

Effect of genetic relatedness among parents on gain in salt tolerance in progeny of crosses of *Eucalyptus occidentalis*

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Abstract

Genetic diversity of a *Eucalyptus occidentalis* breeding population screened for salt and waterlogging tolerance was examined using eight microsatellite loci. Mating using an immature style 'one stop pollination' method between parents was carried out to produce progeny for testing under 500 mM salt waterlogging. The effect of parental genetic distance on seed production and early seedling survival was examined and inheritance of salt/or waterlogging tolerance was assessed by testing performance of progeny in comparison to parents. Diversity was moderate among the nine provenances and the families, and most of the diversity was distributed within rather than between families. Genetic relationships showed no structure in relation to provenance indicating any adaptation to saline environments has not affected genetic similarity. Breeding for salt and water tolerance may be achieved without decline in genetic diversity. There was a significant correlation between capsule production and parental genetic distance and a positive trend between increasing parental genetic distance and increasing number of germinated seeds/capsule, and seedling survival at 2 weeks and 9 months. These trends indicate expression of inbreeding depression in crosses between genetically similar parents. Heritability values, under 500 mM salt-waterlogging treatment, indicated that height had moderate heritability ($h^2 = 0.5$). Crosses with the widest parental genetic distance produced progeny with considerable height increase above parents and this trend was evident even with moderate genetic distance indicating crossing at this level of differentiation may achieve optimum breeding gain.

Key words: eucalypts, salt tolerance, genetic diversity, flat topped yate, microsatellite, inbreeding.

Introduction

Breeding involves selection of individuals with superior performance followed by mating between superior plants to achieve further genetic gain. Genetic diversity of the base population is a fundamental basis for breeding and improvement programs since selection of superior genotypes for tolerance, growth or other traits may

erode genetic variation in the breeding population (ELDRIDGE et al., 1994). Limited diversity in the base population increases the likelihood of producing closely related or inbred progeny with reduced fitness.

Generally there is an optimum genetic distance for mating at which maximum genetic gain is obtained. Breeding between closely related plants commonly results in expression of inbreeding depression caused by combining deleterious recessive genes in the homozygous state, leading to reduced fitness. Conversely, breeding between widely differentiated individuals can lead to outbreeding depression due to disruption of local adaptation, under-dominance or epistatic interactions, and the breakdown of co-adapted gene complexes (EDMANDS, 2007). Inbreeding depression is commonly expressed in forest trees as they tend to have highly outcrossed mating systems. In *Eucalyptus* this outcrossed mating system masks the effects of post zygotic lethal systems that eliminate the effects of selfing in natural populations. In eucalypt breeding programs inbreeding depression has been shown to have significant effects on seed production and on progeny growth performance (GRIFFIN and COTTERIL, 1988; HARDNER and TIBBITS, 1998), therefore breeding is best carried out between genetically diverse parents. Generally in eucalypt breeding, there is little or no artificial mating as it is very costly. However, controlled pollination between superior individuals is an effective way to combine the best alleles in future generations and maximise genetic gain through breeding (MONCUR, 1995; HARBARD et al., 1999).

Eucalyptus occidentalis Endl. (flat-topped yate), is a tall timber species reaching 10–20 m in height with diameter at breast height up to 0.5 m (FAO, 1981; MARCAR et al., 1995). The timber is suitable for building poles, pilings, posts, heavy construction and for pulp (CLARK et al., 1999; HARWOOD, 2001; MARCAR and CRAWFORD, 2004). It has a narrow natural distribution in southern wheatbelt and subcoastal areas of Western Australia at an elevation of 50–300 m and occupies a wide variety of habitats, from riparian areas, including swamps and salt lakes, to xeric landscapes (CHIPPENDALE, 1973; BROOKER and KLEINIG, 1990). This species has moderate to high tolerance to salt and waterlogging (PEPPER and CRAIG, 1986; MARCAR et al., 1995, 2003; BENYON et al., 1999) and there is interest in planting *E. occidentalis* for rehabilitation and reclamation of saline areas especially for agricultural land suffering from waterlogging and increasing dryland salinity. *Eucalyptus occidentalis* was selected as one of six species for tree improvement by the Australian Low Rainfall Tree Improvement Group (HARWOOD et al., 2001). Thus, genetic improvement of salt tolerance in

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E. occidentalis would facilitate its deployment as a suitable plant for management of saline agricultural land in southern Australia.

Previous screening of an *E. occidentalis* breeding population of 30 families from nine provenances for salt and/or waterlogging tolerance (up to 550 mM NaCl) resulted in differential response for growth and survival (HENDRATI, 2008). Breeding among these families will be undertaken and this may be facilitated by knowledge of genetic diversity within the provenances and families to avoid the possibility of inbreeding depression in the future. Analysis of genetic diversity in natural populations of *E. occidentalis* using RFLP markers indicated a moderate level of genetic diversity with no evidence of inbreeding and little differentiation among populations (ELLIOTT and BYRNE, 2003). Microsatellite markers detect high levels of diversity in eucalypts (BYRNE, 2008) and are thus useful for assessing genetic diversity between parents in a breeding population.

The objective of this study was to assess genetic diversity among 30 families selected from nine provenances in *E. occidentalis* and to examine the heritability and effect of genetic distance between parental individuals on the performance of progeny under salt/waterlogged conditions. Controlled crossing with the immature style 'one stop pollination' method was used to produce progeny from selected *E. occidentalis* parents for evaluation of performance under salt/waterlogging conditions. The effect of parental genetic distance, assessed using microsatellites, on seed production and early seedling survival was examined and inheritance of salt waterlogging tolerance was assessed by testing performance of progeny in comparison to parents.

