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Statistical associations between morphology, physiology and AFLP DNA markers enable selection of a putative eucalypt hybrid able to tolerate salt affected floodplains

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Abstract

A naturally occurring putative hybrid between *Eucalyptus largiflorens* F. Muell and *Eucalyptus* gracilis F. Muell called Green Box tolerates saline conditions of the River Murray floodplains better than *E. largiflorens*. Revegetation strategies utilizing seedlings of Green Box have had limited success because only a few are Green Box and the majority are throw backs to *E. gracilis* and *E. largiflorens*. Therefore, the purpose of this study was to identify traits characteristic of Green Box and AFLP markers associated with the traits enabling selection at the seedling stage. This was done by non-linear canonical correlation analysis (OVERALS) to test for statistically significant associations between morphological and physiological traits with 232 AFLP markers from 9 primer combinations. OVERALS with all markers produced 1st and 2nd dimensions accounting for 80 and 74% of variation respectively. Green Box plants were placed intermediate between *E. gracillis* and *E. largiflorens* according to leaf colour, gloss and nitrogen with compo-

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nent loadings (l_c) of 0.340, 0.615 and 0.294 respectively. A second approach of simple linear regression of morphological and physiological traits against all 232 AFLP markers singled out 17 with significance $P<0.05$. Thirteen of these were also identified by OVERALS. Four occurred with high frequency in Green Box and *E. largiflorens* distinguishing them from *E. gracilis*. In order to separate Green Box and *E. largiflorens*, the segregation of a further three markers can be used to align Green Box with *E. gracilis*. Therefore, the segregation of 7 markers can be utilized to select Green Box.

Key words: Eucalyptus, Australia, salt, re-vegetation, hybrid selection, AFLP marker.

Introduction

Prior to Murray River regulation, inundation of the Chowilla floodplain in South Australia (136 000 ML day-1) occurred every 13 years whereas now for the same return period, flows of 76 000 ML day^{-1} flood less than half of the floodplain (OVERTON and DOODY, 2010; SHARLEY and HUGGAN, 1995). The lack of floods has caused drought, and salt inherent in these ancient river soils is no longer regularly transported away from the floodplain. Instead salt has built up to detrimental concentrations. Rainfall is less than 350 mm annually therefore plants rely on flooding for replenishment and leaching of salt from the root zone (KINGSFORD, 2000). Along the length of the Murray River in Australia, it is estimated that approximately 18 000 ha of floodplain vegetation is severely degraded with saline ground water (MARGULES AND PARTNERS *et al.,* 1990). High incidence of dieback amongst *E. largiflorens* and *E. camaldulensis* woodlands was a striking aspect of a biological survey conducted by O'MALLEY and SHELDON (1990).

Amongst deteriorating stands of *E. largiflorens*, Green Box plants were identified because of their healthy appearance. Green Box is characterised by bright, dense, glossy green foliage distinctly different to large glaucous grey-green leaves of *E. largiflorens* (PARSONS and ZUBRINICH, 2010; ZUBRINICH *et al.,* 2000). Although Green Box are less common, several hundred have been catalogued extending the length of the Murray Darling Basin (J. Seekamp unpublished). Green Box are hypothesized to be hybrids between *E. largiflorens* and *Eucalyptus gracilis* F. Muell, a mallee eucalypt inhabiting sandy escarpments up off the floodplain. Characteristics of *E. gracilis* include multiple stems, lignotubers, rough bark at the base and smooth whitish bark throughout the remainder, glossy green leaves (JESSOP *et al.,* 1986) and flowers with staminodes (BROOKER, 2000).

With respect to physiological traits, Green Box had more negative water potentials, pre-dawn and midday, compared to *E. largiflorens* growing at the same sites, indicating they were able to uptake water from drier or more saline soil (ZUBRINICH *et al.,* 2000). Green Box also has smaller xylem vessels. It is likely that Green Box inherited both of these traits from *E. gracilis* which have more negative water potentials and small xylem vessels. Mallees have lignotubers which were shown by MYERS (1995) to resist water flow and accounted in part for persistently more negative predawn water potentials in *Eucalyptus behriana* F. Muell.

There is an inherent salt tolerance of *E. largiflorens* specimens sampled from Clear Lake, western Victoria. These plants tolerated 380 mM NaCl, when salt tolerance was defined as withstanding 300 mM NaCl (BLAKE, 1981). Green Box had significantly higher leaf Na^+ and Cl^- compared to *E. largiflorens* ($P<0.01$) (ZUBRINICH *et al.,* 2000). There was less Na^+ compared to Cl^- and this has been similarly observed in *Eucalyptus microtheca* F.J. Muell, from Marree, South Australia, the lowest rainfall in Australia and *Eucalyptus microcorys* F. Muell, particularly at the highest salt level of 150 mM (CHEN *et al.,* 1998; MORABITO *et al.,* 1994).

Saline affected areas of the Chowilla floodplain have been re-vegetated with seedlings and clones of mature Green Box. Adult Green Box are readily distinct but seedlings are not and because the majority were throwbacks to either *E. largiflorens* or *E. gracilis* they suffered from the salinity of the floodplain. Clones tolerated the saline floodplain however a drawback is limited genetic diversity. Therefore a combined approach of traits associated with molecular markers will help to identify Green Box seedlings.

