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A molecular assessment of the identity of regenerating mallees on the Tropicana Mine Access Rd, in relation to the DRF *Eucalyptus articulata* (Myrtaceae).

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Executive Summary

The objective of this research was to apply the molecular tools of DNA sequence analysis and DNA fingerprinting to identify whether the DRF *Eucalyptus articulata* occurs along the proposed Tropicana Gold Project Mine Access Road Pinjin Option road route. Molecular testing was applied, as confident identification from morphology was difficult due to the burnt and only just regenerating nature of the eucalypt specimens of interest. Leaf samples were collected by Mattiske Consulting staff, and included 6 specimens of the DRF *E. articulata*, as well as various samples of known and questioned identity, from along a 60km stretch of the proposed access road route. DNA sequence variation was assessed for these samples, and compared to eucalypt sequences obtained from GenBank, for two regions - the Internal Transcribed Spacer (ITS) and External Transcribed Spacer (ETS) of nuclear ribosomal DNA (nrDNA). Genetic variation was also assessed with the multi-locus DNA fingerprinting technique Amplified Fragment Length Polymorphism (AFLP). Using the Neighbor-Joining procedure with bootstrapping, both ITS and ETS data generated trees with significant support for a cluster of *E. articulata* individuals that excluded all samples of questioned identity. Ordination of AFLP data revealed a cluster of the known *E. articulata* samples that was strongly differentiated from all other samples. Collectively, these results strongly suggest that *E. articulata* was not amongst the questioned samples. We are currently sequencing a further two regions (matK and rbcL) for these samples, and conducting a "blind" test on a further 2 samples, to assess the robustness of these conclusions, after which a final report will be submitted.

Introduction

Molecular tools such as DNA sequencing and DNA fingerprinting potentially enable the unambiguous identification of specimens that are otherwise taxonomically ambiguous. This DNA barcoding approach is of particular utility when the quality and/or quantity of material is poor or fragmentary, and confident, positive identification is difficult from morphological features alone. These molecular tools facilitate the taxonomic identification of samples through the detection of genetic polymorphisms that are consistent, or fixed, among samples within taxa, but differ among taxa. DNA barcoding, and specifically the identification of suitably universal markers for DNA barcoding, has been a significant area of intense activity recently (Cowans et al 2006; Lahaye et al. 2008; Hollingsworth 2008; Valentini et al. 2008; Hollingsworth et al. 2009). While a universal marker(s), such as the mitochondrial cytochrome oxidase I gene (COI) for animals, remains somewhat elusive for plants (Hollingsworth et al. 2009), DNA sequencing and DNA fingerprinting are powerful molecular forensic tools of general utility for more narrowly defined objectives such as the comparison of unknown specimens to a known taxon.

In August 2008, AngloGold Ashanti commissioned the conservation genetics laboratory at Kings Park and Botanic Garden to undertake a molecular forensic analysis of the identity of plant specimens in relation to the DRF *Eucalyptus articulata* Brooker and Hopper, otherwise known as the Ponton Creek Mallee (Fig. 1). *Eucalyptus articulata* is a low straggly mallee to 3m, with smooth coppery or bronze bark throughout, and forming a lignotuber. It is known only from three populations of approx 120 plants near Mulga Rock, north-east of Kalgoorlie, where it occurs on red sand dunes with arkose rubble. *Eucalyptus articulata* belongs to the *Eucalyptus* subgenus *Symphyomyrtus* section *Bisectae* subsection *Glandulosae* because the cotyledons are bisected, buds have an operculum scar and the branchletts have oil glands in the pith. Within this subsection, *E. articulata* belongs to a small subgroup of four species, series *Loxophlebae*, with *E. loxophleba* (with 4 subspecies), *E. blaxellii*, and *E. semota* (Brooker et al. 2006; Hines & Byrne 2001).

The objective of this research was to apply the molecular tools of DNA sequence analysis and DNA fingerprinting to identify whether the DRF *Eucalyptus articulata* occurs along the proposed Tropicana Gold Project Mine Access Road Pinjin Option road route. Molecular tools were applied as the geographic area had been recently extensively burnt, and confident taxonomic identification of newly regenerating mallee eucalypts was often difficult from morphology alone. Specifically, for each sampled specimen, we asked whether it was taxonomically distinct from *E. articulata*. This objective is distinguished from that of positive taxonomic identification of the unknown specimens, which we did not pursue, and for which the limited sampling was not specifically designed for.

Fig. 1: *Eucalyptus articulata* habit. Photo from Brooker et al (2006).

Materials and Methods

Sampling

With the exception of *E. loxophleba*, all material was sampled by Mattiske Consulting staff in August 2008 or January 2009 (*E. trivalva 1-5*), and these samples of fresh leaf material were delivered to Kings Park, where they were stored briefly at 4^{o}C prior to the extraction of DNA. Three *Eucalyptus loxophleba* subsp. *supralaevis* PERTH herbarium specimens were also sampled and included in analyses, as *E. articulata* is a member of the *E. loxophleba* complex (Hines and Byrne 2001). Samples included 6 specimens identified as *E. articulata*, 5 specimens identified as *E. trivalva*, specimens with taxonomy identified, and specimens with taxonomy tentatively determined and/or undetermined (Table 1). Voucher specimens of samples have been collected and will be deposited at PERTH prior to the final report.

Table 1: Collection details of samples supplied to Kings Park for DNA analysis. ? indicates a tentative identification made by Mattiske staff from limited morphological material from burnt mallees.

Label	Species	EASTING	NORTHING	NOTES
EA1	*E. articulata*	528412	6665115	roadside
EA2	*E. articulata*	528412	6665115	5 m from EA1
EA3	*E. articulata*	526957	6665458	?4.5 m
EA4	*E. articulata*	526931	6665471	DEC tag # 67123
EA5	*E. articulata*	526875	6665490	~60m W of EA4 on track
EA6	*E. articulata*	526817	6665492	DEC rare flora # 9.1.20
EC1	*E. concinna*	498212	6673091	for comparison, on Pinjin Rd
EC@EA2	*E. concinna*	528414	6665118	
2	*? E. oleosa*	520452	6680957	red sand
2a	*? E. rosaceae*	515846	6681057	in mulga
2b	*E. pimpiniana*	514913	6681064	open heath
3a	*? E. transcontinentalis*	522330	6682365	
4a	*? E. oleosa*	548756	6703806	
4b	*?E.*	547463	6703663	burnt mallee, yellow-red sand
4c	*?E. platycorys*	544126	6703272	burnt mallee
4d	*?E. rigidula*	544001	6703244	mallee
4e	*? E. platycorys*	541746	6703662	burnt mallee
4f(1)	*?E. ceratocorys*	538399	6702131	burnt mallee
4f(2)	*?E. rigidula*	538399	6702131	burnt mallee
4g	*? E. mannensis*	536667	6700014	unburnt, yellow sand
4g(2)	*E. youngiana*	536667	6700015	
4h	*?E. gongylocarpa*	535270	3396960	burnt, yellow sand
4i	*?E.*	531517	6690669	burnt mallee, yellow sand
4i(a)	*?E. concinna*	531517	6690669	burnt mallee
4i(b)	*?E. trivalva*	531517	6690669	burnt mallee
4j	*E. trivalva*	531010	6689813	
Etriv1	*E. trivalva*	647940	6762196	
Etriv2	*E. trivalva*	647940	6762196	
Etriv3	*E. trivalva*	647940	6762196	
Etriv4	*E. trivalva*	647940	6762196	
Etriv5	*E. trivalva*	647940	6762196	
Elox1	*E. loxophleba*			PERTH05546699
Elox2	*E. loxophleba*			PERTH07122187
Elox3	*E. loxophleba*			PERTH07111800

DNA extraction

DNA was extracted from fresh leaves (or leaves from herbarium specimens for *E. loxophleba*) using the protocol described in Glaubitz *et al* (2001) with a wash buffer described by Wagner *et al* (1987). The extracted DNA was visualised on agarose gel and quantified using a Nanodrop® spectrophotometer (Appendix 1).

Sequencing (ETS and ITS regions)

Sequences were generated for two regions of nuclear ribosomal DNA (nrDNA) – the internal transcribed spacer region (ITS) and the external transcribed spacer region (ETS). ITS comprised the entire region between the nuclear ribosomal 26S and 18S genes, and has been widely used in reconstructing plant phylogeny (Baldwin et al. 1995). ETS has been demonstrated to have potentially 30% more informative sites than ITS (Baldwin and Markos, 1998). Sequence primers used are shown in table 2.