Materials and Methods

Plant material

A previous salt and waterlogging screening trial where salt concentration was increased to sea salt level (550 mM), held for 2 weeks and slowly reduced to 0 mM salt over 4 weeks resulted in survival of 310 seedling individuals of 30 families from nine provenances of *E. occidentalis* (Beaufort River, Gordon, Grass Patch, Katanning, Lake Magenta, Red Lake Siding, Stirling Range, Truslove and Young River). For this study, these plants were potted out and assessed for genetic diversity. Plants that flowered at 22 months were used as parents in controlled crosses in low x low, high x low and high x high salt tolerance combinations to produce progeny that were then screened for salt waterlogging tolerance. However, very few individuals survived within low x low crosses so these were not assessed further.

Genetic analysis

Genomic DNA was extracted from leaves of the plants following a CTAB protocol for large scale DNA extraction (BYRNE et al. 1998). DNA concentration was assessed using a low range assay solution (10–500 ng/μL) in a Fluorometer DyNA Quantum 200 (Pharmacia Biotech, Hoefer, San Francisco, USA) and samples were diluted to 20 ng/μL. Nuclear microsatellite loci for

eucalypts (En 6, 10 and 16; EMBRA 2-19; EMCRC 1a, 6, 12; BYRNE et al. 1996; RONDANI et al. 1998; STEANE et al. 2001) were tested for amplification and polymorphism in the 310 selections. DNA template (30 ng) was amplified through PCR in a final volume of 15 μL, containing 50 mM KCl, 20mM Tris HCl pH 8.4, 0.2 mM each dNTP, 0.3 μM of each primer, 1.5 mM MgCl₂ and 0.5 units of *Taq* DNA polymerase (Life Technologies Invitrogen). PCR was performed in a Bio-Rad thermocycler (London, UK) with a denaturing step at 96°C for 2 min followed by 30 cycles of 95°C, 56°C, 72°C for 30 seconds at each step and one final cycle of 72°C for 5 min. PCR products were size-fractionated and visualized by fluorescence in an ABI 3730 automated capillary sequencer using Genescan-500 LIZ size standard.

Eight primers that revealed interpretable polymorphisms were selected for further analysis of *E. occidentalis* (EMBRA 3, 5, 8, 11, 17, En 6, EMCRC 6, 12). The number of alleles, and observed and expected heterozygosity (*Ho* and *He* respectively) were calculated for each provenance and family using Powermarker (LIU and MUSE, 2005). Relationships between geographic (altitude, longitude (°E)) or climatic variables (annual rainfall (mm)) and genetic diversity (*He*) were investigated using ordinary linear regression analysis of untransformed data. Genetic distances between individuals were determined according to GOLDSTEIN et al. (1995) using a stepwise mutation model as this is suitable for microsatellite data (LOWE et al. 2004). Relationships among individuals based on genetic distance were determined using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) using POWERMARKER v3.25.

Controlled crosses

The seedlings that survived the original salt/waterlogging treatment were transplanted into potting mix (Premium P/CM, Baileys Fertilizers, Perth) in 15 cm diameter pots. Complete element NPK Blue fertilizer at 0.5 gram/pot (N = 12%, P = 5.2%, K = 14.1%, S = 7%, Ca = 3.5%, Mg = 1.2%, Fe = 0.1%, Zn = 0.01% and Bo = 0.02%, Baileys, Western Australia) was added at 3 months to stimulate plant growth. Plants were arranged under a 16-h light/8-h dark regime. Families of seedlings were classified as high, medium or low salt tolerance based on the combined trait values from each individual (survival, height, relative growth and leaf number) in the original salt/waterlogging treatment. A total of 102 plants initiated flowers at 22 months old.

Controlled crosses were carried out for 45 combinations of high x high, high x low and low x low salt tolerance, with at least 3 individual flowers per plant per cross. Pollen was collected from flower buds (male parents) with yellowish and/or lifted, but attached opercula to ensure pollen was not contaminated. Anthers were trimmed, dried using silica gel and stored in sealed petri dishes at –20°C (0–15 days) for use when female parent flowers were ready for pollination. Pollen was thawed for 1 h before pollination. Pollination followed the 'one-stop pollination' technique of HARBARD et al. (1999). This method had not been used previously in *E. occidentalis*, but was expected to be suitable for maximizing the seed

Table 1. – Details of families of *Eucalyptus occidentalis* used to assess parental and progeny performance under controlled and salt-waterlogged (500 mM salt) conditions. Seedling progeny of five check-families and eight controlled crosses (A-H) were tested.

Treatment	Family or cross combination	Family of female parent	Family of male Parent	Salt tolerance of female	Salt tolerance of male	No. of seedlings
C	87-Beaufort	-	-			14
C	136-Grass P	-	-			7
C	149-Red Lake	-	-			15
C	151-Red Lake	-	-			15
C	153-Red Lake	-	-			13
C	A	36-Katanning	124-Red Lake	Low	High	10
C	B	96-L Magenta	20-Young	Low	High	10
C	C	110-Young	108-Young	High	High	3
SW	87-Beaufort	-	-			14
SW	136-Grass P	-	-			7
SW	149-Red Lake	-	-			15
SW	151-Red Lake	-	-			15
SW	153-Red Lake	-	-			13
SW	A	36-Katanning	124-Red Lake	Low	High	20
SW	B	96-L Magenta	20-Young	Low	High	20
SW	C	110-Young	108-Young	High	High	4
SW	D	20-Young	30-Katanning	High	Low	3
SW	E	20-Young	96-L Magenta	High	Low	4
SW	F	110-Young	150-Red Lake	High	Low	3
SW	G	96-L Magenta	76-Truslove	Low	High	5
SW	H	76-Truslove	124-Red Lake	High	High	5

C = control, SW = salt-waterlogging.

