Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Clones identification of *Sequoia sempervirens* (D. Don) Endl. in Chile by using PCR-RAPDs technique*

Manuel TORAL IBAÑEZ†[1], Margarita CARU[2], Miguel A. HERRERA[3], Luis GONZALEZ[1], Luis M. MARTIN[4], Jorge MIRANDA[1], Rafael M. NAVARRO-CERRILLO[3]

([1]*Department of Forest Management, Faculty of Forestry;* [2]*Department of Ecological Sciences, Faculty of Sciences, University of Chile, Santa Rosa 11315, La Pintana, Santiago, Chile*)

([3]*Department of Forestry;* [4]*Department of Genetic, School of Agriculture and Forestry, University of Córdoba, 14080 Córdoba, Spain*)

†E-mail: mtoral@uchile.cl

Received May 5, 2008; Revision accepted Oct. 28, 2008; Crosschecked Dec. 25, 2008

Abstract: A protocol of polymerase chain reaction-random amplified polymorphic DNAs (PCR-RAPDs) was established to analyse the gene diversity and genotype identification for clones of *Sequoia sempervirens* (D. Don) Endl. in Chile. Ten (out of 34) clones from introduction trial located in Voipir-Villarrica, Chile, were studied. The PCR-RAPDs technique and a modified hexadecyltrimethylammonium bromide (CTAB) protocol were used for genomic DNA extraction. The PCR tests were carried out employing 10-mer random primers. The amplification products were detected by electrophoresis in agarose gels. Forty nine polymorphic bands were obtained with the selected primers (BG04, BF07, BF12, BF13, and BF14) and were ordered according to their molecular size. The genetic similarity between samples was calculated by the Jaccard index and a dendrogram was constructed using a cluster analysis of unweighted pair group method using arithmetic averages (UPGMA). Of the primers tested, 5 (out of 60) RAPD primers were selected for their reproducibility and high polymorphism. A total of 49 polymorphic RAPD bands were detected out of 252 bands. The genetic similarity analysis demonstrates an extensive genetic variability between the tested clones and the dendrogram depicts the genetic relationships among the clones, suggesting a geographic relationship. The results indicate that the RAPD markers permitted the identification of the assayed clones, although they are derived from the same geographic origin.

Key words: *Sequoia sempervirens* (D. Don) Endl., Random amplified polymorphic DNAs (PCR-RAPDs), Molecular markers, Clone assays, Chile, Genetic relationships
doi:10.1631/jzus.B0820162 **Document code:** A **CLC number:** Q7

INTRODUCTION

Sequoia sempervirens (D. Don) Endl. is a conifer originating from the west coast of California (USA) and the southwestern coast of Oregon (USA). Characteristics of *S. sempervirens* biology, such as seed germination, seedling survival, phenology, pollen dispersal, and seed crop production, have been reported elsewhere (Noss, 2000). *S. sempervirens* is the only hexaploid (2*n*=6*x*=66) amongst the members of Taxodiaceae (Ahuja and Neale, 2002), which has been challenging genetic studies of the species. In *Sequoia*, the existence of a high degree of polymorphism has been reported using chemical indicators (Hall and Langenheim, 1987), allozyme (Rogers, 1999; Rogers and Westfall, 2007), and microsatellite markers (Bruno and Brinegar, 2004), within the same population as well as among different populations. A study of allozyme diversity in the species (Rogers, 1999) revealed genetically distinct groups corresponding largely to natural regions. Although this allozyme analysis has been an effective tool in the study of redwood inheritance (Rogers, 1997; 1999)

* Project (No. CONICYT-FONDEF PROYECT D01 I 1008) supported by the National Commission of Science and Research of Chile

and diversity (Rogers, 2000), new DNA-based genetic markers have been applied (Bruno and Brinegar, 2004).

Sequoia was introduced into Chile by Albert (1908) in 1908 to stabilize the dunes of Chanco-Constitución and recommended as a promising species for the development of commercial forest plantations in this country. However, plantations for commercial use have started only in 1952, with yields ranging between 17 and 28 m^3/ha, according to stand quality (Toral *et al*., 2005). There is evidence that some of the seed sources used in Chile have markedly different performances in the field in terms of growth and other commercial traits of interest (Toral *et al*., 2005). Information on the levels and patterns of genetic diversity, based on both molecular markers and quantitative traits, facilitates the development of used strategies for forest tree species (Moran and Bell, 1987). Such strategies also highlight the plant material that is potentially used in plantation programs. Most of the plantations of *Sequoia* in Chile arise from uncontrolled seed sources. Morphological evidence indicates that poor selection material was present in forest plantations (Toral *et al*., 2005). These data, together with the evidence that some seedlings perform poorly in plantations (Osses, 2004), are consistent with an inadequate provenance maybe used.

Selected clones are superior for growth and hence have been the main source of material for breeding populations in recent years (Kuser *et al*., 1995; Kuser, 1996**)**. However, there are other requisites for material in a breeding program of *S. sempervirens*, such as disease resistance, good form, and pulp and wood properties. The Kuser collection comprises 180 clones, obtained from 90 *Sequoia*-growing areas and natural regions in California (USA). This collection is being tested in assays of provenances and clones from several regions and countries, among which the following can be cited: four trials in USA, two in France, one in Spain and one in New Zealand (Kuser *et al*., 1995). In Chile, the two assays have been established in Villarrica and Lanco, respectively (Toral *et al*., 2005). The selection of the provenances or clones with the best aptitude, highest yield, and best timber quality will permit the future establishment of the best-adapted genetic material for specific objectives such as gain in volume or straightness of the wood.