Table 2: Sequence Primers: All sequences are given 5' to 3'. F, forward primer; R, reverse primer.

PRIMER		Sequence	Reference
ITS-4	R	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS-5	F	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
ETS-18S	R	GAGCCATTCGCAGTTTCACAG	Wilson et al. (2007)
ETS-Kunzea	F	CGTGCTGGTGCACCGAA	Wilson et al. (2007)

Sequences were amplified by polymerase chain reaction (PCR), and the products visualized on a 2% agarose electrophoresis gel (stained with SYBERsafe dye). Amplification products were cleaned up using the Argencourt® AMPURE PCR cleanup kits according to the manufacturers protocol. A standard cycle sequence reaction was performed using the Beckman-Coulter cycle sequence dye terminator reaction kit (see Protocol in Appendix 4). DNA sequences were visualised on a CEQ8800 Genetic Analysis System (Beckman Coulter) according to the manufacturer's specifications. Sequences were analysed using the CEQ8800 default sequence analysis parameters. Forward and reverse electropherogram profiles were combined and edited manually in CodonCode Aligner v. 2.0.4 (CodonCode Corporation). Edited sequences were then manually aligned in Se-Al v. 2.0a11 (Rambaut, 1996) along with additional eucalypt ITS and ETS sequences obtained from GENBANK (Appendix 3). Where ambiguities in the alignment occurred, the alignment chosen was the one generating the fewest potentially informative characters. As we were concerned with phenetic similarity rather than phylogenetic relationships, overall sequence similarity among samples was assessed by a Neighbor-Joining (NJ) tree from aligned sequences using PAUP* 4.0b10 for Macintosh (Swofford 2002). Support

for clusters was estimated by using the bootstrap option in PAUP*. Bootstrap values >50% were interpreted as support for clusters.

Amplified Fragment Length Polymorphism (AFLP)

The DNA fingerprinting technique Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995, Mueller & Wolfenbarger 1999) uses stringent PCR protocols for highly reproducible multi-locus DNA fingerprints. The AFLP procedure is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Oligonucleotide adapters are ligated to the ends of restriction fragments, which then serve as priming sites for an initial PCR amplification. A second selective PCR amplification uses primers of complementary sequence to the ligated adapter plus additional arbitrary nucleotides to amplify fragments for subsequent electrophoresis. Detailed protocols are listed in appendix 4. Six primer pair combinations were used (Table 2). *Pst*I and *Mse*I primer sequences are from Muluvi *et al.* (1999). Selective PCRs were amplified separately prior to visualizing with a Beckman CEQ8800 capillary machine, with internal size standard 400. Fragments were scored by eye using Beckman software for the unambiguous presence (1) or absence (0) of fragments between 60 and 400 base pairs in length. Replicate samples were run on each 96-well plate to ensure consistent scoring of reliable markers. Of the six primer pairs tested, two (m-CAG/p-AC and m-CAG/p-CG) generated strong, reliable and reproducible fingerprints.

Table 2. AFLP primer pairs assessed for polymorphism, reliability, and reproducibility.

m-CTC/p-AC	m-CTC/p-CA	m-CTC/p-CG
m-CAG/p-AC	m-CAG/p-CA	m-CAG/p-CG

Genetic dissimilarities among all pairwise combinations of samples was estimated as the sum of alternate marker states (presence/absence) across all markers for each of two primer pairs, using Genalex 6 (Peakall & Smouse 2006). Pairwise genetic dissimilarities was visualized by ordination using principal co-ordinates analysis in Genalex 6.

Results

Sequencing (ETS and ITS regions)

We assessed ITS sequences for 54 samples, including 21 taxa obtained from GENBANK (Appendix 4). ITS failed to amplify sufficiently for all *E. loxophleba* samples, presumably due to poor quality DNA from herbarium samples. The final matrix contained 640 characters, with 85 (13.3%) variable characters. The NJ tree generated a strongly supported cluster (bootstrap support 77%) containing all *E. articulata* samples, all *E. trivalva* samples, *E. wandoo*, and the unknown samples 4i and 4i(b) (Fig. 2). Further strong support (bootstrap support 80%) was found for this cluster with the addition of *E. dundasii*. A single base polymorphism at 398 bases (G instead of A) was diagnostic for this cluster, for the samples analysed. All other samples, with known and uncertain identification, were excluded from the *E. articulata* cluster (Fig. 2).

We assessed ETS sequences for 41 samples, including 8 taxa obtained from GENBANK (Appendix 4). ITS failed to amplify sufficiently for 1 of 3 *E. loxophleba* samples, presumably due to poor quality DNA from herbarium samples. The final matrix contained 467 characters, with 58 (12.4%) variable characters. The NJ tree generated a strongly supported cluster (bootstrap support 59%) containing all *E. articulata* samples and the 2 *E. loxophleba* samples (Fig. 3). A single base polymorphism at 362 bases (T instead of C) was diagnostic for this cluster, for the samples analysed. The *E. loxophleba* samples were identical, and significantly (bootstrap support 72%) clustered within the *E. articulata* cluster. All other samples, with known and uncertain identification, were excluded from the *E. articulata* cluster (Fig. 3).

Amplified Fragment Length Polymorphism (AFLP)

For AFLP primer pair 1 (m-CAG/p-AC), we assessed 31 samples for presence/absence variation across 152 markers, of which 150 (99%) were polymorphic. For AFLP primer pair 1 (m-CAG/p-CG), we assessed 31 samples for presence/absence variation across 131 markers, of which 129 (98%) were polymorphic. Ordination of data from each primer pair showed clustering of all *E. articulata* samples, and marked differentiation (non-overlap) between the *E. articulata* cluster and all other samples (Fig 4, 5). Plotting the first and third principal co-ordinate axes of the m-CAG/p-CG data matrix revealed a cluster of all known *E. trivalva* samples with samples 4i and 4i(b) (Fig. 6), suggesting that these 2 samples are *E. trivalva*, in agreement with the ITS sequence data (Fig. 2). Fixed marker differences between *E. articulata* and all other samples, or *E. articulata/E. loxophleba* and all other samples, were found at numerous AFLP markers (Table 3).

Fig. 2: Neighbor-joining tree of ITS sequence data. Bootstrap support values above 50% are indicated. Note significant cluster (indicated) containing all *E. articulata* samples, all *E. trivalva* samples, 4i and 4i(b), and *E. wandoo*.