Note: Low x Low crosses did not produce surviving seedlings for tolerance testing.

output of the limited number of available flowers and parents to produce as many progeny seeds as possible.

Capsules were harvested at 9 months after pollination and air dried to extract seeds. Seeds were germinated and potted out for salt tolerance testing. Capsule production, seed production and seedling survival were calculated for each cross and correlated with parental genetic distance ordinary linear regression analysis of untransformed data.

Salt tolerance test

Progeny from controlled crosses (Table 1) were grown to 9.5 months then exposed to salt-waterlogged conditions. Progeny from three crosses (A, B and C) were included as controls and progeny from five 9.5-month old families used in the salt screening of the original parent trees were included as check-families to compare both testing environments and to adjust the progeny performance.

A total of 215 seedlings from the five control families and the progeny of eight crosses were randomly placed in two separate tanks (from Oct 2007 to Jan 2008), control (drained) and salt/waterlogging (treatment), with 3-5 replicates per tank, prior to addition of salt to allow for plants to acclimatize. The control tank pump was set for 30 min watering (3 x daily) to bring water levels up to 1 cm below the potting media, and then allowed to drain. In the other tank salt solutions were replaced weekly and salt concentration was increased in equal weekly increments (50 mM) up to 500 mM NaCl. The salt solution was at a ratio of NaCl:CaCl₂=14:1, to equate to natural calcium content (CRAIG et al., 1990;

VAN DER MOEZEL et al., 1991). The fertilizer used was a half concentration of complete hydroponics nutrients (Three Part Perfection, Wanneroo, WA).

Plant survival was recorded every week. Height of seedlings were measured in the second week (before salt was applied), at 5 weeks (250 mM salt) and at 11 weeks (500 mM salt). Relative growth was calculated between 1 week and 11 weeks (((height 11 at weeks- height at 1 week)/height at 1 week))+100%). All leaves were marked and counted to differentiate new from old leaves. Height data from the 5 check-families were correlated with data from the original experiment to assess similarity of environments and analyzed using two way-ANOVA in Excel. Data for progeny were adjusted using check-families. Mid-parent performance under the first screening experiment were compared to progeny performance under the salt tolerance test. Heritability was assessed using parent-progeny correlation instead of parent-progeny regression due to the potentially different testing environments (BOS and CALIGARI, 1995). Analysis of survival could not be carried out because of the limited material. A one-tailed t-test was used to compare progeny mean height with genetic distance between parents for different crosses.

Results

Genetic diversity

A high level of genetic diversity was evident among the original nine provenances using the eight selected microsatellite loci, and all loci were polymorphic (Table 2). The number of alleles per locus ranged from 17 (En6)

Table 2. – Genetic diversity parameters of *Eucalyptus occidentalis* families from nine provenances. Na, average number of alleles per locus. *P*, proportion of polymorphic loci. *Ho*, observed heterozygosity. *He*, expected heterozygosity. *F_{IS}*, fixation index. s.e., standard error.

Provenance	No. individuals	No. families	Na (s.e.)	<i>P</i> (%)	<i>Ho</i> (s.e.)	<i>He</i> (s.e.)	<i>F_{IS}</i> (s.e.)
Beaufort	47	5	16.5 (0.334)	100	0.526 (0.009)	0.838 (0.003)	0.398 (0.009)
Gordon	9	1	7.0 (0.248)	100	0.474 (0.033)	0.717 (0.008)	0.451 (0.034)
Grass Patch	38	3	12.6 (0.168)	100	0.471 (0.008)	0.765 (0.005)	0.412 (0.009)
Katanning	40	3	16.5 (0.207)	100	0.553 (0.001)	0.859 (0.002)	0.381 (0.011)
L. Magenta	22	2	10.4 (0.176)	100	0.512 (0.011)	0.776 (0.004)	0.372 (0.017)
Red Lake Siding	82	8	17.6 (0.148)	100	0.474 (0.005)	0.842 (0.003)	0.448 (0.004)
Stirling	15	2	9.3 (0.280)	100	0.431 (0.016)	0.741 (0.006)	0.478 (0.017)
Truslove	16	1	8.3 (0.203)	100	0.538 (0.013)	0.706 (0.008)	0.282 (0.020)
Young River	45	5	17.5 (0.247)	100	0.496 (0.008)	0.820 (0.004)	0.412 (0.008)
Mean			12.85 (0.225)	100	0.497 (0.002)	0.784 (0.001)	0.431 (0.002)

to 55 (EMCRC6). The maximum number of alleles across all loci in a provenance was 26. The mean number of alleles per locus (Na; Table 2) was high but varied among provenances relative to sample size so comparisons among provenances cannot be made. Across provenances, the number of rare alleles (frequency <0.1) was very high (72%), while the number of common alleles (frequency >0.5) was very low (2.5%). Observed heterozygosity (*H_o*) and expected Hardy-Weinberg panmictic heterozygosity (*H_e*) were moderate and were generally similar in all provenances. The inbreeding coefficient (*F_{IS}*) was high, as expected, due to the assessment of families rather than unrelated individuals (Table 2).

