



## Analysis of the genetic diversity of *Eucalyptus cladocalyx* (sugar gum) using ISSR markers

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**ABSTRACT.** *Eucalyptus cladocalyx* F. Muell is a tree endemic to southern Australia and is distributed across four isolated regions: Kangaroo Island, southern Flinders Ranges, and two geographical zones in Eyre Peninsula. *E. cladocalyx* is capable of growing under extreme environmental conditions, including dry and saline soils. The objective of this study was to analyze genetic diversity in 45 half-sib families planted in northern Chile that are distributed across five different zones (provenances). Genetic variability was assessed using ISSR (Inter Simple Sequence Repeat) molecular markers. The results showed low levels of genetic diversity within populations ( $H_e = 0.113$  to  $0.268$ ) in contrast with other *Eucalyptus* species. In addition, there was a significant genetic differentiation among provenances ( $\Phi_{st} = 0.14$ ); populations from the Kangaroo Island provenance showed more differentiation than any other population. These results are in agreement with previous studies of the species. Our study revealed that Chilean resources are a representative sample of Australian populations; therefore, the germplasm planted in northern Chile would be sufficient for the development of improvement programs. ISSR-Marker technology could be an alternative to identify genotypes of interest in material selection.

**Keywords:** DNA pooling, half-sib families, AMOVA, clustering analysis.

## Análise da diversidade genética de *Eucalyptus cladocalyx* usando marcadores ISSR.

**RESUMO.** *Eucalyptus cladocalyx* F. Muell é distribuída em quatro regiões isoladas do sul da Austrália: Kangaroo Island, Flinders Ranges e em duas zonas da Eyre Peninsula. A espécie tem a capacidade de crescer em condições ambientais extremas como ambientes secos e solos salinos. O objetivo do presente estudo foi examinar a diversidade genética em 45 famílias de meio-irmãos cultivadas no norte do Chile, as quais são originárias de cinco localidades diferentes (procedências). A variabilidade genética foi avaliada usando marcadores moleculares ISSR (Inter Simple Sequence Repeat). Os resultados mostraram baixo nível de diversidade genética dentro de populações ( $H_e = 0,113$  a  $0,268$ ) em contraste com outras espécies de *Eucalyptus*. Adicionalmente, houve uma significativa diferenciação genética entre procedências ( $\Phi_{st} = 0.14$ ), coincidindo com a reportada em estudos anteriores. Kangaroo Island é a procedência mais diferenciada. O presente estudo evidenciou que os recursos existentes no Chile são uma amostra representativa das populações Australianas e, portanto, o germoplasma cultivado no norte do Chile seria suficiente para os programas de melhoramento. A tecnologia de marcadores ISSR poderia ser uma alternativa para identificar genótipos de interesse no processo de seleção.

**Palavras-chave:** pool de DNA, famílias de meio-irmãos, AMOVA, análise de agrupamento.

### Introduction

*Eucalyptus cladocalyx* F. Muell (sugar gum) is a tree endemic to South Australia, where it is distributed discontinuously across the southern Flinders Ranges, Eyre Peninsula and Kangaroo Island (CLARKE et al., 2009; JOVANOVIĆ; BOOTH, 2002; WOODROW et al., 2002). This species is also cultivated outside of its natural range, particularly in areas with 400-1010 mm of annual precipitation and altitudes ranging between 0 to 600 m (JOVANOVIĆ; BOOTH, 2002); the species

thrives in a wide variety of soils, including gravels, clay loams, sandy loams and sands, but grows poorly on very fine sandy soils (HOBBS et al., 2009). Commercially sustainable *E. cladocalyx* plantations have been successfully developed in several Mediterranean countries, Israel and the Central Highlands (South Africa). In Australia, this species has been considered for breeding programs because of its suitability for growth in sites of low rainfall sites for the production of timber and poles as well as environmental remediation (HARWOOD et al., 2001). In Chile, it is also being genetically improved

for honey and pole production (MORA et al., 2009). It is preferred over other species because of its ability to grow in arid environments (GLEADOW; WOODROW, 2000; MCDONALD et al., 2003; WOODROW et al., 2002).