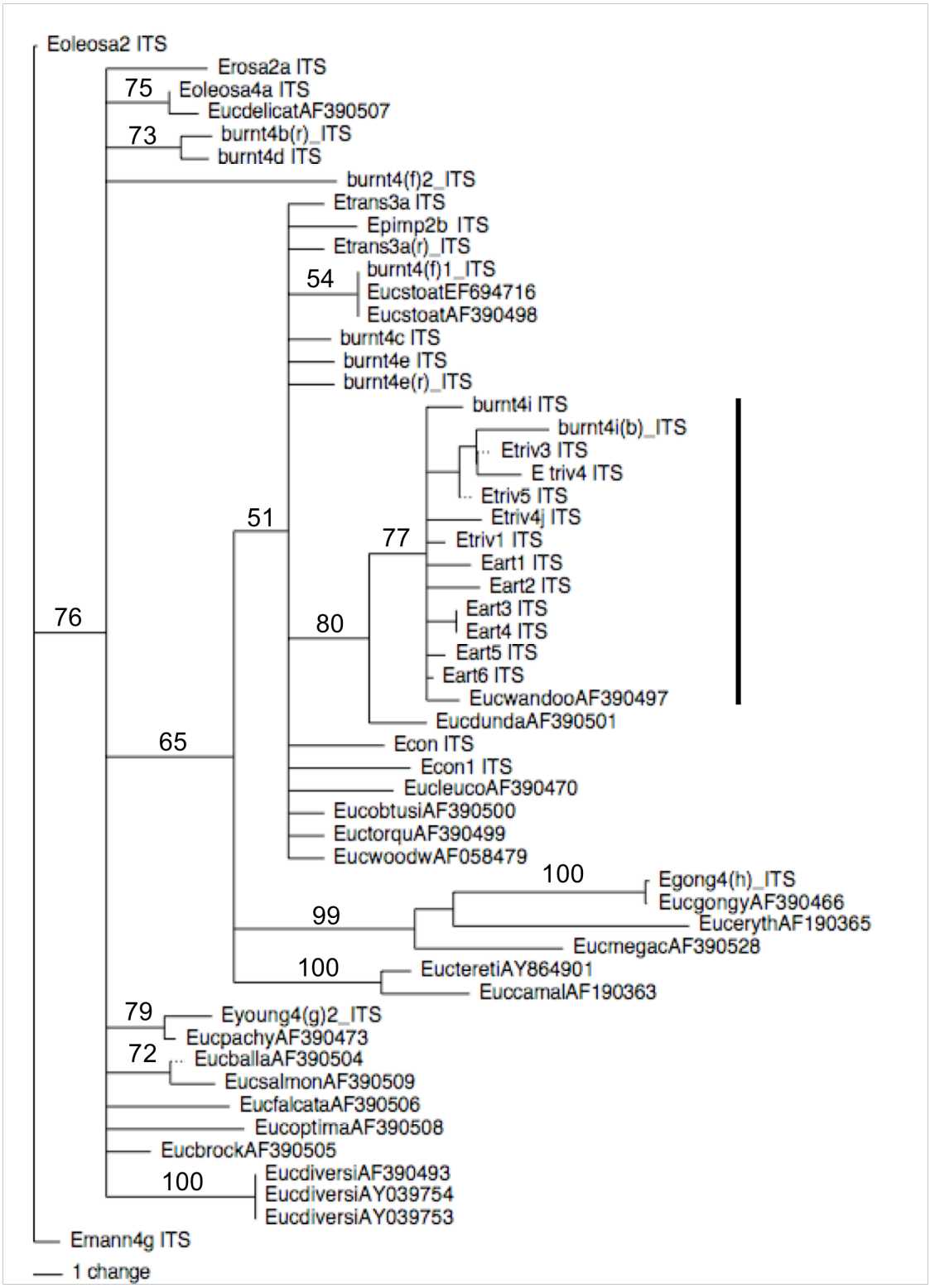

Fig. 3: Neighbor-joining tree of ETS sequence data. Bootstrap support values above 50% are indicated. Note significant cluster (indicated) containing all *E. articulata* samples, and *E. loxophleba* samples.

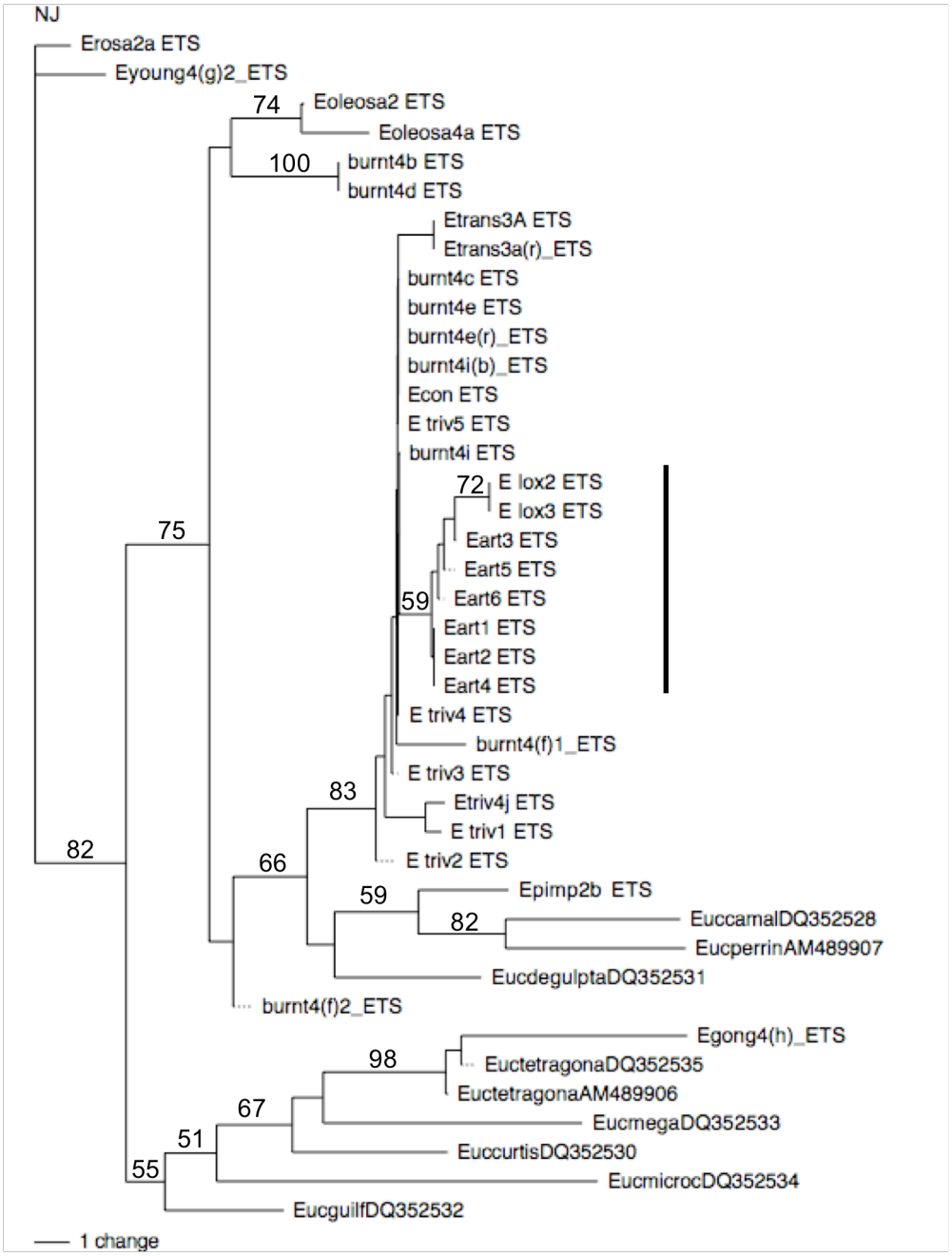

Fig. 4: Ordination showing genetic similarities among eucalypt samples for AFLP products from primer pairs m-CAG/p-CG. Plotted are the first two principal co-ordinates, which account for 48% of the total variation. Note "EA1" to EA6" are *Eucalyptus articulata* samples that are strongly differentiated from all other samples. See also Table 1 for sample codes.

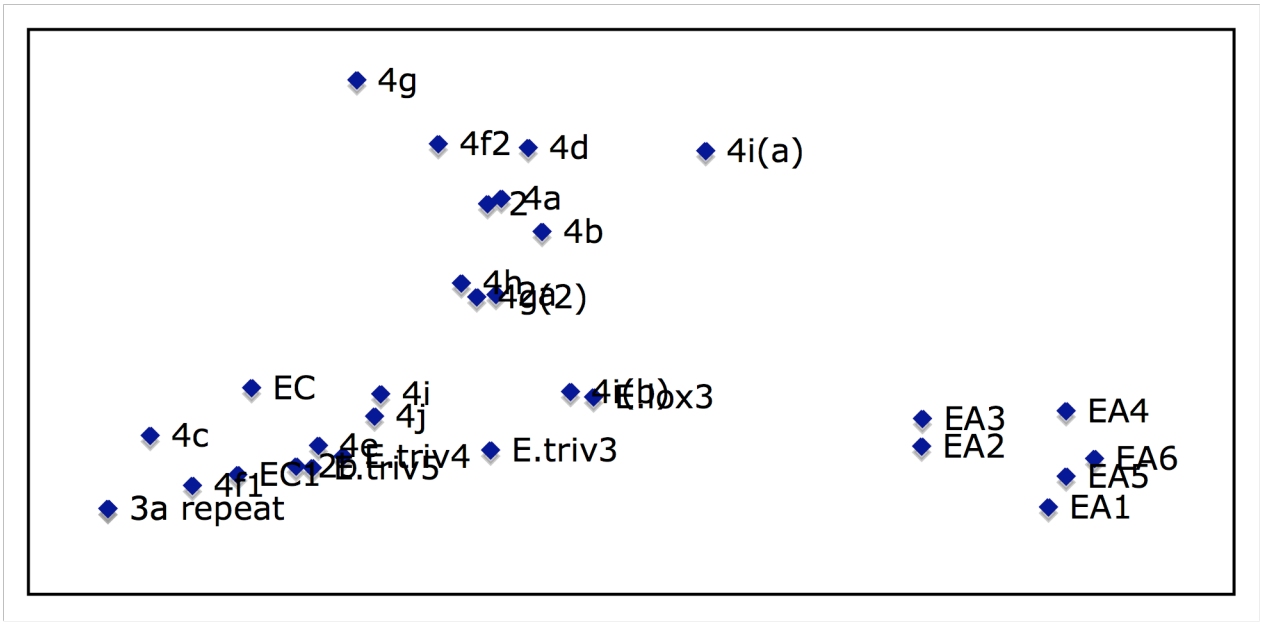

Fig. 5: Ordination showing genetic similarities among eucalypt samples for AFLP products from primer pairs m-CAG/p-AC. Plotted are the first two principal co-ordinates, which account for 46% of the total variation. Note "EA1" to EA6" are *Eucalyptus articulata* samples that are strongly differentiated from all other samples. See also Table 1 for sample codes.