To screen for Green Box, characteristic morphological and physiological traits will be measured among natural stands of *E. largiflorens*, *E. gracilis* and Green Box. To enhance identification of suitable traits, links will be explored with amplified fragment length polymorphisms (AFLP) DNA markers (VOS *et al.,* 1995). AFLP DNA markers have been utilised extensively for screening purposes (MCKINNON *et al.,* 2008; WANG *et al.,* 2005). DNA markers or morphological traits segregating with physiological traits may be easier and quicker to score as physiological traits are time consuming to measure routinely in a screening process. For example, a correlation between leaf size and absisic acid (ABA) in rice enabled selection for drought resistance such that plants with smaller leaf sizes were more likely to have higher levels of ABA (QUARRIE *et al.,* 1997).

In this study, traits characteristic of Green Box identified by ZUBRINICH *et al.* (2000) will be utilised. Morphological traits include leaf colour, gloss and bark. In addition, new traits will be explored that indicate resource partitioning away from plant growth when plants are experiencing salinity stress. These traits determine the growth potential of a species (LAMBERS *et al.,* 2008; POORTER and EVANS, 1998) and include specific leaf area (SLA), leaf total nitrogen and carbon, and natural abundance of carbon and nitrogen isotopes (KOERBER *et al.,* 2012). Lower SLA is hypothesised to be a result of evolutionary selection pressure for leaves with long life spans, thus retaining nutrients longer in nutrient poor environments (SCHIEVING and POORTER, 1999; WRIGHT *et al.,* 2004). Other hypothesised strategies involving reduced SLA are accumulation of secondary compounds to detract herbivores and accumulation of lignin to facilitate survival during dry or cold environmental conditions (POORTER and GARNIER, 1999). Measurement of biomass composition of carbon and nitrogen gives an indication of resource partitioning, either into photosynthetic apparatus or structural components (LAMBERS *et al.,* 2008; LLOYD *et al.,* 1992; NIINEMETS, 1999; REICH *et al.,*

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1998). In a comparison of evergreen and deciduous species, evergreen species were hypothesised to possess a higher cost-benefit ratio attributed to longer-lived leaves, lower SLA, lower nitrogen content, and lower assimilation on a mass basis (EAMUS *et al.,* 1999). Although investment in growth of leaves construction of leaves/growth is more expensive, maintenance costs are lower and affordable over a longer payback interval (longer-lived leaves) (EAMUS *et al.,* 1999).

Nitrogen isotope ($\delta^{15}N$) abundance in plant tissue is hypothesised to be affected by changes in assimilation activity when salinity was imposed on barley plants (HANDLEY *et al.,* 1997; ROBINSON *et al.,* 2000). Furthermore, HANDLEY *et al.* (1997) speculated $\delta^{15}N$ patterns might be produced under natural conditions for plants with slower growth habits and that the patterns would overlay variations in source (soil) $\delta^{15}N$.

The objective of this study is to screen for Green Box seedlings using characteristic morphological and physiological traits and AFLP DNA markers.

Methods and Materials

Location

The Chowilla floodplain is centred on the South Australia-Victoria-New South Wales Borders (140°52'E 33°59'S) covering approximately 200 km^2 (JARWAL *et al.,* 1996). It is the largest floodplain forest on the lower Murray River (KINGSFORD, 2000) and is part of 'Riverland Wetlands' listed under the UNESCO Ramsar Convention (Section 14.5) as wetlands of international importance because of unique bird-life and woodlands of *E. camaldulensis* and *E. largiflorens* (NEC, 1988). The area is located away from the moderating influence of the ocean and experiences generally clear skies allowing free heat exchange. Yearly rainfall is low, averaging 250–300 mm, and potential evaporation is about 2000 mm (JARWAL *et al.,* 1996).

Sites and Trees

The Chowilla floodplain is located within the Riverland Biosphere Reserve (RBR). Throughout RBR, 66

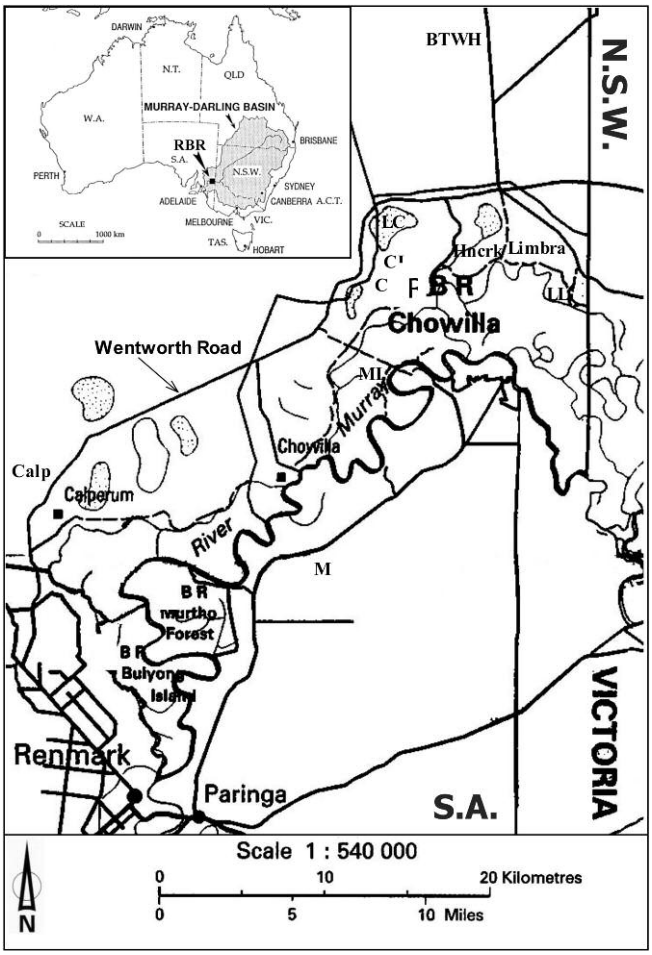

Figure 1. – Local-area sites within the Riverland Biosphere Reserve (RBR). Inset shows location of RBR. The table below shows abbreviations for sites and their GPS coordinates.