Studies of the genetic variation among trees are essentials for the discipline of forest genetics (MORA; SERRA, 2014) and are the main basis for the application of tree improvement programs that use selection and breeding to identify existing natural variation (ACQUAAH, 2006; WHITE et al., 2007). The extent to which genetic diversity is partitioned within and among populations influences where sampling efforts should be directed to capture the maximum amount of variation for breeding purposes. Traditionally, morphological observations and progeny tests have been used as descriptors of genetic variability and biodiversity (MARCUCCI-POLTRI et al., 2003). These data provide fundamental information on the genetic variability of different traits, but their description of overall genetic variability is limited because they do not cover the complete genome and are usually not neutral to breeding selective pressures.

In the case of *E. cladocalyx*, investigations of physiological, biochemical and genetic variation have been carried out by many studies (BUSH et al., 2011; GLEADOW; WOODROW, 2000; MCDONALD et al., 2003; MORA et al., 2009), and some spatial genetic structuring may be expected among populations as the species has a discontinuous natural distribution (MCDONALD et al., 2003; STEANE et al., 2011), unlike other eucalypts species that have a relatively continuous distribution across part or all of the species' natural distribution where geographic partitioning was not observed (i.e., *E. grandis*, *E. nitens* and *E. pilularis*; STEANE et al., 2011).

Data obtained from Australian Low Rainfall Tree Improvement Group trials (BUSH et al., 2009) illustrate the progress that has already been made for the evaluation genetic resources. These data have revealed provenance differences in growth and form that have allowed the recommendation of the best provenances for the development of seed production areas and seed stands. However, more information about the genetic basis of this variation is needed to optimize these genetic resources and introduce suitable germplasms for operational plantations.

Another useful procedure is the use of molecular marker technology, which has proved to be a valuable tool in the characterization and evaluation of genetic diversity within and among species and populations (BALASARAVANAN et al., 2005;

CHEZHIAN et al., 2010). Several marker-based diversity studies have been carried out for tree improvement programs and conservation of forest genetic resources (FINKELDEY et al., 2010). Although DNA-based molecular markers provide valuable information on genetic diversity and genetic relationships among populations, they have not been used for the analysis of genetic diversity in a species with such economical potential species as *E. cladocalyx*. In the present study, ISSR markers were used to examine the levels of genetic diversity in germplasm accessions of *E. cladocalyx* from Chile.

## Material and methods

An open-pollinated progeny trial of *E. cladocalyx* that represents the entire natural distribution of this species was established in June 2001 in the Choapa Province of northern Chile. It is located in the rural community of Tunga Norte (31°38' Lat. S, 71°19' Long. W, 297 m altitude, annual rainfall 243.7 mm average, 15°C average annual temperature).

The present study considered forty-five families from three regions-of-provenance (ROPs) of the species: Flinders Ranges, Kangaroo Island and Eyre Peninsula. Seeds were collected from five locations (provenances) in South Australia, the center of the species' origin (RUTHROF et al., 2003) (Table 1). To study the genetic diversity among and between regions, juvenile leaf samples were collected randomly in the trial. Five trees per family were randomly selected and analyzed in the present study (Table 1).

**Table 1.** Provenances of *E. cladocalyx* evaluated in the Tunga Norte trial, northern Chile.

Provenances	Families (n)	Latitude (S)	Longitude (E)
Flinders Chase (Kangaroo Island)	8	35°57'	136°42'
Marble Range (Eyre Peninsula)	4	34°30'	135°30'
Mt. Remarkable (Flinders Ranges)	15	32°43'	138°06'
Cowell (Eyre Peninsula)	10	33°38'	136°40'
Wirrbarra (Flinders Ranges)	8	33°06'	138°14'

At least 50 ng of pooled total DNA samples were extracted from juvenile fresh or frozen leaf tissue (MICHELMORE et al., 1991) using the CTAB extraction protocol as described by Doyle and Doyle (1987) with minor modifications. The integrity and concentration of the DNA were visualized on 0.8% agarose gels and compared with the Promega® 50-8.000 kb lambda ladder (Promega, Madison, WI, USA). ISSR-PCR amplifications were performed in a 20 µL reaction volume containing 2 µL of template DNA, 1X Green GoTaq reaction PCR Buffer (Promega, Madison, WI, USA), 2.5 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, 100 nM of each primer and 0.3 U Taq

DNA polymerase (Promega, Madison, WI, USA). Eight primers that have been used previously by others for genetic analyses in eucalyptus were used in the present study (Table 2) (BALASARAVANAN et al., 2005; CHEZHIAN et al., 2010; OKUN et al., 2008). Two different PCR amplification programs were carried out, and different annealing temperatures were used for each primer (Table 2).