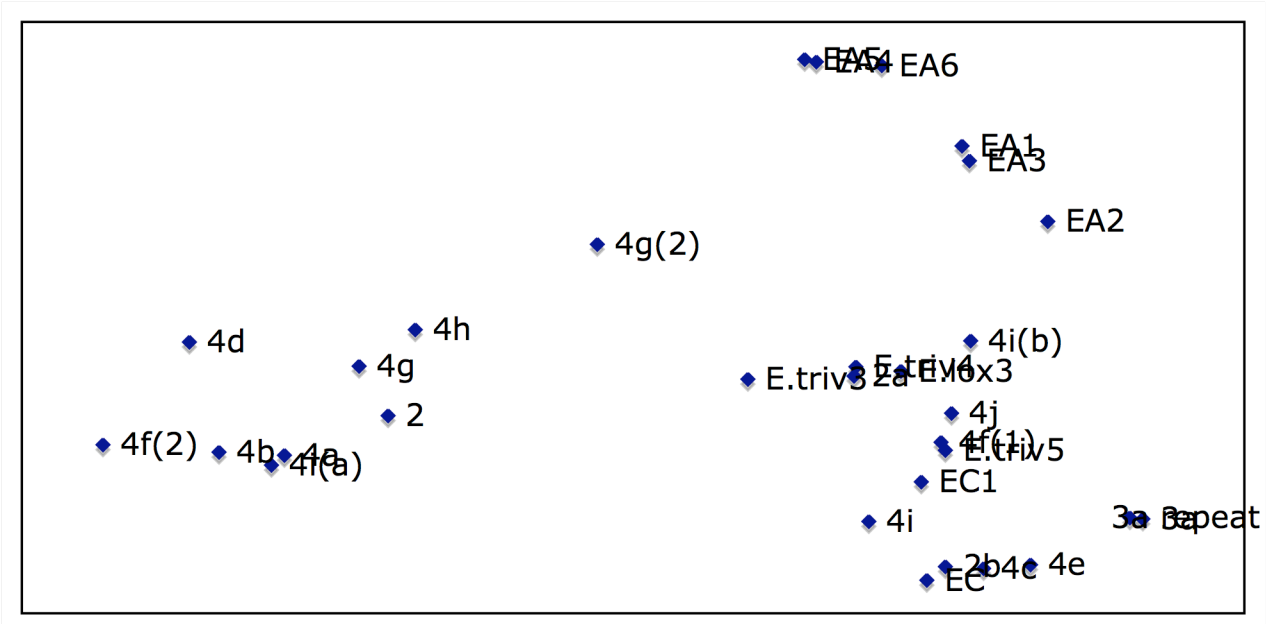

Fig. 6: Ordination showing genetic similarities among eucalypt samples for AFLP products from primer pairs m-CAG/p-CG. Plotted are the first and third principal co-ordinates, which account for 46% of the total variation. Note strong overlap of "4i", "4ib", and "4j" with known *E. trivalva* samples ("E.triv3-5"). See also Table 1 for sample codes.

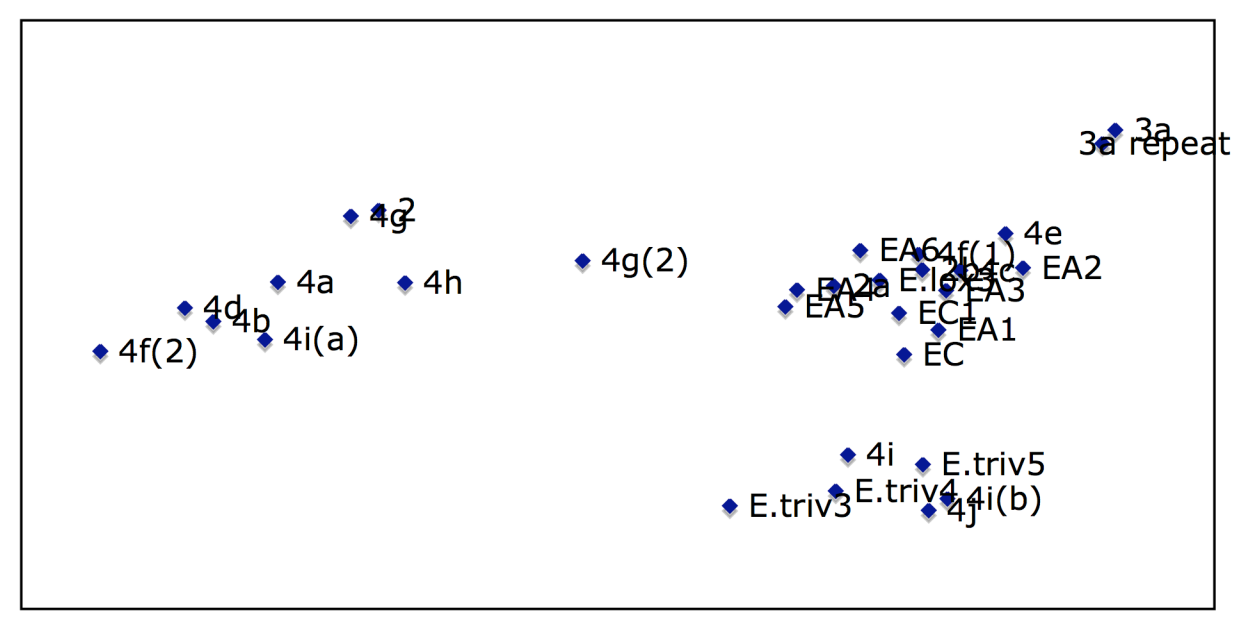

Table 3: Diagnostic AFLP markers for *E. articulata* from primer pairs m-cag/p-ac and m-cag/p-cg

PRIMER	MARKER	DIAGNOSTIC FEATURE
m-cag/p-ac	79	Absent in all *E. articulata*, present in all other samples
m-cag/p-ac	95	Present in all *E. trivalva*, 4i, 4i(b), absent in all other samples
m-cag/p-cg	101	Absent in all *E. articulata, E. trivlava, E.loxophleba*, 4i & 4i(b), present in all other samples.
m-cag/p-cg	263	Present in all *E. articulata & E. loxophleba*, absent in all other samples
m-cag/p-cg	272	Present in all *E. articulata*, absent in all *E. trivalva, E. loxophleba*, 4i & 4i(b)
m-cag/p-cg	277	Present in all *E. articulata & E. loxophleba*, absent in all other samples

Conclusions

A strong feature of this molecular assessment is the congruence in the results with regards *E. articulata* from three independent molecular marker sets – ITS sequence data, ETS sequence data, and AFLP DNA fingerprinting data. All three data sets identified genetic clusters of the *E. articulata* samples that, with the partial exception of ITS sequencing, significantly excluded all taxonomically unknown or uncertain samples. For the ITS data, only samples 4i and 4i(b) clustered with *E. articulata*, along with *E. trivalva*. Ultimately, diagnostic sequence variation was found that uniquely defined the E. *articulata* samples from all others, and numerous diagnostic AFLP markers were identified that differentiated *E. articulata* samples from all others.