Site	Label	GPS	Site	Label	GPS
Coombool	C	S 33^0 55' E 140^0 54'	Hancock Creek	Hncrk	S 33^0 55' E 140^0 55'
Coombool Inlet	CI	S 33^0 55' E 140^0 54'	Box Tree Waterhole	BTWH	S 33^0 50' E 140^0 56'
Monoman Island	MI	S 33^0 57' E 140^0 52'	Calperum	Calp	S 34^0 02' E 140^0 40'
Lake Coombool	LC	S 33^0 55' E 140^0 54'	Limbra	Limbra	S 33^0 53' E 140^0 58'
Lake Littra	LL	S 33^0 55' E 140^0 59'	Murtho	M	S 34^0 03' E 140^0 51'

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adult trees were sampled from 'local-area' sites (23 *E. largiflorens*, 16 Green Box, 18 progeny of Green Box and 9 *E. gracilis*). The sites Coombool (C), Coombool Inlet (CI), Monoman Island (MI), Lake Coombool (LC) and Lake Littra (LL) were chosen because Green Box naturally occurs there. At Hancock Creek (Hncrk), there were approximately 50 seedlings each of Green Box (Hancock creek progeny) and *E. largiflorens* planted in 1991. The *E. largiflorens* seedlings were purchased from Wand F Nursery at Berri (J.V. Seekamp, pers. comm.) and their seed source is unknown. The sources of the Green Box seedlings were mature Green Box plants located on the floodplain. They were also the source of approximately 700 clonal Green Box plantlets planted in 1994. The clones were produced by tissue culture carried out by Dr T.C. Lee, Adelaide Botanic Gardens (J.V. Seekamp, pers. comm.). From each of these three types of revegetation plantings, five plants growing under similar soil and environmental conditions were chosen. In addition, two nearby mature *E. largiflorens* plants and one mature Green Box plant were chosen. Box Tree Waterhole (BTWH) was chosen because it is an isolated population of Green Box and *E. largiflorens* surrounded by *E. gracilis* mallee scrub. All three taxa were available for comparison within approximately 100 m^2 and were thus growing under similar soil and environmental conditions. There were four Green Box plants and the four closest *E. largiflorens* and *E. gracilis* plants were chosen for comparison. Calperum (Calp) and Limbra were chosen because they were stands of *E. gracilis* located close to the floodplain. A further site, Murtho (M), was chosen because there was a mature *E. largiflorens* and a mature *E. gracilis* with several younger trees, greater than 10 years old (Murtho progeny), occurring on the edge of a field and isolated from other trees. The locations of local-area sites are shown in *Fig. 1*.

In addition, 10 trees of *E. largiflorens* and *E. gracilis* were sampled from allopatric populations using the Australian National Herbarium web site (http://www.anbg.gov.au/cpbr/anhsir/anhsir-manual/index.html, accessed 3rd October 2012). *E. largiflorens* were sampled from Wilcannia approximately 450 km NE of the local-area, and *E. gracilis* were sampled from Port Lincoln, approximately 450 km SW of the local-area.

Morphological Traits

From each tree, colour and gloss of 25 leaves was recorded from small branches while fresh. Leaf colour was recorded using a Royal Horticultural Society (R.H.S. London) colour chart produced in conjunction with the Flower Council of Holland (Leiden). Each tree was assigned to one of six colour categories ranging from Yellow Green through Green to Grey Green. Leaf gloss was recorded as Dull, Intermediate or Shiny. Rough bark extends to the stem tips in *E. largiflorens* whereas in *E. gracilis* it terminates above the butt and is intermediate in Green Box. Therefore, the diameters of the main trunks/stems at the point where rough bark (Rb_d) terminated were measured for all plants as a distinguishing trait.

Physiological Traits

From each tree four healthy mature leaves located at the fourth node below the stem tip were sampled and kept fresh for measurement of leaf area with a portable area meter (Model LI-3000 Li-Cor, U.S.A). Specific leaf area (SLA) was calculated from the ratio of leaf area to dry leaf weight ($cm^2\ g^{-1}$). Leaves were then dried and ground to fine powder consistency for determination of carbon and nitrogen isotope discrimination by mass spectrometry (Model GEO 20 –20 Dual Inlet, Europa Scientific Ltd., England). Sample weights required for

Table 1. – Nucleotide sequences for adaptors, preamplification primers and selective primers. The reverse of the core primer sequences minus the last nucleotide, corresponds to the reverse adaptor sequences (5'→3'). Sequences for EcoRI are according to (Vos *et al.,* 1995) except for A in brackets was a C in the current study.

Label		Sequence (5'→3')
	Adaptor sequences	
*Eco*RI	Forward	CTC GTA GAC TGC GTA CC
	Reverse	AAT TGG TAC GCA GT(A) TAC
*Mse*I	Forward	GAC GAT GAG TCC TGA G
	Reverse	TAC TCA GGA CTC AT
*Pst*I	Forward	CTC GTA GAC TGC GTA CAT GCA
	Reverse	TGT ACG CAG TCT AC
	Core primer sequences	
*Eco*RI		GAC TGC GTA CCA ATT C
*Mse*I		GAT GAG TCC TGA GTA A
*Pst*I		GAC TGC GTA CAT GCA G

PCR primer sequences (core primer · nucleotide(s))	
Pre amplification PCR	Selective PCR
*Eco*RI core primer + A	*Eco*RI core primer – ACT
	*Eco*RI core primer – ATG
*Mse*I core primer + C	*Mse*I core primer + CCC
	*Mse*I core primer + CAG
	*Mse*I core primer + CCA
*Pst*I core primer – A	*Pst*I core primer + AG