**Table 2.** ISSR primer sequences and annealing temperatures.

Name	Sequence	Annealing temperature
R(CA) <sub>7</sub>	5'-GRTRCYGRTRCACACACACACA-3'	55°C
T(GT) <sub>9</sub>	5'-CRTAYGTGTGTGTGTGTGTGTGT-3'	50°C
TA(CAG) <sub>4</sub>	5'-ARRTYCAGCAGCAGCAG-3'	55°C
RA(GCT) <sub>6</sub>	5'-AYARAGCTGCTGCTGCTGCTGCT-3'	55°C
(GA) <sub>8</sub> , R	5'-GAGAGAGAGAGAGAGAAGY-3'	45°C
UBC810	5'-GAGAGAGAGAGAGAT-3'	48°C
ISO <sub>1</sub>	5'-CACACACACACAGG-3'	45°C
ISO <sub>2</sub>	5'-CTCTCTCTCTCTCTCTAC-3'	50°C

For primers (RCA)<sub>7</sub>, T(GT)<sub>9</sub>, TA(CAG)<sub>4</sub>, RA(GCT)<sub>6</sub>, GA<sub>8</sub>, and UBC810, the following PCR program was used for template DNA amplification: initial denaturation (3 min., 94°C), 35 cycles consisting of denaturation (30 sec., 94°C), annealing (30 sec.), and extension (1 min., 72°C), and a final extension (10 min., 72°C). In the case of ISO<sub>1</sub> and ISO<sub>2</sub>, the thermal cyclers was programmed as follows: initial denaturation (7 min., 94°C), 45 cycles of denaturation (30 sec., 94°C), annealing (45 sec.), and extension (2 min., 72°C), and a final extension (7 min., 72°C). Amplification products were resolved on a 2% agarose gel with 1X TAE buffer at 30 V for 50 min. and then 70 V for 1h and were compared against a 1 Kb ladder (Axygen®). PCR products were visualized in a Vilbert Loumart Super-Bright UV transilluminator after staining with ethidium bromide and photographed with a Nikon CoolPix 4300 camera.

Each ISSR amplicon generated was considered as a locus, was scored as present (1) or absent (0) and was used for the statistical analysis. First, non-neutral loci or outliers were discerned using the Arlequin v 3.5 software (EXCOFFIER; LISCHER, 2010) as previously shown by Excoffier et al. (2009). Nei's genetic distances were calculated following the method of Lynch and Milligan (1994) after estimating the frequencies of the alleles accountable for the presence or absence of bands. The GenAlEx version 6.41 software (PEAKALL; SMOUSE, 2006) was used to calculate the percentage of polymorphic ISSR loci (% P), number of effective alleles (*Ne*), number of unique alleles (*Na*), Nei's

genetic diversity or expected heterozygosity (*He*) and Shannon-Weaver's index (*I*). Nei's genetic diversity is a simple measure of genetic variability and is defined as  $He = 1 - \sum x_i^2$ , where  $x_i$  is the population frequency of each allele (1 and 0) at locus *i* (in the case of dominant molecular markers). Shannon-Weaver's index (*I*) is defined as  $I = -\sum p_i \ln(p_i)$ , where  $p_i$  is the proportion of the *i*th allele in the population.