Allelic diversity in families was generally high and the maximum number of alleles per locus in any one family was 12. Across families, there were a similar proportion of rare alleles (50%) and common alleles (49%). The number of individuals per family ranged from 4 to 17 and mean number of alleles per family was generally associated with family size. *H_o* and *H_e* were moderate to high and had values between 0.563–0.695 and 0.395–0.768, respectively. Highest allelic diversity (>0.683) occurred in some families from the northern range of the natural distribution including all families from Katanning, three families from Red Lake Siding (122, 124 and 153), two families from Beaufort River (86 and 87), and one family from each of Young (108), Lake Magenta (94) and Grass Patch (137). Most of the diversity was distributed within (84%) rather than between families (16%). Families from the two eastern provenances that originate from calcareous areas, Red Lake Siding and Grass Patch, possessed 37% of the diversity. Overall there were only weak correlations between family genetic diversity and geographic parameters, such as longitude, where there was more diversity in the west (Family *H_e* = –11.1 x longitude + 127, *R*² = 0.121, *p* < 0.5) and with increasing altitude (Family *H_e* = 245 x altitude + 81, *R*² = 0.108, *p* < 0.5) or climatic variables where increasing annual rainfall was associated with

greater diversity (Family *H_e* = 233 x rainfall + 290, *R*² = 0.032, *p* > 0.5).

Analysis of the relationships between families showed little clustering of families from the same provenances

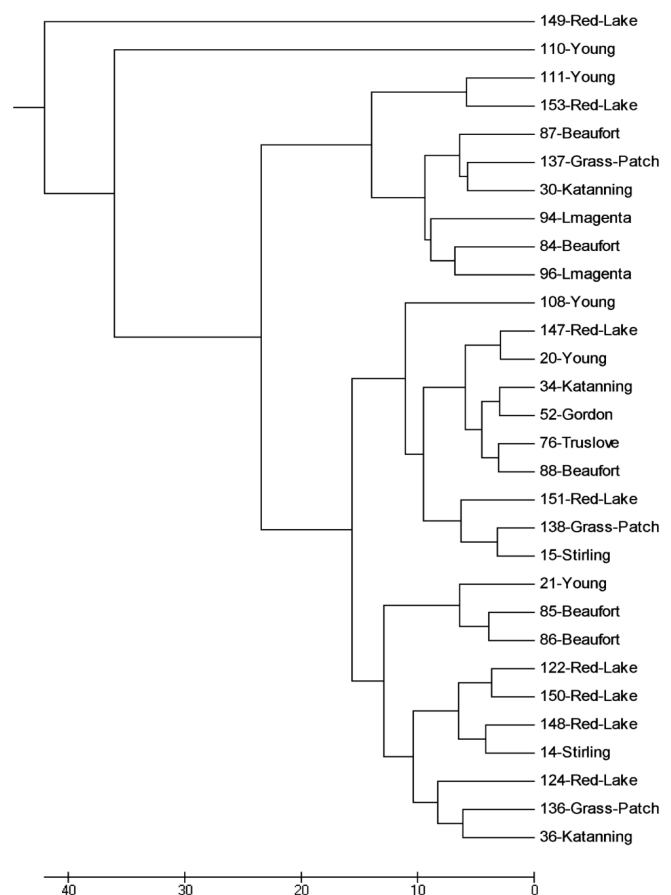


Figure 1. – Genetic relationship between families from nine provenances of *Eucalyptus occidentalis* based on Unweighted Pair Group Method with Arithmetic mean analysis of Goldstein's genetic distance.

except for three families from Red Lake Siding (*Figure 1*). Two families, 149-Red Lake Siding and 110-Young, showed differentiation from the rest of the families.

Controlled crosses

Success of pollination in the 45 crosses was low and 8% of pollinated flowers involving 25 crosses produced capsules at harvest. Seed germination occurred for 22 crosses but early seedling mortality was high and only eight crosses produced seedlings that survived to 9 months

months (*Table 3*). There was a very weak but significant correlation ($p=0.001$) between capsule production and parental genetic distance (*Figure 2a*). There was a positive trend between increasing parental genetic distance and increasing number of germinated seeds/capsule, seedling survival at 2 weeks and at 9 months (*Figure 2*). When one outlier was excluded, association was greater ($R=0.67$, $p=0.001$) for number of germinated seed per capsule and seedling survival at 2 weeks (*Figure 2B* and *C*) but decreased for 9 month seedling survival.

Table 3. – Cross combinations of *Eucalyptus occidentalis* male and female parents, their pairwise genetic distances, number and proportion (%) of germinating seeds/capsule, and number and proportion (%) of seedlings surviving at 9 months.

Female family provenance	Male family provenance	Cross combination (level of salt tolerance)	Individual genetic distance of parents	Family genetic distance of parents	Provenance genetic distance of parents	Germinated seeds/capsule (%)	Seedling survival at 9 months (%)
36-Kat	124-Red	A (L x H)	7727	268	55	41 (68)	30 (50)
96-LMag	20-Young	B (L x H)	3437	371	106	59 (96)	38 (62)
110-Young	108-Young	C (H x H)	83	568	0	11 (19)	7 (12)
20-Young	30-Kat	D (H x L)	2138	379.3	103	5 (10)	3 (6)
20-Young	96-LMag	E (H x L)	5550	371	106	23 (25)	4 (4)
110-Young	150-Red	F (H x L)	2187	242	46	7 (24)	3 (10)
96-LMag	76-Trus	G (L x H)	6145	159	97	7 (23)	5 (16)
76-Trus	124-Red	H (H x H)	4904	245	97	10 (10)	5 (5)

Young = Young river, LMag = Lake Magenta, Kat = Katanning, Red = Red Lake Siding, Trus = Truslove, H = High, L = Low salt and waterlogging tolerance.