While our objective was specifically focussed on confirming or rejecting the identity of eucalypt samples as *E. articulata*, the association of samples 4i and 4i(b) to known *E. trivalva* from ITS sequence data and AFLP data suggests that we are able to conclude the identification of these samples as *E. trivalva*. In addition, there is some DNA sequence support for many of the tentative identifications made from morphology. However, more detailed sampling of these other species is required for a confident identification of these non-*E.articulata* species.

A limitation of this study was the relatively limited sampling, of *E. articulata* and particularly other species of interest. This was in some part a consequence of the practical challenges posed in achieving robust sampling in this region. To address these concerns in a feasible manner, we are currently assessing DNA sequence variation at a further 2 regions, re-doing failed reactions and/or adding missing taxa (eg. *E. loxophleba* ITS sequencing), as well as conducting a blind test on a further 2 samples provided to us, one of which is *E. articulata*. These further assessments will provide a test of the robustness of the conclusions drawn from the current study, and results of which will be included in a final report.

Within the limits of the current study, we conclude that the taxonomically uncertain or unknown eucalypts sampled and provided to us for genetic assessment do not include *E. articulata*. This conclusion is supported by the limited morphological material provided for voucher specimens, and is supported by the tentative conclusions drawn in the field by Mattiske staff undertaking the sampling (pers comm).

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Appendix 1: DNA extraction

Eucalyptus DNA Extraction Protocol
(Glaubitz *et al.* 2001)

1. Add Na metabisulfite to proportion of extraction buffer and wash buffer and keep on ice.
2. Grind 1-2g leaf material in liquid nitrogen
3. Add 10ml cold extraction buffer (it will freeze in the mortar- allow to thaw before the next step).
4. Filter through muslin (in a funnel) into 10 ml tube, and keep on ice.
5. Mix and spin at 2000 rpm for 10 min to pellet.
6. Pour off supernatant; invert tubes to drain for 1 minute.
7. Add 1 ml wash buffer, resuspend pellet
8. Add 100 μl 20% SDS (final concentration 2%). Mix.
9. Add 100 μl 20% Triton X-100 (final concentration 2%). Mix.
10. Mix gently, incubate at room temperature for 5 minutes, with occasional mixing by inversion.
11. Add 200 μl 5M NaCl. Mix. (final concentration 0.7M)
12. Add 500 μl 8.6% CTAB/0.7M NaCl. Mix.
13. Incubate in 65^{O}C water bath for 20-30 minutes.
14. Add 2 volumes (3.6 ml) chloroform:isoamyl alcohol (24:1); place on the shaker for at least 2 minutes.
15. Centrifuge at 5000 rpm for 10 minutes.
16. Transfer supernatant (top layer) to new tube.
17. Add 2/3 volume isopropanol. Mix.
18. Hook out DNA with the tip of a sealed glass pipette. (If unhookable, centrifuge for 8 minutes at 2000 and remove supernatant.)
19. Place pipette in an eppendorf tube and wash in 1 ml of 50% isopropanol/0.3M Ammonium acetate (NH_4Oac) overnight (or for several hours) at 4^{O}C.
20. If not on a pipette re-centrifuge to pellet DNA and pour off supernatant.
21. Air dry DNA
22. Re-suspend in minimal volume of warm (65^{O}C) TE

Eucalyptus Extraction Buffer

	For 1 litre	For 500 ml
0.35M Sorbitol	64 g	32 g
100 mM Tris	12.1 g	6.05 g
100 mM Boric Acid	6.2 g	3.1 g
25 mM EDTA pH 8.0)	50 ml (0.5M pH 8.0)	25 ml (0.5 ml
1M NaCl	58.4 g	29.2 g
Adjust pH to 8 before adding PEG and PVP		
10% PEG 8000[1]	100 g	50 g
2% PVP 40 000[2]	20 g	10 g
0.5% BSA[3]	5 g	2.5 g

0.1% spermine	1 g	0.5 g
0.1% spermidine[4]	1 g	0.5 g
0.2% sodium metabisulphite[5]	2 g	1 g

Notes:
[1]PEG may need to be placed on the stirrer to dissolve
[2]Make a paste of PVP in a little dH_2O before adding
[3]Buffer containing BSA must be soaked in bleach after use
[4]Spermidine is optional
[5]Add sodium metabisulphite on the day of use

Eucalyptus Wash Buffer (From Wagner et al 1987)

	500 ml	50 ml	100 ml
50 mM Tris	3.025 g	0.3 g	0.6 g
25 mM EDTA (use 0.5M, pH 8)	25 ml	2.5 ml	5 ml
0.35 M sorbitol	32 g	3.2 g	6.4 g
0.5% spermine	2.5 g	0.25 g	0.5 g
0.5% spermidine	2.5 g	0.25 g	0.5 g
0.2% sodium metabisulphite*	1 g	0.1 g	0.2 g

*Add on the day of use

Notes

- Always keep extraction and wash buffer in the fridge
- SDS (sodium dodecyl sulfate), to make 10 ml of 20% SDS, use 2 g and make up to 10 ml with dH_2O.
- Triton –X 100, to make 10 ml of 20%, use 2 ml and add 8 ml dH_2O.
- To make 100 ml of 50% isopropanol/0.3M ammonium acetate. Make up in the fume hood and store in the fridge. Make up 0.6M ammonium acetate and add 50 ml of this to 50 ml 100% isopropanol for a total of 100ml.
- To make 100 ml 8.6% CTAB/0.7M NaCl: Add 8.6 g CTAB and 4.09 g NaCl and make up to 100 ml with dH_2O.

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Fig. 1.1 Agarose gel of demonstrating quantity and quality of DNA extracted from *Eucalyptus* species (lanes from left to right correspond to numbers 1- 19 in table). The final lane (right) is a DNA ladder.

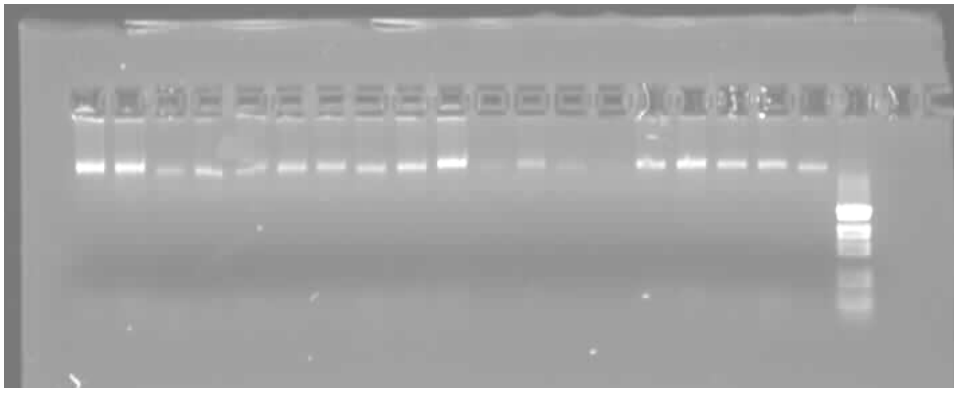


Table 1.1: Quantity of DNA extracted from *Eucalyptus* leaf material

Number/symbol		Species	DNA ng/μL
1.	Ec1	*E. concinna*	14.59
2.	4(f)1	Burnt mallee	32.02
3.	Ea5	*E. articulata*	19.99
4.	Ea6	*E. articulata*	26.51
5.	Ec	*E. concinna*	10.86
6.	Ea2	*E. articulata*	25.32
7.	Ea1	*E. articulata*	37.31
8.	Ea3	*E. articulata*	28.32
9.	2b	*E. pimpiniana*	25.67
10.	2	*? E. oleosa*	21.55
11.	4c	Burnt mallee	6.73*
12.	4b	Burnt mallee	5.19*
13.	4e	*? E. concinna,* burnt mallee	4.12*
14.	4d	mallee	0.48*
15.	4(f)2	Burnt mallee	11.72
16.	4g	*? E. mannensis*	19.56
17.	4i	Burnt mallee	18.69
18.	3a	*? E. transcontinentalis*	15.9
19.	4j	*E. trivalva*	5.73*
20.	Ea4	*E. articulata*	52.99
21.	2a	*? E. rosaceae*	38.62
22.	4a	*? E. oleosa*	59.39
23.	4i(b)	Burnt mallee	26.28
24.	4i(a)	Burnt mallee	241.27
25.	4h	*E. gongylocarpa*, burnt	14.21
26.	4(g)2	*E. youngiana*	9.04*
27.	E.lox1	*E. loxophleba* (herbarium)	2.01

28.	E.lox2	*E. loxophleba* (herbarium)	4.87
29.	E.lox3	*E. loxophleba* (herbarium)	2.17
30.	E.triv1	*E. trivalva*	36.77
31.	E.triv2	*E. trivalva*	20.07
32.	E.triv3	*E. trivalva*	20.35
33.	E.triv4	*E. trivalva*	44.2
34.	E.triv5	*E. trivalva*	57.45

* These specimens were re-extracted due to the low yield (data not shown).