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accurate measurements, determined from preliminary runs, were 1.5 mg and 4 mg for carbon and nitrogen respectively. Carbon isotope deviations ($\delta^{13}C$) were measured against 'Vienna'-Pee Dee Belemnite (PDB) (DAWSON and BROOKS, 2001). Nitrogen isotope deviations ($\delta^{15}N$) were measured against the standards N1, N2 and N3. These standards are relative to atmospheric N_2 according to the International Atomic Energy Agencies (IAEA) standards (DAWSON and BROOKS, 2001). Precision for nitrogen and carbon was quoted as $\leq \pm 0.2‰$ and $\leq \pm 0.1‰$ respectively (Europa Scientific Ltd.). Carbon deviation (δ) was converted to standard discrimination (Δ) values using the equation:

$$\delta = ((\delta_a/1000) - (\delta_p/1000)) / (1 + (\delta_p/1000))$$

Units: ‰ (per mil)

Where δ_a is free atmospheric CO_2 on the PDB scale and has an approximate value of –8‰.

δ_p is CO_2 of plant material, typical C_3 plant on the PDB scale has a value of –27.6‰ (FARQUHAR *et al.,* 1989). Nitrogen is commonly expressed as deviation units in per mil (‰) (HÖGBERG, 1997). Carbon and nitrogen discrimination was measured from adult ($\Delta^{13}C_{ad}$ and $\delta^{15}N_{ad}$) and juvenile ($\Delta^{13}C_j$ and $\delta^{15}N_j$) leaves. Mass spectrometry also provides total carbon and nitrogen of adult (TC_{ad} and TN_{ad}) and juvenile leaves (TC_j and TN_j).

Extraction of DNA

Mature leaves at the fourth node with minimal blemishes or signs of insect attack were chosen to avoid red pigments (anthocyanins) and tannins present in younger leaves (SKABO *et al.,* 1998); (Dr. R.E Vaillancourt and Dr. B. M. Potts, University of Tasmania, Australia, Pers. Com.). Leaves were picked into 50 mL centrifuge tubes (Sarstedt Australia Pty. Ltd.) and placed into liquid nitrogen then stored at –80 °C upon return from the field. DNA was extracted according to standard procedures (BYRNE *et al.,* 1996; BYRNE *et al.,* 1993). Some modification to the procedures are derived from DOYLE (1991) and DOYLE and DOYLE (1990) to account for high concentrations of hydrophobic compounds in *Eucalyptus* leaves.

AFLP Analysis

AFLP was carried out using protocols derived from VOS *et al.* (1995) and PARKER (1998). The only modification was addition of 2 µL RL DNA instead of 4 µL to improve clarity of marker banding profiles. A total of 9 primer combinations were screened and markers ranged from 50 to 600 bp (*Table 1*). *Eco*RI/*Mse*I primer sequences were chosen according to published studies involving *Eucalyptus* species (GAIOTTO *et al.,* 1997; MARQUES *et al.,* 1998). *Pst*I/*Mse*I primer sequences were chosen based on recommendations by G. Parker and the fact that *Pst*I is highly methylation sensitive to target coding regions (POWELL *et al.,* 1997; YOUNG *et al.,* 1999). *Eco*RI or *Pst*I selective primers were radioactively labelled with $^{33}P\gamma ATP$. PCR products were visualised on 6% polyacrylamide gels (19:1) [40mL SequaGel-6, 10 mL SequaGel Complete Buffer and 500 µL 10% APS] cast between 35 × 43 cm glass plates for running in a Heofer® SQ3 Sequencer apparatus. Marker ladder was radioactively labelled pUC19 *Msp*I. Vacuum dried gels were exposed onto Kodak Diagnostic Film (35 × 43 cm) X-Omat™ K XK-1 for 3 days then developed in a KODAK X-OMAT 1000 Processor, KODAK (Australasia) Pty. Ltd. AFLP marker presence or absence data was collected by blind sampling conducted by two volunteers with no knowledge of sample identities.

Data Analysis

All data analysis was carried out in SPSS (IBM SPSS Statistics 19 USA). All data was checked for normality and homogeneity of variance by carrying out Levene's test and transformations were applied if necessary. To examine associations between physiological and morphological traits with AFLP markers, two statistical approaches were undertaken: non-linear Canonical Correlation Analysis (OVERALS) and linear regression with randomization (HANCOCK, 2002).

OVERALS is similar to Principal Component Analysis (PCA), however two or more sets of variables with cases (trees) in common are correlated (CONNOLLY, 1997). OVERALS is suitable for scale traits such as physiological traits and discrete traits such as genotypes of molecular markers. In this study, the two sets are traits (morphological and physiological) and AFLP markers. OVERALS produces "dimensions" maximally correlating the sets. Eigenvalues from OVERALS can be interpreted as r^2 (fit) and their square root, canonical correlations, can be interpreted as Pearson's r, with values greater than 0.30 explaining acceptable variance (GARSON, 2002). Output includes "component loadings" (l_c) for interpreting correlations between variables and each dimension. Plotting 1st and 2nd dimensions, using object scores (trees, cases) and component loadings (variables) produces a diagrammatic representation of the relationship between traits and AFLP markers. Variables positioned further from the origin contribute more to variation between trees. Type II error or underestimation was minimized by including at least 20 times as many cases as variables for interpreting the 1st dimension reliably (STEVENS, 1986).