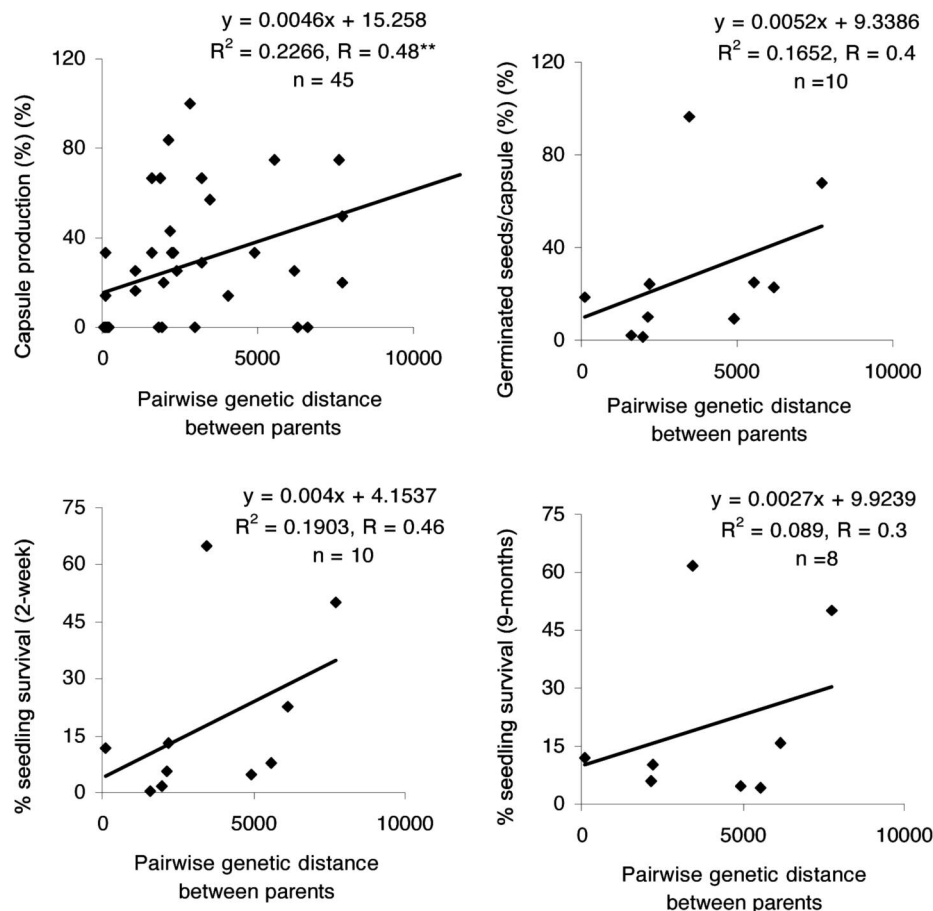


Figure 2. – Relationship between genetic distances between *Eucalyptus occidentalis* parents and fitness traits. (a) capsule production; (b) germinated seeds/capsule; (c) seedling survival at 2 weeks; (d) seedling survival at 9 months. ** $p = 0.001$.

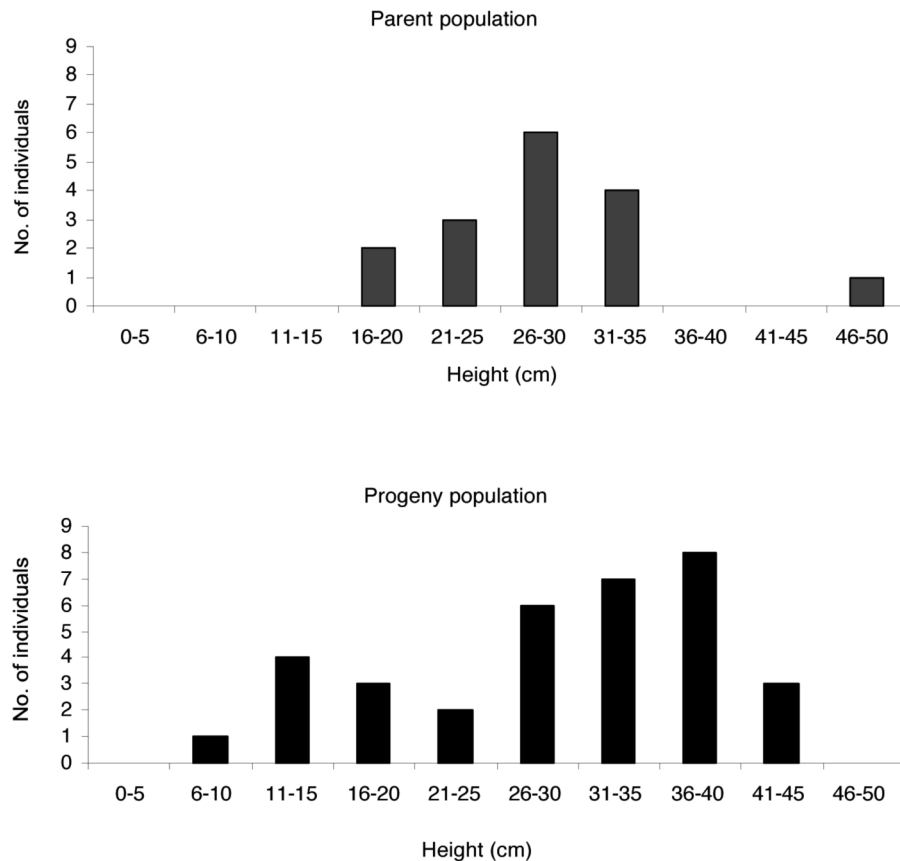


Figure 3. – Comparison of height in *E. occidentalis* parent and progeny populations under 500 mM NaCl treatment.

Table 4. – Survival, height, relative growth and final leaf proportion for eight *Eucalyptus occidentalis* crosses (A-H) under salt-waterlogged (500 mM salt) conditions. Analysis of variance was carried out for crosses with ≥ 3 progeny to determine significance of differences among crosses for each trait. For cross combination details refer to Table 3.

	lsd #	Cross (no. progeny)							
		A (9)	B (9)	C (2)	D (1)	E (3)	F (1)	G (5)	H (4)
Survival (%)		45	45	50	33	75	33	100	80
Height (cm) **	8	36	28	30	12	21	28	30	27
Rel.Growth (%) ***	55	179	230	170	303	248	137	205	249
Final.Leaf (%)*	26	131	167	114	150	121	109	126	90

lsd = least significant difference; significance of differences among crosses given.