Appendix 2: DNA sequencing protocol

Gene Amplification – PCR 1

Concentrations of some of the following vary between protocols (usually obtain them from a published protocol for your region): below is mine for ETS. Standard modifications are: changing $MgCl_2$ concentration to vary primer specificity, and slight changes in concentration of primers and Taq [add more or less H_2O as required to make to 45 μL]. You may also wish to double amounts to do a 100 μL total volume.

Mix a stock batch for N samples (+10% of N) xf each reagent below [the 10% is an extra amount ensure you don't run out of stock master mix before reaching the last tube]

EXAMPLE STOCK MASTER MIX ONLY:
Stock:

Distilled H2O	22.9 μL
5X polymerisation buffer	10 μL
$MgCl_2$ (50 mg/μL)	2 μL
Fwd Primer (5 μM)	5 μL
Rev Primer (5 μM)	5 μL
Taq (5.0 U/μL)	0.1 μL

Vortex stock and spin briefly in centrifuge.

Add 45 μL of stock to each well of your PCR plate.
Add 5 μL of your sample DNA.

Vortex the plate to mix reagents and spin the plate in the lettuce centrifuge.

Run your relevant gene amplification PCR profile (usually obtain from a published procedure for the region you are using – slight changes to annealing temperature (48-55^{o}C) may help amplify uncooperative samples).

EXAMPLE PCR ONLY:
Hold: 95^{o}C for 90 sec
35 cycles of:
    95^{o}C for 30 sec
    55^{o}C for 60 sec
    72^{o}C for 90 sec
Hold 72^{o}C for 7 min
Hold indefinitely at 4^{o}C

Check product on agarose gel. Check that products are around the expected size range, and that there is only a single band (sometimes weak secondary bands

will make no difference, but if you see equally strong bands or common secondary bands you need to re-optimise the PCR conditions).

If you don't get any, or only partial amplification (and are sure your DNA is ok), try the following modifications to the PCR:

(1) Add DMSO and BSA to the PCR reaction if not already used. DMSO assists the primer to bind to the template and BSA helps bind PCR inhibitors.
(2) Lower the annealing temperature.
(3) Increase the $MgCl_2$ concentration.
(4) Try a "touchdown" PCR [often these don't work as reliably as standard PCR, but some templates seem to preferentially amplify with touchdown]

PCR 1 cleanup [using Agencourt AMPure Kit]

ASSUMING 46 μL PCR Volume [50 μL reaction minus 4 μL for agarose gel]

*Make new labelled tubes IF you are going to transfer the cleaned product to eppendorf tubes rather than leave them in the plate.

1. Transfer successfully amplified PCR products to a new plate so there are no gaps (not necessary if all samples successfully amplified). **NOTE:** there is a high probability of sample mislabelling at this step, which can have major interpretation problems later – plan it well.
2. Gently shake AMPure bottle to resuspend magnetic particles.
3. Add 82.8 μL AMPure to each well [ie 1.8 x reaction volume].
4. Seal tubes with lids* and vortex strongly for *at least* 30 s. Check all reagents are thoroughly mixed and homogenous before continuing. *Note: if a plate cover is used it MUST be able to seal isopropanol during heavy vortexing, and be VERY careful of contamination between wells – lids are much safer but will still leak if not completely closed.
5. Spin plate briefly in lettuce spinner. Incubate samples OFF THE MAGNET for minimum 3-5 mins to bind extension products.

Binding

6. Place plate on magnet for 5-10 mins (or until solution is very clear).
7. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible. [Use a multichannel pipette, but don't touch the bead ring].

First ethanol wash.

8. WHILE ON MAGNET, Add 200 μL of 70% ethanol to each well. [Mixing and resuspension is NOT necessary.]
9. Incubate at room temp for 30 seconds.
10. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible.

Second ethanol wash.

11. WHILE ON MAGNET, Add 200 μL of 70% ethanol to each well.

12. Incubate at room temp for 30 seconds.
13. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible.
14. Place plate on bench (off the magnet) to air-dry for 10-20 minutes (sometimes longer). Plate must be completely dry. [**Note**: the plate can be sealed and stored dry at –20°C indefinitely here.]
15. Add [20-40 μL] 30 μL elution buffer (TE, DI H_2O or Tris-Acetate) to each well. **Note:** 30 μL seems to be a good balance, but particularly weak bands on the agarose gel should be concentrated by adding only 20 μL (the difference between getting acceptable vs too weak and noisy sequences). Extremely strong bands could be diluted by using 40 μL, but it is safer to dilute them later after measuring DNA concentration.
16. Vortex strongly for 30 seconds [critical if using less than 40 μL to elute].
17. Place plate on magnet for 2-5 mins until solution is clear [**Note**: with 30 μL of liquid you can't actually see the ring of magnetic beads from the side, only from the top]. [NOTE: the plate can be sealed and stored at 4°C or –20°C indefinitely here.]
18. OPTIONAL: Transfer liquid to labelled eppendorf tube. [**Note**: beads carried over do not inhibit further reactions – but they CAN affect quantification]

Cycle Sequence PCR

Note: CEQ dyes are light sensitive and degrade over time (within a day or two in formamide). Keep any dye-containing reagents or mixes on ice. Plan to do the dye cleanup of the cycle sequence reaction within 18-24 hours of starting the cycle sequence thermocycling. Samples can be left dry indefinitely at –20°C AFTER the dye removal step if necessary.

Note: DNA concentration is an important variable for sequencing reactions. It is best, at least initially, to quantify your *cleaned* PCR products on the nanodrop spectrophotometer. Ideally you want concentrations in the order of:
[6]10-40[60] ng/μL for PCR products 400-800 bps long
[10]15-65[80] ng/μL for PCR products 900-1200 bps long
[15]20-80[120] ng/μL for PCR products 1300-2000 bps long
Concentrations lower than the lower bracketed limit will definitely NOT give good product, with variable success up to the lower non-bracketed limit. Concentrations higher than the upper-bracketed limit could cause potential problems, especially in larger size PCR products, and should ideally be diluted, at least until you have a feel for how your sequences work.

NOTE: Beckman-Coulter (CEQ) process uses a different dye chemistry to ABI systems used elsewhere. If you plan to send your sequences off to be run elsewhere you will probably need to use ABI BigDye reagents, not our standard reagents.

Note: we usually use a 1/4 reaction, and it works reliably in all cases so far. If you are planning to send your sequences to be run elsewhere or are getting consistently bad reactions, using half reactions MAY be a better option: see Matt for suggestions and correct protocol.

NOTE: For a sequence reaction you must add only one primer to get readable sequence. A short (< c. 5-600 bps) region is usually sequenced in two reactions using the same primers for the PCR. Longer regions need internal primers as well to get all of the sequence. In our experience Beckman sequence data is prone to more random noise than ABI chemistry, making it (usually) essential to sequence both fwd and reverse strands.

The cycle sequence reaction requires the following reagents per reaction [for a 1/4 reaction, 10 μL total volume]:

5X Cycle Sequence Buffer	1.0 μL
Primer (5 uM)	0.64 μL [ie 3.2 pmoles of primer in 10 μL reaction]
Dye Terminator Mix	2.0 μL
DNA	[20]40-80[200] ng SEE BELOW
Distilled H_20	to 10 μL total volume

There are many ways to achieve this. Typically we mix as many reagents as practical into one or many "stock mixes", aliquot them to each well then add DNA and any other missing reagents as required.

PROTOCOL

How much DNA do I add?