The distribution of the hierarchical ISSR frequency was evaluated using analysis of molecular variance (AMOVA) performed with Arlequin version 3.5 (EXCOFFIER; LISCHER, 2010) to estimate the variations within and between populations. Population genetic differentiation was estimated using the Arlequin software that calculates the  $\Phi_{st}$  parameter, which is equivalent to a F-st statistic when the degree of relatedness among the genetic variants is evaluated. This parameter was calculated from a matrix of Euclidean metric distances (PEAKALL; SMOUSE, 2006). The statistical significance was obtained using null distributions generated by random permutation of individuals (1,000 bootstrap permutations). Clustering analysis of families was performed by the Unweighted Neighbor-Joining algorithm using the DARwin 5.0 software (PERRIER; JACQUEMOUD-COLLET, 2006) with a Jaccard's dissimilitude coefficient with 10,000 bootstrapping samples. The provenance and ROPs dendrograms were generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm in Popgene 1.32 (YEH et al., 1997). A dissimilarity matrix was generated using Nei's genetic distance. In addition, Principal Coordinates Analyses (PCoAs) were performed in GenAlex for the clustering analyses of the provenances and ROPs.

## Results and discussion

A total number of 93 scorable loci were detected within a size range of 100–2,500 bp. The total number of amplified markers per primer for all provenances varied from 8 (with GA<sub>8</sub>) to 15 (with ISO<sub>2</sub>; Table 3). Seventy-eight loci (83.87% of the total loci) were considered neutral according to Excoffier et al. (2009). UBC810, ISO<sub>1</sub> and R (CA)<sub>7</sub> were the most polymorphic primers (100%), while RA(CGT) was only scored in 44% of the polymorphic bands. Genetic variability analysis of the 78 ISSR neutral loci is shown in Table 4.

**Table 3.** Details of the ISSR primers used for the genetic analysis of *E. cladocalyx* natural provenances. *N*: Number of total loci; *Nn*: Number of neutral loci; *Nnp*: Number of neutral polymorphic loci; % *P*: Percentage of neutral polymorphic loci.

Primer name	<i>N</i>	<i>Nn</i>	<i>Nnp</i>	% <i>P</i>
RA(CGT)	9	9	4	44.4
ISO <sub>1</sub>	14	14	14	100.0
ISO <sub>2</sub>	15	12	10	83.3
TA(CAG) <sub>1</sub>	11	8	7	87.5
T(TG) <sub>9</sub>	11	9	8	88.9
GA <sub>8</sub>	8	6	5	83.3
R(CA) <sub>7</sub>	12	9	9	100.0
UBC810	13	11	11	100.0
Total/Average	93	78	68	87.2

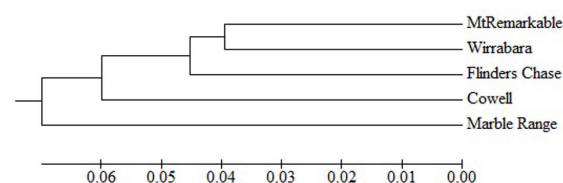
**Table 4.** Genetic diversity indexes of each provenance and ROPs. *Na*: Number of different alleles; *Ne*: Number of effective alleles; % *P*: Percentage of polymorphic loci; *I*: Shannon-Weaver's Index; *He*: Nei's Unbiased Expected Heterozygosity (1978); *SE*: Standard error.

ROPs	<i>Na</i> ± <i>SE</i>	<i>Ne</i> ± <i>SE</i>	% <i>P</i>	<i>I</i> ± <i>SE</i>	<i>He</i> ± <i>SE</i>
<b>Flinders Ranges</b>					
Mt. Remarkable	1.65 ± 0.07	1.44 ± 0.04	74.36	0.39 ± 0.03	0.27 ± 0.02
Wirrabara	1.24 ± 0.09	1.33 ± 0.04	48.72	0.27 ± 0.03	0.20 ± 0.03
ROPs Mean	1.74 ± 0.06	1.46 ± 0.04	80.80	0.40 ± 0.03	0.27 ± 0.02
<b>Eyre Peninsula</b>					
Cowell	1.55 ± 0.08	1.41 ± 0.04	66.67	0.35 ± 0.03	0.25 ± 0.02
Marble Range	1.13 ± 0.08	1.22 ± 0.04	34.62	0.19 ± 0.03	0.15 ± 0.03
ROPs Mean	1.63 ± 0.07	1.44 ± 0.04	71.80	0.38 ± 0.03	0.26 ± 0.02
<b>Kangaroo Island</b>					
Flinders Chase	1.46 ± 0.09	1.36 ± 0.04	62.82	0.32 ± 0.03	0.23 ± 0.02
Mean	1.41 ± 0.04	1.35 ± 0.02	57.44	0.31 ± 0.02	0.22 ± 0.01