* = (p 0.05), ** = (P 0.01), *** = (P <0.01).

Salt tolerance assessment

Five check-families were used to compare the two environments of the first screening experiment, where the performance of the parental trees was determined and the progeny test conducted here. There was a significant correlation between the mean heights of the five check-families in both experiments for the control treatment ($R^2=0.82$) and for the salt waterlogging treatment ($R^2=0.98$) indicating that both test environments of the two experiments were similar. Comparison of families subjected to both the control and salt/waterlogging treatments showed differences indicating an effect of the salt treatment on survival and growth. Progeny under

the 250 mM salt treatment were shorter than those under the control treatment and the observed difference were even greater at 500 mM NaCl.

Heritability values, under 500 mM salt-waterlogging treatment, indicated that height was the only trait with moderate heritability ($h^2=0.5\pm 3.4$). Relative growth ($h^2=0.17\pm 0.47$) and final leaf proportion ($h^2=0.05\pm 20.6$) had low heritability values. It was not possible to compare heritability values across cross combinations between high x high, high x low and low x low because of the limited number of progeny from each combination, particularly in the low x low crossings where very few seedlings survived for further salt test-

ing. Although not significant, overall there was an increase in mean height across the progeny population (29.4 ± 1.7 cm, $n=34$) than in the parents (28 ± 1.7 cm, $n=16$) under 500 mM salt-waterlogging treatment (Figure 3). The distribution of the progeny population was shifted towards the right (taller) compared with the parental population.

Comparison of performance of progeny of the different crosses showed that those with greater genetic distance between parents had greater survival and taller progeny. Only cross combinations with a parental genetic distance of ≥ 3436 (A, B, E, G and H) had ≥ 3 surviving progeny under 500 mM salt (Table 4, Figure 4). Cross combinations with parental genetic distance of ≥ 4904 (E, G and H) had $\geq 75\%$ survival except cross combination A which had only 45% survival. Interactions between treatments and cross combinations were limited and influenced different aspects of growth. At 250 mM salt, cross combinations did not influence height, relative growth or final leaf proportion (data not shown). At 500 mM salt comparisons of cross combinations showed that A (LxH), C (HxH) and G (LxH) were taller; D (HxL) and H (HxH) had greater relative growth; and

B (LxH) and D produced or retained more leaves than other crosses (Table 4).

A one-tailed t-test analysis revealed consistent patterns in comparison of progeny performance from crosses with ≥ 3 individuals (Table 5). Progeny from crosses with similar parental genetic distance were similar in height. However, progeny from parents of medium genetic distance were shorter than progeny from parents with large genetic distances (B vs A and E vs A). Compared to other crosses, Cross A between parents with the widest genetic distance (7727) produced the highest number of progeny with the greatest increase in height (50%) from mid-range parents.

Discussion

Genetic diversity

Maintenance of genetic diversity in breeding populations is essential to avoid deleterious effects of inbreeding that are common in EUCALYPTUS (POTTS and WILTSHIRE, 1997; HARDNER and TIBBITS, 1998). This study has shown that high diversity can be maintained in a breeding population of *E. occidentalis* through selection from

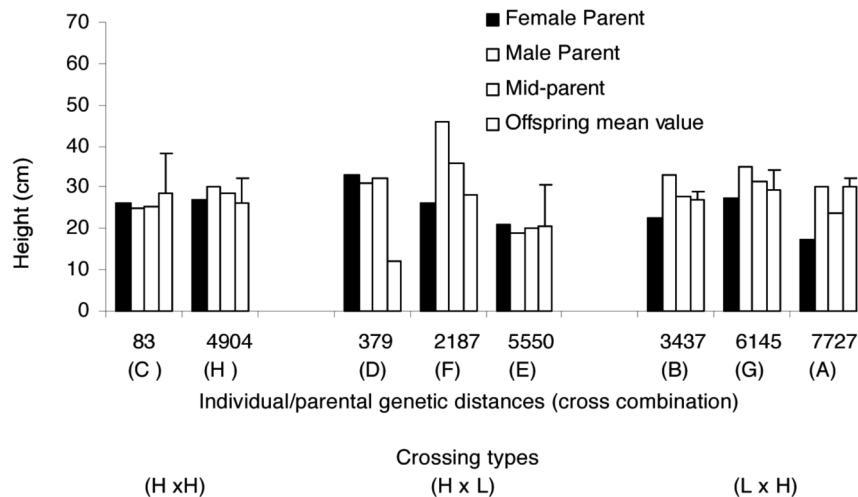


Figure 4. – Performance for height of parents and progeny of crosses of *E. occidentalis*. Performance was tested under 500mM NaCl treatment for crosses with different genetic distance between parents and different salt tolerance of parents.

Table 5. – Comparison of mean-progeny height (t-test) from *Eucalyptus occidentalis* crosses between parents of different genetic distances. For cross combination details refer to Table 3.