You need a total volume 4 μL of DNA + distilled H_2O for the reaction
Initially, you should aim for 40-80 ng of DNA, depending on size of the amplified PCR product. We suggest aiming for [sequence reaction will almost certainly be too weak below the bracketed DNA amounts]:
0.5-0.7 kbases use [20]40 ng DNA (double-stranded)
0.8-1 kbases use [25]50-55 ng DNA (double-stranded)
1.1-1.5 kb use [30]65 ng DNA (double-stranded).
1.5-2 kb use [40]80 ng DNA (double-stranded).
In general it is better to use more rather than less DNA (low DNA concentration is the biggest cause of sequence reaction failure). However, in cases of weak amplification, we have successfully sequenced using as little as 4 μL (maximum available volume) of PCR product with concentrations of 6 ng/μL.

Once you have a feel for how strong your PCR products typically are (in multiple reactions from material from different extractions), you can probably simplify set up considerably by using 4 μL of cleaned PCR product *regardless of concentration* as template for your sequence reaction, especially if you tweak the

final elution volume at the end of PCR cleanup based on strength of agarose gel bands.

1. Stock reagent mix

Mix a stock batch for N samples (+10% of N) x each reagent below [the 10% of N is an extra amount to make sure you don't run out before reaching the last tube].

Remember you typically need 1 forward reaction and 1 reverse reaction for each sample. Stock mixes could be made for EACH primer (as below), or be made for all samples (excluding primers in stock mix, total vol. = 5.36 μL to each well), and adding 0.64 μL of 5 μM primer to each well. Which one is more efficient depends on the number of samples and the number of different primers used for each sample.

Amounts per reaction [for a 1/4 reaction, 10 μL total volume]:

Distilled H20	2.36 μL
5X Cycle Sequence Buffer	1.0 μL
Primer (5 uM)	0.64 μL [ie 3.2 pmoles of primer in 10 μL reaction]
Dye Terminator Mix	2.0 μL

2. Vortex the stock mix and centrifuge briefly.
3. Aliquot the relevant amount to each PCR well (eg 6 μL if you include the primer with the stock mix, 5.36 if you add the primers separately).
4. Add Primer (if necessary): ONLY IF you have not included primers in stock mixes, add 0.64 μL of 5 μM primer to each well.
5. Add DNA: If you have used the AMPure kit AND have not transferred the final eluant to a new tube (ie still contains magnetic beads), place plate on magnet for 2-5 mins until solution is clear. Transfer the appropriate amount of DNA [eg. 4 μL] to the sequence reaction. [**Note**: beads carried over do not inhibit further reactions – but they CAN affect quantification].
6. Seal plate with lids and vortex the plate to mix reagents and spin the plate in the lettuce centrifuge.
7. Run the CEQ cycle sequence PCR profile. This is usually the same for all templates, however occasionally it might need to be changed for difficult primer/templates [never yet in our experience].

This protocol can be found on PCR1 and PCR2 as: ***MattCEQcycseq***

96^{o}C for 20 sec
30 cycles of:
    96^{o}C for 20 sec
    50^{o}C for 20 sec
    60^{o}C for 4 min
Hold 4^{o}C indefinitely

Dye removal [Using Agencourt CleanSEQ protocol]

Note: The protocol takes about 2 hours for a plate, after which EITHER:

(1) If you add formamide at the last step the samples MUST be run on the Beckman immediately (within a few hours) as formamide degrades the dye, OR

(2) DO NOT add formamide after the last 20 min (no more) drying stage and store the dried product at –20°C indefinitely until you are ready to run them.

Make your sample sheet ready for the Beckman if you are going to load them straight after cleanup – you may not have time during the cleanup process.

Note: the following assumes a 10 μL cycle sequence reaction volume.

Before starting, you need:

1. Enough CleanSEQ bead suspension for N x 10 μL (N=no. samples to clean)
2. Make some FRESH 73% Isopropanol (need 455 uL per 10 μL reaction) Dilute as to table below (volumes in μL) **NOTE**: 73% Isopropanol can be kept only 1-3 days in –20°C freezer; best to make fresh each day.

No. Samples	100% isopropanol	H_2O	Total Vol. (455 μL per sample x 1.1 dead volume)
1	365	135	501
8	2923	1081	3640
16	5846	2162	7280
24	8769	3243	10920
32	11692	4324	14560
40	14615	5405	18200
48	17538	6487	21840
56	20460	7568	25480
64	23383	8649	29120
72	26306	9730	32760
80	29229	10811	36400
88	32152	11892	40040
96	35075	12973	43680

3. Make STOP solution (5.5 μL for every 10 μL reaction) as in the table below. Calculate Total vol needed (in μL) is: TV = 5.5 μL x N reactions x 1.1 dead volume.

No. samples	H_2O	3M Sodium Acetate pH 5.2	100 mM Na2EDTA pH 8	20 mg/μL glycogen (in kit)	Total (5.5 μL per sample x 1.1 dead volume)
1	3.0	1.2	1.2	0.6	6.05
8	24.2	9.7	9.7	4.8	48.4
16	48.4	19.4	19.4	9.7	96.8
24	72.6	29.0	29.0	14.5	145.2
32	96.8	38.7	38.7	19.4	193.6
40	121	48.4	48.4	24.2	242.0
48	145.2	58.1	58.1	29.0	290.4
56	169.4	67.8	67.8	33.9	338.8
64	193.6	77.4	77.4	38.7	387.2
72	217.8	87.1	87.1	43.6	435.6
80	242	96.8	96.8	48.4	484.0
88	266.2	106.5	106.5	53.2	532.4
96	290.4	116.2	116.2	58.1	580.8

(Check you have at least 5.5 μL for each sample)

PROTOCOL [*ASSUMING 10 μL sequence reaction*]
NOTE: retain lids for mixing and close them tight when requested to ensure no isopropanol is escaping during vortexing. Alternatively, can use multichannel pipette to pipette-mix 10-15 times to homogenise solutions when required.

Add STOP solution.

1. Add 5.5 μL dilute STOP solution to each sample well (in plate).

Prepare & add CleanSEQ

2. Shake CleanSEQ vigorously to resuspend magnetic beads (should be homogenous and uniform in colour).
3. Add 10 μL of CleanSEQ to each well.

Isopropanol precipitation

4. Add 55 μL of FRESH 73% Isopropanol to each well
5. Seal tubes and vortex strongly for *at least* 30 s. Check all reagents are thoroughly mixed and homogenous before continuing.
6. Incubate samples OFF THE MAGNET AND IN THE DARK for minimum 10 mins to bind extension products.

Binding

7. Place plate on magnet for 3-5 mins or until solution is clear.
8. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible. [Should be able to do this with a multichannel pipette, but don't disturb magnetic suspension]

First Isopropanol wash.

9. WHILE ON MAGNET, Add 200 μL of 73% isopropanol to each well. [Mixing and resuspension is NOT necessary.]
10. Incubate at room temp for 3 mins.
11. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible.

Second Isopropanol wash.

12. WHILE ON MAGNET, Add 200 μL of 73% isopropanol to each well. [Mixing and resuspension is NOT necessary.]
13. Incubate at room temp for 3 mins.
14. WHILE ON MAGNET, remove clear supernatant from wells by placing pipette tip on bottom of plate. Remove as much supernatant as possible.

IF RUNNING SEQUENCES IMMEDIATELY, START THAWING DEIONISED FORMAMIDE.

Air drying

15. OFF THE MAGNET Let samples air-dry for 10-20 mins at room temp. DO NOT OVERDRY! (longer than 30 mins lowers signal)

[Long-term storage]

16. Stop here if you are not running immediately: seal plate with lids and keep DRY at –20^{o}C indefinitely before resuming protocol when ready. Otherwise proceed to (14).

Re-suspension

17. OFF THE MAGNET, add 35 μL de-ionised formamide to each well. **NOTE: multiple re-thawings of deionised formamide may prevent full elution.**
18. Seal and vortex until beads are homogenously resuspended in deionised formamide (about 30 secs).

Binding

19. Separate on magnet for 3-5 mins or until solution is clear.
20. Transfer 30 μL to CEQ plate for running. **NOTE**: need to leave 5-10 μL behind to prevent bead transfer. Some bead transfer is OK, but must take care to minimise it. If too much is sucked up, re-dispense back into the well, wait 1-3 mins and re-transfer.
21. Centrifuge plate if necessary to remove air bubbles.
22. Add a drop of mineral oil to samples.