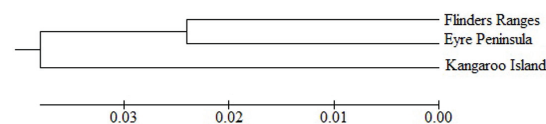
The average gene diversity (*He*) of all the provenances was 0.22, and the Shannon-Weaver Index was 0.31. Among the provenances studied, Mt. Remarkable had the highest rates of genetic diversity (*Na* = 1.65; *Ne* = 1.44; % *P* = 74.36; *He* = 0.27; *I* = 0.39), while Marble Range had the lowest (*Na* = 1.13; *Ne* = 1.22; % *P* = 34.62; *He* = 0.15; *I* = 0.19). Regional analysis (Table 4) showed that Flinders Ranges had the highest genetic diversity (*Na* = 1.74; *Ne* = 1.46; % *P* = 80.8; *I* = 0.40; *He* = 0.27), while Kangaroo Island the lowest (*Na* = 1.46; *Ne* = 1.36; % *P* = 62.8; *I* = 0.32; *He* = 0.23). Figures 1 and 2 show UPGMA dendrograms generated from Nei's genetic distance among the provenances and ROPs, respectively.

Genetic distances among the provenances varied from 0.063 to 0.130 (Table 5) and from 0.048 to 0.102 among the ROPs (Table 6). Consistently, Wirrabara and Mt. Remarkable, which are both within the Flinders Ranges, formed a single cluster while Marble Range (Eyre Peninsula) was the most genetically separated from the other provenances. As shown by ROPs clustering, Flinders Chase (Kangaroo Island) was the most genetically differentiated from Eyre Peninsula and Flinders Ranges. The results from these clustering analyses were consistent with those from principal coordinate analyses (PCoA; Figure 3). In both PCoA graphs, the first axis separates the provenances of Flinders Ranges (Mt. Remarkable and Wirrabara) and Flinders Chase (Kangaroo Island) from provenances of Eyre Peninsula (Marble Range and Cowell). In a), the second axis separates the provenances of Flinders Ranges and provenances of Eyre Peninsula from Kangaroo Island; however, Marble Range was the less genetically distant from Flinders Chase (Kangaroo Island). In b), the third axis also separates Flinders Chase from others provenances; however, it did not reflect the underlying geographical pattern as Marble Range was highly separated from Cowell (both are from Eyre Peninsula), and Mt. Remarkable was highly separated from Wirrabara (both are from Flinders Ranges).

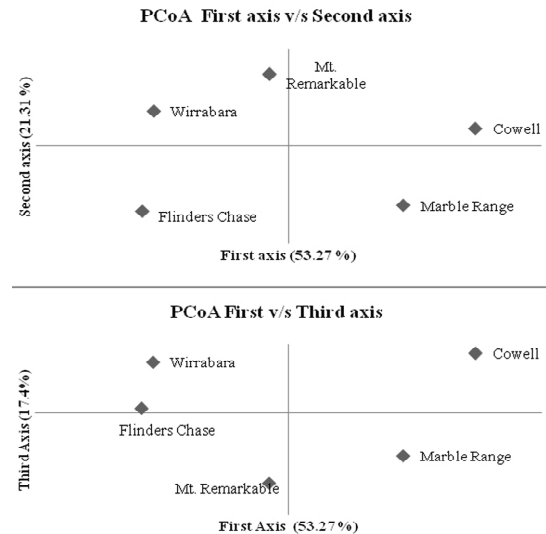
second axis separates the provenances of Flinders Ranges and Eyre Peninsula from Kangaroo Island; however, Marble Range was the least genetically distant from Flinders Chase (Kangaroo Island). In B, the third axis also separates Flinders Chase from others provenances; however, it does not reflect the underlying geographical pattern as Marble Range was highly separated from Cowell (both are in Eyre Peninsula), and Mt. Remarkable was highly separated from Wirrabara (both are in Flinders Ranges). According to these results, Flinders Chase is the most genetically separated from the others provenances.