Cross 1	Cross 2	Progeny height		No. of Progeny cross 1/cross 2	Genetic distance	t test (P 0.001)
		Cross 1 (Mean \pm s.e)	Cross 2 (Mean \pm s.e)			
E	H	21 \pm 6	27 \pm 6	3 / 4	medium – medium 5550 and 4904	ns
E	B	21 \pm 6	28 \pm 2	3 / 9	medium – medium 5550 and 3437	ns
E	G	21 \pm 6	30 \pm 5	3 / 5	medium – medium 5550 and 6145	ns
B	A	28 \pm 2	36 \pm 2	9 / 9	medium vs large 3437 and 7727	**
E	A	21 \pm 6	36 \pm 2	3 / 9	medium vs large – 5550 and 7727	**
G	A	30 \pm 5	36 \pm 2	5 / 9	large - large 6145 and 7727	ns

ns = not significant; ** = (P < 0.01).

a representative range of provenances in the species distribution. This is similar to the high level of diversity maintained in first generation selections of *E. globulus* (JONES et al., 2006) and clonal orchards of *E. dunnii* (MARCUCCI POLTRI et al., 2003). Maintenance of high diversity in breeding populations in eucalypts is likely to be related to the high diversity generally found in most eucalypt species (BYRNE, 2008). A previous assessment of genetic diversity in the natural populations of *E. occidentalis* found moderate levels of diversity consistent with its localised distribution (ELLIOTT and BYRNE, 2003). The diversity found in this study was higher than that of the natural populations due to the different marker systems used in the two analyses, RFLP analysis in ELLIOTT and BYRNE, (2003) versus microsatellites in this study. Comparison of diversity in different markers systems in eucalypts has shown a general pattern of a two-fold increase in measured levels of diversity with microsatellite markers compared to RFLPs (BYRNE, 2008) as was detected here.

High genetic diversity is influenced by the proportion of rare alleles. Inclusion and maintenance of rare alleles in a breeding program is important especially as these alleles may be related to beneficial genes (YANCHUK, 2001). The frequency of rare alleles was high in the families of *E. occidentalis* assessed here indicating that sufficient genetic diversity has been retained in these families for future breeding purposes. Maintenance of diversity even though families were selected from only nine provenances will be related to the low level of genetic differentiation between provenances across the natural distribution of *E. occidentalis* (ELLIOTT and BYRNE, 2003).

Although differentiation among provenances was low there was some correlation between genetic diversity and environmental factors, with higher diversity associated with lower longitude (west) and higher altitude (longitude and altitude were not related). Association of higher diversity with high elevation is consistent with trends in the natural populations (ELLIOTT and BYRNE, 2003). There is a greater occurrence of salinity and waterlogging in lower elevation areas in south-western Australia (HUNT and GILKES, 1992) and lower diversity in *E. occidentalis* from low elevation sites, where they would be exposed to salinity and waterlogging stresses, may be an indication of some adaptation and selection in these saline environments. This is supported by the better performance in the salt and waterlogging trials of families from the two provenances from the most saline environments, Red Lake Siding and Grass Patch. Good performance of families from Grass Patch has also been observed in earlier trials in Australia and Italy (HARWOOD et al., 2001). These provenances may also have advantages in terms of stress tolerance associated with soil characteristics. There were substantial differences in soil characters between the eastern provenances, that grew on calcareous sands (containing calcium carbonate or lime), and most other provenances that grew on various soils including low nutrient soils derived from the Yalgarrin block, loams, clays and duplex soils, some with periodic waterlogging (CONACHER and CONACHER, 1998). Soil with calcium carbonate tends to be alkaline and

nutrient availability is limited due to lack of solubility of many elements under high pH (HUNT and GILKES, 1992). The eastern provenances of *E. occidentalis* may have natural adaptation to high pH and/or low nutrient availability. This requires further examination, but nonetheless it would be a useful adaptation for production in much of the south west of Western Australia, and in other low nutrient or alkaline soils, such as those found in the eastern Mediterranean (ZOHAR, 1991).

Genetic relationships among families were essentially random and showed no structure in relationship to provenance. This suggests that any adaptation to saline environments has not increased genetic similarity indicating that there would be little genetic constraint to further exploration of salt and waterlogging tolerance within the two best performing provenances, Red Lake Siding and Grass Patch. There is a strong possibility that different genes and perhaps different mechanisms are being employed for salt /and waterlogging tolerance and it may be possible to pyramid these traits (BENNET and KUSH, 2003). Low levels of genetic structuring imply that breeding with salt and waterlogging-tolerant individuals from these or other *E. occidentalis* provenances would be expected to result in substantial increases in salt and waterlogging tolerance, but no decline in genetic diversity.

The one stop pollination of immature styles (HARBARD et al., 1999) was suitable for controlled crossing in *E. occidentalis* although the numbers of fruit and seed produced were relatively low. This is most likely related to the young age of the parental plants as they were pollinated at first flowering. Low fruit production is common in young plants following the transition from the juvenile to mature stage as the apportioning of photoassimilates shifts (SEDGLEY and GRIFFIN, 1989). However, there was also evidence of a genetic effect in fruit production. Crosses between parents with small genetic distance had low capsule production, poor seed germination and early seedling death. This is likely to be an expression of inbreeding depression that is common in *Eucalyptus* (ELDRIDGE et al., 1994; HARDNER and POTTS, 1995; SEDGLEY and GRIFFIN, 1989). *Eucalyptus occidentalis* has a high outcrossing rate under open pollination conditions (BYRNE, 2004) and low fitness is expected in crosses between related parents (WILLI and BUSKIRK, 2005). Expression of inbreeding depression has also been observed as reduced wood volume at 4 years of age in *E. globulus* (HARDNER et al., 1998). Molecular measurement of genetic distance and careful selection of parents would help to avoid this late effect on wood production in *E. occidentalis*. Further research will be required to determine the best provenances and families for salt and waterlogging tolerance breeding but certainly microsatellites provide a suitable means to screen parents for adequate genetic distance to avoid the deleterious effects of inbreeding in breeding programs.