Two OVERALS analyses were conducted with the same morphological and physiological traits. Firstly, OVERALS including all 232 AFLP markers proceeded by nominating 10 sets of variables to ensure number of cases (trees) exceeded number of variables. The first set comprised morphological and physiological traits and the remaining 9 were AFLP markers split by primer combination. Accompanying MANOVAs (Multivariate Analysis of Variance) were conducted and Wilks's lambda tested whether the variable sets were significantly correlated (GARSON, 2002). Categorical traits were treated as unordered variables (multiple nominal in SPSS). The second OVERALS was of trees from the local-area with 11 AFLP markers identified within the local-area that were at least 78% specific for either *E. largiflorens* (5 markers) or *E. gracilis* (6 markers).

The second statistical approach of linear regression would be straightforward if only one marker was used with either its presence or absence scored. The observed variance ratio would be tested with a critical value associated with a significance level of 5% (0.05). The difficul-

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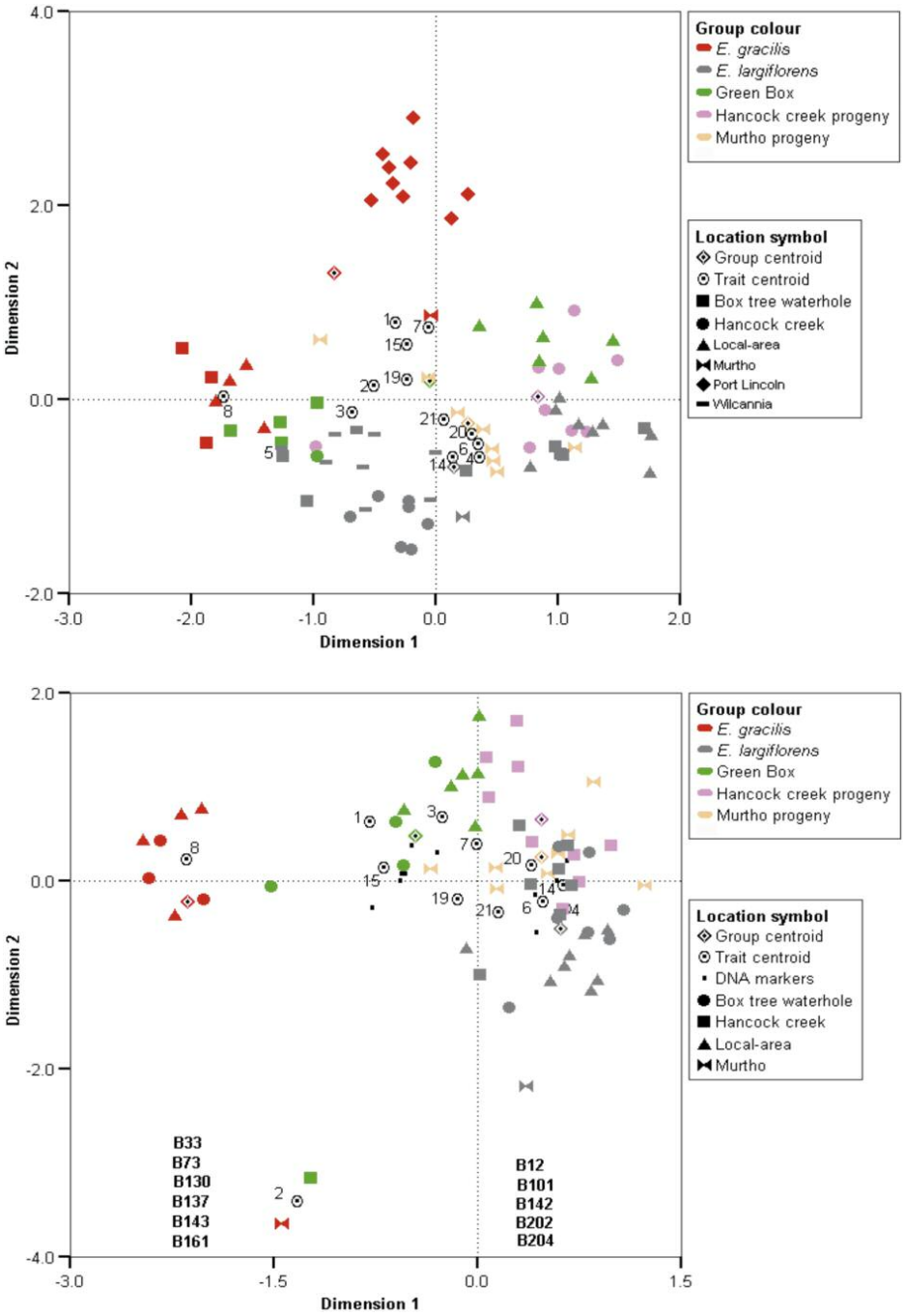

Figure 2. – OVERALS analyses of: a, All 232 AFLP DNA markers and b, eleven at least 78% specific markers within the local-area associated with morphological and physiological traits. First and 2nd dimensions in a, account for 80% and 74% of the total variance respectively, in b, account for 95% and 89% of the total variance respectively. Box Tree Waterhole, Hancock Creek and Murtho are within the local-area and their different symbols are meant to distinguish them from the remaining plants within the local-area. Each of the trait/trait state centroids are labelled with numbers defined in the table below.