**Figure 1.** UPGMA dendrogram of the genetic similarity among the five *E. cladocalyx* populations.



**Figure 2.** UPGMA dendrogram of the genetic similarity among the ROPs of *E. cladocalyx*.



**Figure 3.** Principal Coordinate Analysis (PCoA) of the ISSR data of *E. cladocalyx* using covariance analysis. In both PCoA graphs, the first axis separates the provenances of Flinders Ranges (Mt. Remarkable and Wirrabara) and Flinders Chase (Kangaroo Island) from provenances of Eyre Peninsula (Marble Range and Cowell). In a), the second axis separates the provenances of Flinders Ranges and provenances of Eyre Peninsula from Kangaroo Island; however, Marble Range was the less genetically distant from Flinders Chase (Kangaroo Island). In b), the third axis also separates Flinders Chase from others provenances; however, it did not reflect the underlying geographical pattern as Marble Range was highly separated from Cowell (both are from Eyre Peninsula), and Mt. Remarkable was highly separated from Wirrabara (both are from Flinders Ranges).

**Table 5.** Genetic similarity among provenances as calculated by Nei's genetic distance using polymorphism data generated using eight ISSR primers.

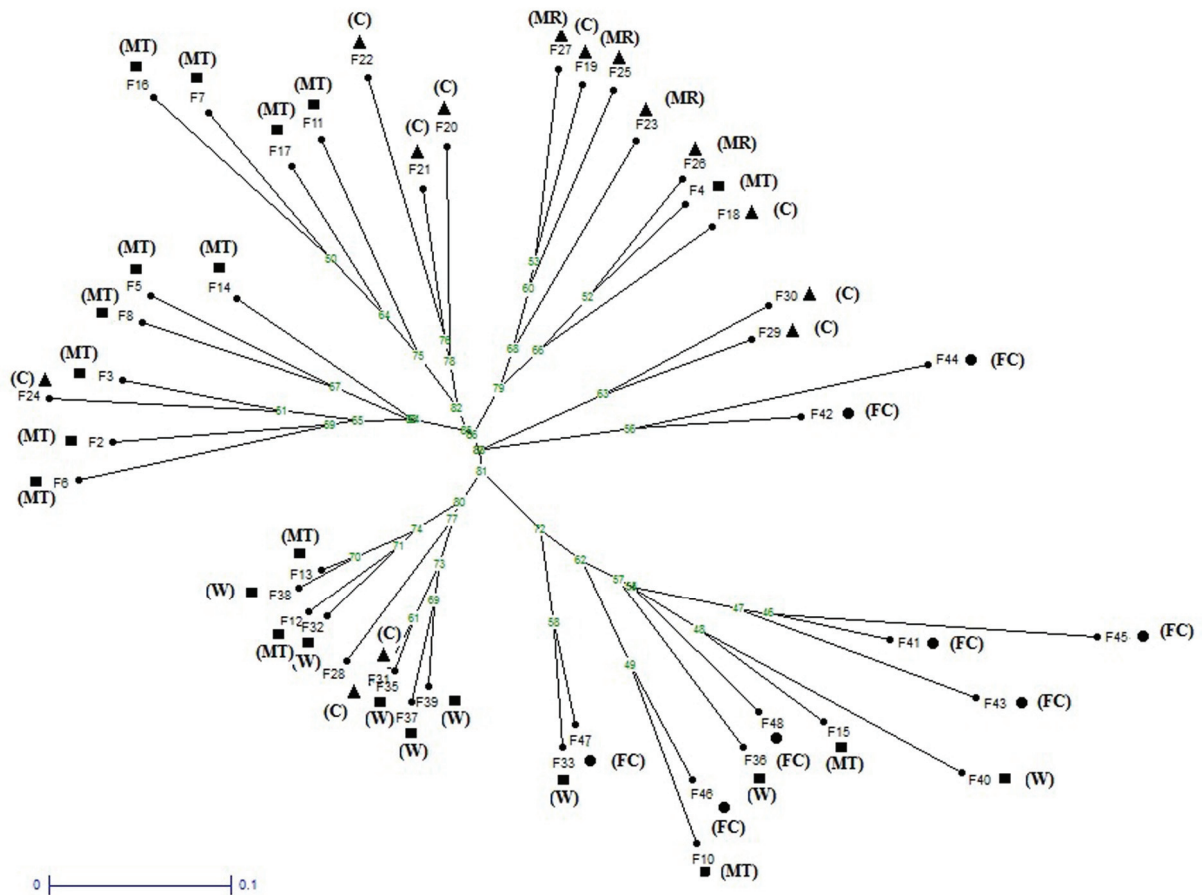
Provenance	Mt. Remarkable	Cowell	Marble Range	Wirrabara	Flinders Chase
Mt. Remarkable	-				
Cowell	0.065	-			
Marble Range	0.106	0.122	-		
Wirrabara	0.065	0.114	0.121	-	
Flinders Chase	0.063	0.130	0.130	0.082	-