Salt and/waterlogging tolerance assessment

Assessment of trait expression requires standardization of screening tests that give repeatable, consistent results. Factors that influence expression of salt-tolerance that need to be controlled in multiple assessments

include salt concentration, macro and micro nutrient levels, timing, length of exposure, and environmental factors such as temperature, humidity, net radiation and potting media (SHANNON, 1997). In this study standardization of test environments was assessed by the inclusion of five check-families in both the original experiment and the analysis of progeny performance reported here. The performance under salt/waterlogged treatment of the parental and progeny populations was highly correlated indicating standardization of conditions. Thus repeated salt/waterlogged testing can be carried out for a species, such as *E. occidentalis*, with many provenances and families, when numbers are too large to be completed in one trial, or over subsequent generations as in this study, as long as control families are included to assess and confirm repeatability.

Determination and measurement of inherited traits is essential for assessment of trait expression. Both progeny and parents exposed to salt/waterlogging stress showed similarity in height, growth and final leaf proportion. However, only height had high heritability and this was supported by ANOVA-based (HENDRATI, 2008), and to a lesser extent parent-offspring regression, heritability values. Heritability values using analysis of variance are often higher than parent offspring regression (ZOBEL and TALBERT, 1984; BOS and CALIGARI, 1995). Here the differences in these tests may have been due to sampling as ANOVA-based analysis was derived from 237 data points from 30 families, while parent-offspring regression used 35 progeny from eight cross combinations. Low crossing success producing very few progeny reduces precision of heritability estimates (LOO-DINKINS, 1992). In addition, parent seedlings were selected for uniformity before screening, increasing the precision of heritability estimation, whereas variation in progeny performance was influenced by differences in seedling size due to irregular seedling emergence following germination (BURDON and SWEET, 1979). Controlled pollination of young plants may also have biased results as only individuals with flowers could be used and these were not necessarily the best performers.

Wide parental genetic distance increased progeny survival and performance under salt-waterlogging conditions in *E. occidentalis*. The mean survival and height of progeny were higher than their parental mean values and the overall mean value for the progeny population was increased compared to that of the parental population. Crosses with the widest parental genetic distance produced progeny with considerable height increase above parents and this trend was evident even with moderate genetic distance. Genetic fitness determines vigor, plant size and the ability to grow under saline conditions (HU et al., 1977; MUNNS and TERMAAT, 1986) especially the harsh, salt-waterlogged conditions used in this study. Lower fitness of progeny from parents with close genetic distance was expected due to inbreeding depression resulting from increasing homozygosity and deleterious recessive alleles (CHARLESWORTH and CHARLESWORTH, 1987). This reflects the strong association between genetic similarity of sexually mated parents and the performance of their progeny. However, the association is represented by a bell-shaped curve such

that performance will be reduced once optimum genetic distance is exceeded. The greatest genetic distances examined here improved progeny performance and this may represent the intermediate genetic distance which achieves optimum fitness (WILLI and BUSKIRK, 2005). When genetic distance between parents is too wide, as in some wide intra-specific crosses and interspecific hybridizations, outbreeding depression can be expressed due to the disruption of local adaptation, underdominance or epistatic interactions, and the breakdown of co-adapted gene complexes (EDMANDS, 2007). However, this end of the association was not explored in this study and further examination of crosses from wide genetic distances may be required to confirm that the optimum genetic distance for breeding in *E. occidentalis* has indeed been obtained. Genetic recombination in progeny may also have added value and this can be exploited in large crossing programs (ZOBEL and TALBERT, 1984).

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Genetic structure and diversity of *Polylepis australis* (Rosaceae) tree populations from central Argentina: Implications for forest conservation

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Abstract

Worldwide, large areas of forest are being transformed to other land cover types and the resulting fragmented populations may suffer from restricted gene flow leading to genetic pauperization and increased inbreeding. To assess the genetic constitution of fragmented *Polylepis australis* mountain forests of central Argentina, analyses of the structure and diversity of ISSR markers were carried out for 90 trees distributed throughout five river basins with differing degrees of fragmentation. Overall, average polymorphism (P) ranged between 87.2 and 94.9% (95% criterion) while marker diversity index (M) varied between 0.35 and 0.39; values which are comparable with other wind-pollinated tree species. Analysis of molecular variance (AMOVA) revealed that most genetic variation occurred within river basins (97.8%), with only a little occurring between river basins (2.2%; $\Phi_{ST} = 0.02$). In addition, Mantel's test indicated that *P. australis* does not follow the usual pattern of isolation by distance; instead the UPGMA method showed that trees from the two most degraded river basins formed a group while trees from the three better pre-

served basins formed another. As such, either effective pollen flow has maintained high levels of genetic diversity, or present day genetic variability is a remnant of a recently fragmented ancestral panmictic population. We conclude that, at present, genetic degradation in *P. australis* populations of central Argentina is not as important as ecological degradation – such as soil loss, intensive browsing by livestock or increased frequencies of wildfires, and that genetic variability is still fully available for forest restoration.

Key words: fragmentation, genetic diversity, *Polylepis australis*, Argentina, conservation, ISSR.

Introduction

Worldwide, large areas of forest are being felled for timber, burnt or clear-cut to produce grasslands for livestock, agricultural lands or urban areas, all of which are changing the face of forest landscapes across the globe (CINGOLANI et al., 2008; ZAK et al., 2004; SPIES, 1998; ELLENBERG, 1979). The resulting fragmented forest landscapes may have tree populations with diminished effective population sizes, decreased gene flow and increased inbreeding and eventually may produce high differentiation among remnant populations (JUMP and PEÑUELAS, 2006). However, this pattern has exceptions, as some species compensate fragmentation with increased gene flow through greater pollen or seed dispersal distances (FRANKHAM et al., 2002). Determining the genetic structure of fragmented tree populations is necessary for detecting any possible genetic loss due to inbreeding within fragments and for planning any adequate restoration action with regards to genetic differentiation and variability. Forest restoration is important

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