Label	Morphological traits/trait states		Label	Physiological traits
1	Colour1	Yellow Green	19	$\Delta^{13}C_{ad}$
2	Colour2	Yellow Green and Green	20	TN_{ad}
3	Colour3	Green	21	SLA
4	Colour5	Grey Green		
5	Colour6	Grey Green and Yellow Green		
6	Gloss1	Dull		
7	Gloss2	Intermediate		
8	Gloss3	Shiny		
14	Leaf Area			
15	Rb_d, Diameter at rough bark height			

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ty in this study is that there are more than 200 markers and repeatedly applying this test greatly increases the chance of type I error (rejecting the null hypothesis, when it is in fact true). To arrive at an alternative critical value that correctly has a significance level of 5%, a randomization study was performed where response variables were randomised and the maximum variance ratio identified, thus ensuring the null hypothesis was in fact true. Randomisation was repeated 1000 times to produce the distribution of the variance ratio under the null hypothesis. This distribution is then used to identify the 95th percentile, or critical value for testing each of the 200 excess repeated analyses against the observed value of the response variables – i.e. morphological and physiological traits (HANCOCK, 2002).

Results

OVERALS with all AFLP DNA markers

OVERALS of morphological and physiological traits with all 232 AFLP DNA markers produced 1st and 2nd dimensions accounting for 80 and 74% of variation respectively (*Fig. 2a*). Trait centroids occurring in the vicinity of the Green Box centroid (–0.0445, 0.191) reinforced their intermediacy between *E. gracilis* and *E. largiflorens*. Green Box leaf colour centroid yellow green (–0.330, 0.794) and intermediate gloss (–0.059, 0.746). Other trait centroids reinforcing the intermedicy of Green Box were Rb_d (–0.239, 0.578), $\Delta^{13}C_{ad}$ (–0.241, 0.204) and SLA (0.066, –0.207). Trait centroids characterizing *E. gracilis* (centroid –0.834, 1.303) were grey green and yellow green leaf colour (–1.252, –0.488) and shiny gloss (–1.733, 0.037). Trait centroids characterizing *E. largiflorens* (centroid 0.1511, –0.685) were grey green leaf colour (0.354, –0.587), dull gloss (0.350, –0.448), leaf area (0.135, –0.585) and TN_{ad} (0.294, –0.357).

There were several component loadings (l_c) for traits and AFLP markers greater than 0.3 therefore strong enough to separate groups. In the 1st dimension, leaf colour and gloss contributed most with l_c of 0.340 and 0.615 respectively. TN_{ad} was not quite as strong, 0.294, and $\Delta^{13}C_{ad}$, SLA, leaf area and Rb_d did not contribute much with l_c of –0.241, 0.066, 0.135 and –0.239 respec-

Table 2. – AFLP markers significantly associated with traits (response variables) from both OVERALS and linear regression. * indicates *E. gracilis* 78% specific markers and ^ indicates 78% specific markers for *E. largiflorens*. For variables: $\delta^{15}N_{ad}$, TN_{ad}, Rb_d and leaf colour; $n = 78$: 17 + 33 + 11 + 9 + 8 *E. gracilis*, *E. largiflorens* and Green Box, Hncrk progeny and Murtho progeny respectively. For $\Delta^{13}C_j$; $n = 43$: 6 + 15 + 5 + 9 + 8 *E. gracilis*, *E. largiflorens* and Green Box, Hncrk progeny and Murtho progeny respectively. Italics indicate markers identified by OVERALS and linear regression but not by MANOVA.

Response variable	Critical value	Number of significant markers ($P < 0.05$)	Markers identified	Markers identified with MANOVA (P)
$\delta^{15}N_{ad}$	15.8	2	B58, B121	B42(0.003), B58(0.001), B71(0.003), B109(0.004), B121(0.006), B134(0.000), B184(0.003), B196(0.005), B208(0.002), B215(0.005)
$\Delta^{13}C_{ad}$	15.5	0		
TN_{ad}	14.6	2	B95, B137*	B95(0.000), B137*(0.001)
SLA	16.0	0		
$\Delta^{13}C_j$	16.6	1	B224	B202^(0.000), B205(0.000) B224(0.000)
Rb_d	14.9	10	B33*, B78, B85, B137*, B143*, B144, B161*, B162, B202^, B204^	B30(0.020), B33*(0.024), B42(0.031), B67(0.001), B73*(0.007), B75(0.008), *B78(0.052)*, B85(0.011), B89(0.033), B128(0.003), B136(0.030), B137*(0.018), B138(0.032), B143*(0.007), *B144(0.274)*, B161*(0.025), B162*(0.001), B182(0.002), B193(0.027), B202^(0.037), *B204^(0.083)*, B207(0.033), B218(0.002)
Colour Categories	14.8	8	B33*, B66, B142^, B143*, B144, B161*, B170, B204^	*B33*(0.803)*, B57(0.011), *B66(0.309)*, B67(0.001), B88(0.002), B89(0.011), B92(0.007), B115(0.008), B128(0.002), B142^(0.003), *B143*(0.622), B144(0.285), B161(0.059)*, B170(0.013), B182(0.001), B194(0.005), *B204(0.128)*

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Table 3. – Quantifications of physiological and morphological traits associated with the presence and absence of AFLP markers identified from linear regression. Plants from local-area and outside-local area populations were included. The frequency of marker present in each of the plant groups is also shown. [*n*]. * indicates *E. gracilis* 78% specific markers and ^ indicates 78% specific markers for *E. largiflorens* plants identified from local area markers.