**Table 6.** Genetic similarity values among the ROPs calculated by Nei's genetic distance using polymorphism data generated using eight ISSR primers.

ROPs	Flinders Ranges	Eyre Peninsula	Kangaroo Island
Flinders Ranges	-		
Eyre Peninsula	0.048	-	
Kangaroo Island	0.050	0.102	-

The clustering analysis among families was not consistent with their respective provenances (Figure 4). However, the NJ unrooted tree displayed high

bootstrapping values in most nodes of the dendrogram (> 50), which indicate consistency and correct separation among the different ROPs of *E. cladocalyx*. The clustering analysis among the ROPs is represented in five different groups. In genetic structure analysis, the hierarchical ISSR frequency distribution was analyzed using analysis of molecular variance (AMOVA), which indicated that a high proportion of total variation is supported by variability within families and less proportionately among provenances (Table 7). Additionally, there was a significant genetic differentiation among provenances ( $\Phi_{st} = 0.14$ ). Moreover, the AMOVA of the ROPs (Table 8) also showed a significant genetic differentiation ( $\Phi_{st} = 0.12$ ). All provenances were significantly different from each other with the exception of Wirrabara and Flinders Chase ( $\Phi_{st} = 0.06$ ; Table 9).



**Figure 4.** Unrooted neighbor-joining tree of 45 families from different natural provenances of *E. cladocalyx*. Bootstrap values at the branch points are shown in branch intersections. The names of provenances are represented by letters: Marble Range (MR), Mt. Remarkable (MT), Cowell (C), Wirrabara (W) and Flinders Chase (FC). The corresponding regional origins are represented by symbols: Eyre Peninsula (▲), Kangaroo Island (●) and Flinders Range (■).

**Table 7.** Analysis of molecular variance for ISSR data of *E. cladocalyx* among provenances.

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among provenances	4	1.0078	14.05
Within provenances	40	6.167	85.95
Total	44	7.131	
$\Phi_{st}$ P-value	0.14 < 0.000		
p < 0.05			

**Table 8.** Analysis of molecular variance for ISSR data of *E. cladocalyx* among the ROPs.

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among ROPs	2	0.887	12.17
Within ROPs	42	6.404	87.83
Total	44	7.131	
$\Phi_{st}$	0.1217		
P- value	<0.000		
p < 0.05			

**Table 9.** Pairwise Differentiation Index ( $\Phi_{st}$ ) among provenances of *E. cladocalyx*.

Provenance	Mt. Remarkable	Cowell l	Marble Range	Wirrabara a	Flinders Chase
Mt. Remarkable	-				
Cowell	0.19*	-			
Marble Range	0.17*	0.11*	-		
Wirrabara	0.11*	0.17*	0.19*	-	
Flinders Chase	0.16*	0.25*	0.19*	0.06	-

\*p &lt; 0.05.