Setup and loading sequencer

Prepare buffer tray, wetting tray and check sample plate has not bubbles and you have added mineral oil. Add gel cartridge and check gel quantity on sequencer. Open sample plate menu. Set run conditions to LFR-1. Select all samples and check analysis parameters and choose DefaultSequenceAnalysisParameters. Run the sample plate.

Fig. 2.1: Agarose gel demonstrating amplification of the ITS region following PCR.

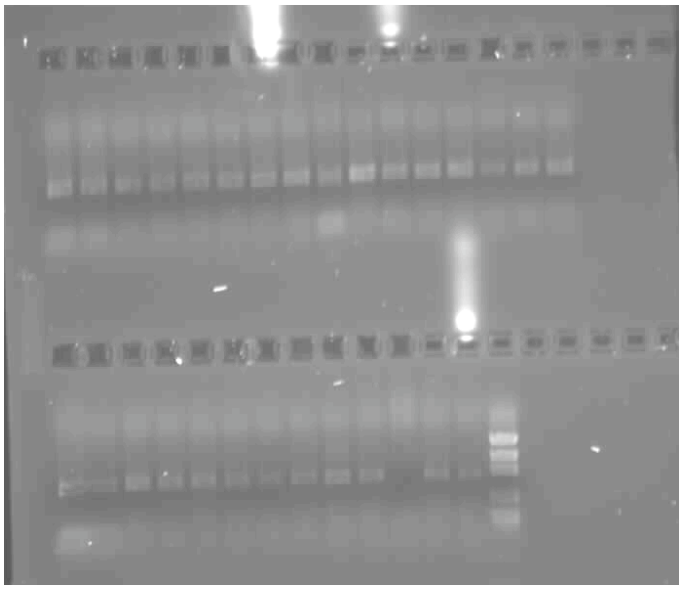
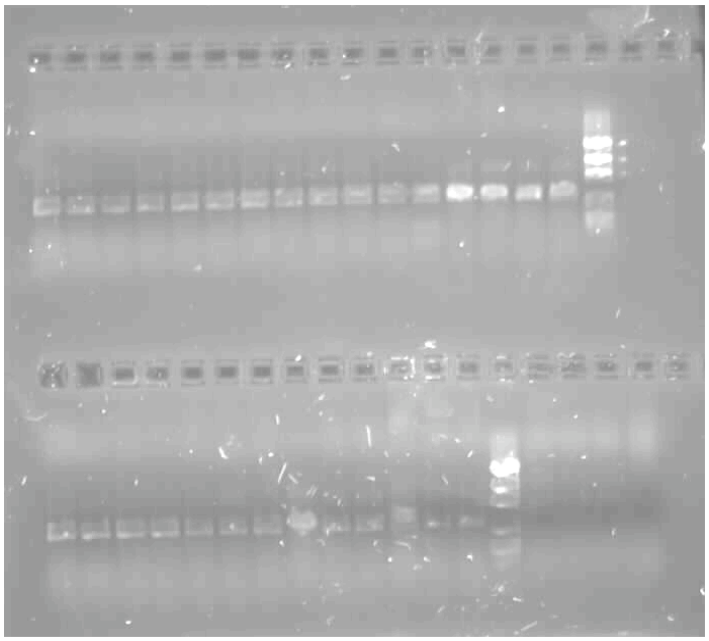


Fig. 2.2: Agarose gel demonstrating amplification of the ETS region following PCR.



Appendix 3: Eucalypt species for which ITS and ETS sequence data was obtained from GenBank

ETS

Eucalyptus degulpta	(DQ352531)
Eucalyptus camaldulensis	(DQ352528)
Eucalyptus curtisii	(DQ352530)
Eucalyptus perriniana	(AM489907)
Eucalyptus megacarpa	(DQ352533)
Eucalyptus guilfoylei	(DQ352532)
Eucalyptus microcorys	(DQ352534)
Eucalyptus tetragona	(AM489906)
Eucalyptus tetragona	(DQ352535)

ITS

Eucalyptus balladoniensis	(AF390504)
Eucalyptus salmonophloia	(AF390509)
Eucalyptus falcata	(AF390506)
Eucalyptus optima	(AF390508)
Eucalyptus delicata	(AF390507)
Eucalyptus brockwayi	(AF390505)
Eucalyptus diversicolor	(AF390493)
Eucalyptus diversicolor	(AY039754)
Eucalyptus diversicolor	(AY039753)
Eucalyptus pachyphylla	(AF390473)
Eucalyptus tereticornis	(AY864901)
Eucalyptus leucophloia	(AF390470)
Eucalyptus camaldulensis	(AF190363)
Eucalyptus stoatei	(EF694716)
Eucalyptus obtusiflora	(AF390500)
Eucalyptus torquata	(AF390499)
Eucalyptus stoatei	(AF390498)
Eucalyptus woodwardii	(AF058479)
Eucalyptus gongylocarpa	(AF390466)
Eucalyptus erythrocorys	(AF190365)
Eucalyptus megacarpa	(AF390528)
Eucalyptus wandoo	(AF390497)
Eucalyptus dundasii	(AF390501)

Appendix 4: Amplified Fragment Length Polymorphism (AFLP) Protocol:

AFLP involved three steps, restriction-digestion, pre-selective PCR amplification, and selective PCR amplification (Vos et al. 1995; Mueller & Wolfenbarger 1999). Restriction of genomic DNA was done at 37^{o} C for 2 hr in a 20µl volume containing approximately 250ng of DNA, 2.5U of *Mse*1 and 5.2U *Pst*1, 2.0µl NE buffer 2 (supplied with *Mse*1 enzyme), 2.0µl 0.1% BSA, and DNA-free water. Next, 5µl of a solution containing 4.0µl *Mse*1*/Pst*1-adapter solution, 0.5µl T4 ligase, 0.5µl ligation buffer (supplied with T4 ligase) was added to the samples and further incubated at 20^{o} C overnight, then diluted 1/20 in DNA-free water. Pre-selective PCR was performed in a 20µl total volume containing 4.0µl 5X PCR buffer containing dNTPs, 1.2µl $MgCl_2$ (25mM), 0.5µl each of *Pst*1 and *Mse*1 primers (5µM), 0.825U *Taq* DNA polymerase (Fisher Biotech), 4.0µl restricted/diluted DNA template and DNA-free water. The PCR was performed in a PerkinElmer Applied Biosystems 9700 thermal cycler for 20 cycles each at 94^{o} C for 30s, 56^{o} C for 2 min, 72^{o} C for 2 min. A final extension step at 72^{o} C for 5 min was performed. PCR products were diluted 1/30 with DNA-free water for subsequent, selective amplification. Selective PCR was done in a 10µl total volume containing; 2.0µl 5X PCR buffer containing dNTPs, 0.6µl $MgCl_2$ (25mM), 0.25µl fluorescently-labelled *Pst*1 primer (1µM), 0.5µl *Mse*1 primer (5µM) (Sigma GenoSys), 0.25U *Taq* DNA polymerase, 2.5µl of diluted pre-selective PCR product, and DNA-free water. The selective PCR cycle consisted of a touchdown cycle for 13 cycles at 94^{o} C for 30s, 65-53^{o} C for 30s, 72^{o} C for 1 min, followed by 25 cycles at 94^{o} C for 30s, 56^{o} C for 2 min, 72^{o} C for 2 min, and a final extension at 72^{o} C for 2 min. *Pst*I and *Mse*I primers sequences are from Muluvi *et al.* (1999). Six primer pair combinations were used (Table 2). Selective PCRs were amplified separately prior to visualizing with a Beckman CEQ8800 capillary machine, with internal size standard 400. Fragments were scored for the presence (1) or absence (0) of peaks unambiguously between 60 and 400 base pairs. Replicate samples were run on each 96-well plate for consistent scoring of bands. Of the six primer pairs used, two (m-CAG/p-AC and m-CAG/p-CG) generated strong, reliable and reproducible fingerprints.

Figure 4.1. Partial AFLP DNA fingerprints generated by the primer pair m-CAC/p-AC: From top to bottom- two burnt mallee's (4i(a) & 4i(b)), two *Eucalyptus articulata* Ea2 & Ea1, two *E. trivalva* (E.triv4 & E.triv3) and one *E. loxophleba* (E.lox3). The peaks in black are the generated fingerprints and the red peaks are the size standard.