Trait	Marker	Trait quantification (± 1 SEM)		Frequency of marker presence				
		Absence	Presence	*E. gracilis* [18]	*E. largiflorens* [33]	Green Box [11]	Hnerk progeny [9]	Murtho progeny [8]
$\delta^{15}N_{ad}$	B58	4.241(0.2904)	2.067(0.3992)	0.78	0.24	0.18	0.22	0.25
(‰)	B121	1.440(0.5642)	3.937(0.2639)	0.44	0.94	1.00	0.78	0.88
TN_{ad}	B95	14.00(0.5083)	11.68(0.3287)	0.67	0.73	0.82	0.56	0.75
($mg.g^{-1}$)	B137*	13.48(0.3654)	11.06(0.3947)	1.00	0.39	0.46	0.00	0.13
$\Delta^{13}C_i$	B224	17.94(0.4164)	15.47(0.2142)	0.67	0.85	0.82	100	0.88
(‰)								
Rb_d	B33*	2.795(1.1730)	11.145(1.2827)	0.72	0.12	0.18	0.11	0.38
(cm)	B85	6.987(1.1983)	2.121(1.2315)	0.17	0.73	0.36	0.11	0.25
	B78	3.0771(1.1730)	11.145(1.2827)	0.39	0.06	0.55	0.22	0.13
	B137*	2.425(1.2068)	7.768(1.2218)	1.00	0.39	0.46	0.00	0.13
	B143*	2.221(1.1842)	9.670(1.2147)	0.94	0.18	0.55	0.00	0.63
	B144	8.855(1.2688)	2.832(1.1872)	0.28	0.85	0.46	1.00	0.63
	B161*	2.347(1.1748)	10.740(1.2286)	0.83	0.03	0.55	0.56	0.38
	B162	3.219(1.1672)	12.794(1.3763)	0.39	0.06	0.09	0.00	0.63
	B202^	7.941(1.2348)	2.578(1.2012)	0.11	0.73	0.55	0.67	0.88
	B204^	10.475(1.2619)	2.578(1.2012)	0.06	0.91	0.82	0.67	0.88
Leaf	B33*	3.750(0.2401)	2.000(0.3746)	0.72	0.12	0.18	0.11	0.38
colour	B66	3.955(0.2704)	2.343(0.3032)	0.72	0.21	0.73	0.33	0.50
	B142^	2.125(0.3065)	4.000(0.2529)	0.28	0.88	0.36	0.44	0.63
	B143*	4.067(0.2560)	2.147(0.2945)	0.94	0.18	0.55	0.00	0.63
	B144	2.111(0.3441)	3.827(0.2479)	0.28	0.85	0.46	1.00	0.63
	B161*	4.061(0.2368)	1.900(0.3026)	0.83	0.03	0.55	0.56	0.38
	B170	3.615(0.2221)	1.500(0.4786)	0.33	0.00	0.55	0.22	0.00
	B204^	1.885(0.3369)	3.906(0.2359)	0.06	0.91	0.82	0.67	0.88

tively. Of AFLP markers, 70 out of 232 had l_c greater than 0.3, thirty of these could be used to predict leaf colour and ten could be used to predict leaf gloss, as their l_c occurred with similar magnitudes and in the same direction.

Analysis with MANOVA revealed 8 out of the 9 AFLP primer combinations significantly correlated with traits, reinforcing that they were a good choice. Significance values were: 0.001 and 0.033 for *Eco*ACT/*Mse*CAG and *Eco*ACT/*Mse*CCA respectively, 0.000 for each of the *Pst*AG primer combinations then, 0.000, 0.003 and 0.031 for each of the *Eco*ATG primer combinations respec - tively.

OVERALS with 78% specific AFLP DNA markers

OVERALS of morphological and physiological traits with 11 AFLP DNA markers that were 78% specific for either *E. gracilis* or *E. largiflorens* produced 1st and 2nd dimensions of 95 and 89% suggesting variance in traits (response variables) was adequately predicted (*Fig. 2b*). There was clear separation of plants into groups with Green Box intermediate. MANOVA confirmed that all 11 AFLP markers significantly correlated with traits in the 1st dimension (Wilks's lambda, $P = 0.000$) and all had l_c greater than 0.3 indicating they adequately predicted variation in traits. Markers, B101 and B137 contributed most with l_c of 0.658 and –0.772 respectively. Leaf area and colour in the 1st dimension with l_c of 0.629 and 0.641 respectively, loaded similarly to B101, indicating its presence (an *E. largiflorens* 78% specific marker) can predict variation in leaf area and colour. Conversely, variation in Rb_d with a negative l_c of –0.692, can be predicted by presence of B137, also with a negative l_c of –0.772. Physiological traits did not contribute greatly with the exception of TN_{ad} (0.395). Markers behaving similarly for predictive purposes were B12 (0.426) and B142 (0.437).

Simple Linear Regression and Comparison with MANOVA of OVERALS component loadings

Simple linear regression identified 17 AFLP DNA markers shown in *Table 2*. There was agreement between linear regression and MANOVA of OVERALS l_c for markers B58 and B121 significantly associated with $\delta^{15}N_{ad}$ (*Table 2*). The presence of B58 is associated with low $\delta^{15}N_{ad}$, indicative of *E. gracilis* while its absence is associated with higher $\delta^{15}N_{ad}$, indicative of *E. largiflorens* (*Table 3*). The reverse situation applied to B121,

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with its presence associated with high $\delta^{15}N_{ad}$, indicative of *E. largiflorens*. Both linear regression and MANOVA of OVERALS l_c identified two markers associating with TN_{ad}: B95 and B137 (*Table 2*). The presence of both markers is indicative of lower concentrations of nitrogen (*Table 3*). Marker B224 associated with $\Delta^{13}C_j$ according to linear regression and MANOVA of OVERALS l_c. There were 7 markers identified from linear regression and MANOVA of OVERALS l_c for Rb_d (*Table 2*) and their quantifications with either presence or absence are displayed in *Table 3*. Linear regression identified 8 AFLP markers significantly associated with leaf colour and two of these were also identified by MANOVA of OVERALS l_c (*Table 2*). Their corresponding quantifications are shown in *Table 3*.