The results indicated that Mt. Remarkable had the highest genetic diversity, while Marble Range had the lowest. In contrast, McDonald et al. (2003) found that Wirrabara and Marble Range were the most diverse provenances, while Mt. Remarkable and Flinders Chase had the lowest diversity. The current study determined that the diversity indexes are mildly higher than those calculated by McDonald et al. (2003). This difference could result from the fact that McDonald et al. used isoenzyme markers, and these molecular markers could underestimate the genetic diversity due to their lower resolution (WEISING et al., 2005; MONDINI et al., 2009). Both studies are in agreement with the hypothesis that *E. cladocalyx* has a low genetic diversity in comparison to other *Eucalyptus* species. Many investigations of other *Eucalyptus* populations have reported genetic diversity indexes over 0.6 (MIMURA et al., 2009; PAYN et al., 2008; JONES et al., 2005).

Breeding systems of *Eucalyptus* spp. show a high probability of self-pollination (HARDNER; POTTS, 1995). In fact, *E. cladocalyx* has post-zygotic self-incompatibility systems (ELLIS; SEDGLEY, 1992). Nevertheless, McDonald et al. (2003) reported a high level of inbreeding and low genetic diversity within populations, which is consistent

with the present study. Therefore, other factors are responsible for the low genetic diversity of *E. cladocalyx*. The genetic structure can break Hardy-Weinberg equilibrium due to genetic and non-genetic factors. Non-genetic factors, such as physical barriers and the geographical isolation of populations (FRANCOIS; DURAND, 2010) could, block to gene flow among populations. Migration among populations tends to homogenize their allele frequencies, and therefore, the absence of significant genetic differentiation among populations indicates that the populations maintain continuous gene flow.

The results of the present study indicated that Kangaroo Island is the most genetically distant region, which is according to McDonald et al. (2003) and Bush and Thumma (2013), and it would be expected due to its geographical isolation. In addition, McDonald et al. (2003) reported a positive relationship between geographic distance and genetic divergence among populations in *E. cladocalyx*. The genetic differentiation among populations could be the result of an adaptation to environmental gradients. In particular, *E. cladocalyx* is distributed across differential environmental conditions with respect to soil composition, altitude and annual rainfall (MCDONALD et al., 2003; CLARKE et al., 2009), which could drive the acquisition of local adaptations. Moreover, the environmental conditions could influence flowering times and restrict the gene flow through pollination and seeds dispersion among populations. In fact, according to the findings of Contreras-Soto et al. (2011), Mora et al. (2009) and Cané-Retamales et al. (2011), there are significant differences in flowering times among natural provenances of *E. cladocalyx*. These three sets of authors reported that Cowell has the higher flowering intensity and precocity, and trees from Flinders Chase were the worst for both traits. In the present study, Cowell and Flinders Chase had the highest genetic differentiation. The provenances of Eyre Peninsula were most genetically separated from the Kangaroo Island provenance.

On the other hand, in accordance with the PCoA and clustering analyses, there was a significant genetic differentiation among provenances of the same region (e.g., Marble Range and Cowell, Eyre Peninsula) that could be due to the conservation status of the provenances. According to McDonald et al. (2003), most Eyre Peninsula subpopulations are relatively small and fragmented due to clearance for agriculture, while the largest and most continuous populations occur in the southern

Flinders Ranges, where they are largely present in conservation parks, which is consistent with the clustering analysis of the present study.

Finally, the results showed a significant genetic differentiation among natural provenances consistent with previous studies of phenotypic variation in morphological traits (MORA et al., 2009; 2007; CANÉ-RETAMALES et al., 2011; CONTRERAS-SOTO et al., 2011; VARGAS-REEVE et al., 2013) and chemical compounds in wood durability (BUSH et al., 2011). This information allows for the establishment of a positive relationship between genetic controls and morphometric traits that could be useful for breeding programs.

### Conclusion

This article showed that the status of genetic resources of *Eucalyptus cladocalyx* in Chile is similar to that reported in studies of natural populations of the species, which indicates that the introduction of genetic material is not necessary for the development of breeding strategies. Despite the fact that the germplasm of the same origin has a low genetic diversity in contrast to the variability among provenances, there is a significant variability among populations of different origins, which is consistent with the previous phenotypic variation that has been observed in natural populations and provenance studies.

### Acknowledgements

The authors thank the financial support provided by the Chilean National Science and Technology Research Fund (FONDECYT), Projects 11090129 and 1130306.

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Received on November 28, 2012.

Accepted on April 6, 2013.

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