phenology.

Family, genus, and plausible alternative species-level identifications	Number of samples
Annonaceae Genus undetermined Species undetermined	1
Fabaceae <i>Erythrina</i> Species undetermined	18
<i>Bauhinia</i> <i>carronii/hookeri</i>	16
Species undetermined	19
Lamiaceae <i>Vitex</i> Species undetermined	10
Moraceae <i>Ficus</i> <i>subpisocarpa/virens</i>	1
Myrtaceae <i>Callistemon</i> Species undetermined	13
<i>Corymbia</i> <i>aparrerinja/dallachiana</i>	29
<i>aspera</i>	2
<i>brachycarpa/clarksoniana/erythrophloia/nesophila/xanthope</i>	28
<i>clarksoniana/hendersonii/intermedia/rhodops</i>	19
<i>confertiflora</i>	23
<i>dallachiana</i>	43
Species undetermined	51
<i>polycarpa</i>	4
<i>stockeri</i>	7
<i>tessellaris</i>	31
<i>Eucalyptus</i> <i>camaldulensis</i>	59
<i>camaldulensis/tereticornis</i>	65
<i>coolabah</i>	19
<i>coolabah/leucophylla/microtheca/raveretiana</i>	21
<i>crebra/shirleyi</i>	20
<i>megasepala</i>	14
<i>melanophloia/shirleyi/xanthoclada</i>	25
Species undetermined	94
<i>platyphylla</i>	27

<i>populnea</i>	35
<i>Melaleuca leucadendra</i>	51
Species undetermined	47
<i>viridiflora</i>	33
<i>Syzygium</i> Species undetermined	1
Rubiaceae <i>Nauclea orientalis</i>	5
Sapotaceae <i>Planchonella arnhemica</i>	3
Verbenaceae <i>Citharexylum</i> Species undetermined	2

Table S6: Taxonomic identifications based on *rbcL* long sequences, using the AssignTaxonomy and AssignSpecies functions in dada2. Sequences with fewer than 10,000 reads across the study removed. Samples with fewer than 1000 reads removed. Taxa not identified to species can be placed to species level using local knowledge of species distribution and flowering phenology. Samples from run 1 (2x250bp paired-ends) have been excluded due to poor resolution of unmerged single-end sequences.

Family, genus, and plausible alternative species-level identifications	Number of samples
Anacardiaceae <i>Mangifera indica/sylvatica</i>	7
<i>Tapirira mahagoni/timoriense</i>	8
Annonaceae <i>Annona cherimola/pruinosa/reticulata/squamosa/urbaniana</i>	3
<i>muricata</i>	2
Apocynaceae <i>Secamone afzelii/elliptica/emetica/wightianus</i>	3
Asparagaceae <i>Agave americana/angustifolia/geminiflora/ghiesbreghtii/schottii/sisalana/victoriae-reginae/virginica</i>	3
Caricaceae <i>Carica cauliflora/chilensis/cundinamarcensis/glandulosa/palandensis/papaya/quercifolia/sphaerocarpa/sprucei</i>	1
Fabaceae <i>Erythrina acanthocarpa/humeana/numerosa/sousae/vespertilio/zeyheri</i>	10
<i>Bauhinia carronii/gilvum/hookeri</i>	34
Species undetermined	19
Lamiaceae <i>Vitex acuminata/doniana/ferruginea/grandifolia/harveyana/leucoxydon/payos/petersiana/rehmannii</i>	13
<i>melicopea</i>	7
Moraceae <i>Ficus abelii/abutilifolia/altissima/americana/ampelas/aurea/auriculata/benghalensis/benguensis/benjamina/bernaysii/binnendijkii/bullenei/bussei/cahuitensis/callosa/cardiophylla/carica/citrifolia/colubrinae/congesta/cordata/coronata/costaricana/craterostoma/crocata/curtipes/cyrtophylla/deltoidea/destruens/drupacea/elastica/erecta/esquiroliana/fistulosa/formosana/fraseri/fulva/gasparriniana/glaberrima/glumosa/gracilior/gul/hederacea/heterophylla/heteropleura/hirta/hispida/hispidioides/hondurensis/Ilicina/ingens/irisana/ischnopoda/jimenezii/laevis/lanceolaria/langkokensis/longifolium/lutea/lyrata/maclellandii/macrophylla/melinocarpa/microcarpa/mollior/morazaniana/natalensis/negundo/nervosa/nodosa/nymphaeifolia/obtusifolia/oligodon/opposita/orthoneura/ovalis/pandurata/pedunculosa/pertusa/petersii/popenoei/pseudojaca/pubigera/pumila/pungens/pygmaea/racemosa/religiosa/retusa/rubiginosa/rumphii/sagittata/sarmentosa/semicordata/semivestita/septica/sinuata/stenophylla/stricta/stuhlmannii/subcuneata/subincisa/subpisocarpa/</i>	19

<i>subulata/sur/ sycomorus/ thonningii/tikoua/ tinctoria/ trachypison/ trichopoda/ trigona/ trigonata/ trivia/ tumpahensis/ urens/ variegata/ variolosa/ vasculosa/ virens/ wassa</i>	
Myrtaceae <i>Corymbia</i> <i>citriodora/ dallachiana/ eximia/ henryi/ tessellaris/ torelliana</i>	48
<i>Eucalyptus</i> <i>camaldulensis/ deglupta/ diversicolor/ globulus/ gomphocephala/ grandis/ major/ pellita/ platyphylla/ tereticornis</i>	68
<i>cladocalyx/ coolabah/ crebra/ globulus/ lehmannii/ leucophylla/ melanophloia/ melliodora/ microtheca/ polybractea/ populnea/ pruinosa/ raveretiana</i>	43
<i>megasepala</i>	14
<i>spathulata/ torquata</i>	35
<i>Melaleuca</i> <i>cajuputi/ leucadendra/ nervosa/ quinquenervia/ stenostachya/ viridiflora</i>	69
<i>Syzygium</i> Species undetermined	1
Genus undetermined Species undetermined	80
Proteaceae <i>Grevillea</i> Species undetermined	10
Sapotaceae <i>Pouteria</i> <i>chartacea/ cotinifolia/ gongrijpii/ grandifolia/ myrsinifolia/ pohlmaniana/ venulosa</i>	6
Verbenaceae <i>Citharexylum</i> <i>berlandieri/ costaricense/ fruticosum/ spinosum</i>	3