Markers for selecting Green Box

Of the 13 AFLP markers significant for both linear regression and MANOVA of OVERALS l_c, five had high frequencies (>0.82) in Green Box: B95, B204, B224 and B121 (*Table 3*). The presence of B95 is associated with lower TN_{ad} indicative of Green Box and *E. largiflorens* as *E. gracilis* had higher TN_{ad}. The presence of B204 was associated with both Rb_d and the grey green colour category, occurring in 91% of *E. largiflorens*, 82% of Green Box and only 6% of *E. gracilis* (*Table 3*). The presence of B224 was associated with low $\Delta^{13}C_j$ having highest frequency in *E. largiflorens* (85%), lower frequency in Green Box (82%) and lowest frequency in *E. gracilis* (67%). Lastly, the presence of B121 is associated with higher $\delta^{15}N_{ad}$ indicative of Green Box and *E. largiflorens* (100 and 94%) whereas *E. gracilis* displayed lower $\delta^{15}N_{ad}$ (44%).

Discussion

In this study, none of the AFLP DNA markers were 100% diagnostic for either species. Instead, analyses were conducted using all AFLP markers and markers that were at least 78% specific for *E. largiflorens* (6 markers) and *E. gracilis* (5 markers). Justification for using DNA markers that were not diagnostic is provided by GOODMAN *et al.* (1999) who stated, "Generally, genetic markers should never be treated as absolutely diagnostic because it is impossible to determine the effects of genetic heterogeneity or ancient introgression between sympatric species.

Of the 11 78% specific DNA markers, one occurred beyond the local-area in 8 out of 10 Port Lincoln *E. gracilis* (B143) and another one in 9 out of 10 Wilcannia *E. largiflorens* (B204). Both were also present in the majority of Green Box therefore supporting their assignment as putative hybrids. This study recommends screening for more AFLP markers with high specificity for *E. gracilis* and *E. largiflorens* with the ultimate goal of encompassing natural variation extending from local-area hybrid zones to allopatric populations. Working outwards from a hybrid zone fulfils the requirement of no past introgression between putative parental species within the hybrid zone, as their specificity will have been tested against allopatric populations (TRIEST *et al.,* 2000).

The alternative approach of linear regression was effective at singling out 17 AFLP DNA markers significantly associated with physiological and morphological traits. The remaining 215 paled into comparison indicated by much lower critical values. Linear regression has been applied elsewhere to identify markers for prediction of response variables (traits). Twelve AFLP DNA markers were associated with shoot Na^+, $\delta^{13}C$ and site-of-origin of ecogeographic data measured on 39 genotypes of wild barley (*Hordeum spontaneum*) (PAKNIYAT *et al.,* 1997).

The frequency of AFLP markers was highest in either *E. gracilis* or *E. largiflorens* with Green Box displaying mostly varying frequencies. There were four markers high in frequency in Green Box and *E. largiflorens* and traits significantly associated with these markers were TN_{ad} (B95), $\delta^{15}N_{ad}$ (B121), Rb_d and leaf colour (B204) and $\Delta^{13}C_j$ (B224). These markers occurred with much lower frequencies in *E. gracilis* and, therefore, can be utilized to select for Green Box attributes over *E. gracilis* attributes. However, because these 4 markers occurred with similar frequencies in Green Box and *E. largiflorens*, they cannot be utilized to select for Green Box attributes over *E. largiflorens* attributes. To overcome this limitation, similar presence/absence of additional markers is necessary to align Green Box with *E. gracilis*. One such marker is B66 occurring with high frequencies in *E. gracilis* and Green Box and low frequency in *E. largiflorens* (*Table 3*). In addition the absence of markers B85 and B142 in both Green Box and *E. gracilis* distinguishes them from *E. largiflorens*. Therefore, the segregation of 7 markers can be utilized to select for Green Box. It is important to qualify that there would be other attributes of Green Box not identified in this study. More salt tolerance traits could be foliar concentrations of Cl^- and ratio of sub-stomatal to ambient CO_2 concentration.

It is interesting that the physiological traits identified for Green Box correspond to reduced growth potential (LAMBERS *et al.,* 2008; POORTER and EVANS, 1998). An exception was that SLA was not identified as significantly associating with Green Box or AFLP markers. The reason why is because it behaved in an intermediate manner, whereas the 7 markers identified above correspond to extremes of traits aligning Green Box with either *E. largiflorens* or *E. gracilis*. The presence of B95, B204, B224 and B121 were associated with lower TN_{ad}, smaller Rb_d, the grey green colour category, lower $\Delta^{13}C_j$ and higher $\delta^{15}N_{ad}$ in Green Box and *E. largiflorens.* Whereas, the presence of B66 and B142 was associated with low frequencies of the grey green colour category, and the presence of B85 was associated with low frequency of smaller Rb_d in Green Box and *E. gracilis* (*Table 3*).

In conclusion, we have found molecular markers that correlate with physiological and morphological traits measured in *E. largiflorens*, Green Box and *E. gracilis*. The majority of these traits correspond to reduced growth potential. Green Box were hypothesized by ZUBRINICH (1996) to grow at slower growth rates because *E. largiflorens* had significantly higher total plant and shoot biomass. Morphological comparisons between

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E. gracilis, Green Box and *E. largiflorens* under saline conditions, indicated *E. gracilis* was slower growing at the seedling stage and maybe Green Box have inherited this quality. The seven AFLP markers identified can be used to select Green Box increasing the efficiency of revegetation strategies by selecting for favourable seedlings and thus limiting the number of seedlings that are throw backs to *E. gracilis* and *E. largiflorens*.

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