In recent years, biotechnological methods, the application of molecular markers such as RAPD markers, have been fairly successfully used for identifying genetic patterns of specific clones selected in improved populations. These techniques allow the complementation or, even, the replacement of plant or clone identification based on other analytical techniques. RAPD markers have been successfully employed in several plant genera for the reconstruction of phylogenetic relationships (Furman *et al*., 1997; Nkongolo *et al*., 2002; Syring *et al*., 2005). Moreover, RAPDs have also been used for quantifying the intra- and interpopulation gene diversities in several forest species (Grotkopp *et al*., 2004). In Chile, there is a lack of information about the genetic pattern and field response of the *Sequoia* clones selected from the Kuser collection (Toral *et al*., 2005).

Therefore, in the present study we aimed to test an effective protocol of polymerase chain reaction-random amplified polymorphic DNAs (PCR-RAPDs) for *Sequoia sempervirens* (D. Don) Endl. using 10 clones that are representative of those used in *Sequoia* plantations in Chile, and to apply the results to the analysis of gene diversity and genotype identification in clones of the species introduced into Chile.

MATERIALS AND METHODS

Plant material

The material used was a fraction of the Kuser collection that comprises 180 clones representing 90 sites of *Sequoia* provenances (Fig.1) (Kuser *et al*., 1995). For this study, 10 from 34 clones used in the assay located in Voipir-Villarrica, Chile (39°19′20″ S, 72°17′08″ W) were analyzed by PCR-RAPDs (Table 1). The 10 clones selected had the best percentages of nursery rooting, growth rate, and survival after plantations (Toral *et al*., 2005).

Genomic DNA extraction process and amplification by PCR

Needles from the clones in the last growth period were collected in November 2004, flash-frozen in liquid nitrogen, and ground in a mortar to a fine powder. Total genomic DNA was extracted following a modified hexadecyltrimethylammonium bromide

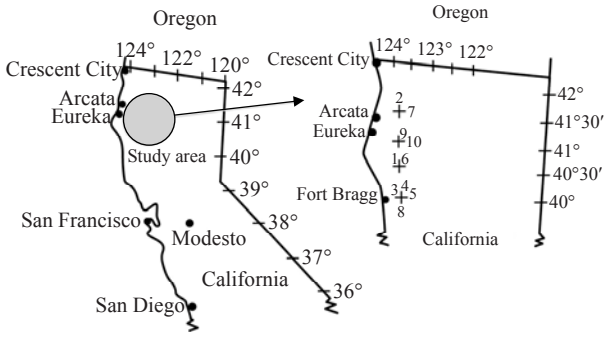

Fig.1 Geographical origin of the 10 clones selected for the study

Table 1 Genetic material selected for molecular analysis of *Sequoia sempervirens* (D. Don) Endl. in Chile*

Original code	Latitude	Longitude	Altitude (masl)
PLUS C	40°56′ S	123°58′ W	91~140
RB-236-10	41°45′ S	123°56′ W	90~140
RB2-238-16	40°01′ S	123°49′ W	91~140
RB54-225-17	40°01′ S	123°49′ W	91~140
RB54-225-14	40°01′ S	123°49′ W	91~140
RB54-225-19	40°56′ S	123°58′ W	91~140
COMMERCIAL	41°45′ S	123°56′ W	90~140
RB2-236-18	40°01′ S	123°51′ W	91~140
PLUS B	41°08′ S	123°56′ W	91~140
RB3-57-77-7	41°09′ S	123°56′ W	91~140

*Source: forestry databases, Simpson Korbel-Arcata-California, 2002; masl: metre above sea level

(CTAB) method (Doyle and Doyle, 1990; Tsumina *et al*., 1995). For DNA extraction, the needles were exhaustively rinsed with distilled sterile water, homogenized with a mini-grinder in TENC buffer (100 mmol/L Tris, 1.4 mol/L NaCl, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.055 mol/L CTAB, 0.038 mol/L mercaptoethanol), preheated at 60 °C with 10 mg/ml polyvinylpyrrolidone (PVP), and incubated at 60 °C for 1 h. Then, 500 µl of chloroform-octanol (24:1, v/v) was added and centrifuged at 6000 r/min for 5 min. The aqueous phase was extracted and DNA was precipitated by adding 1/10 volume of 3 mol/L sodium acetate and 2 volumes of 95% (v/v) ethanol, incubated at −20 °C for 1 h, and then centrifuged at 13000 r/min for 5 min. After drying, the pellet was resuspended in 200 µl of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA; pH 8), treated with 300 µl of chloroform-isoamilic alcohol, and then homogenized. DNA was rinsed with 70% (v/v) ethanol, resuspended in TE buffer, and then incubated with RNase at 37 °C for 1 h. Finally, DNA was cleaned with 1/2 volume of 8 mol/L LiCl and then left at room temperature for 1 h. DNA was precipitated with 1 volume of 95% (v/v) ethanol at −20 °C and then resuspended in 50 µl of TE buffer.

PCR reactions were conducted according to Williams *et al*.(1990) using 10-mer random primers (Operon Technologies, Alameda, CA, USA). 30~50 µg of chromosomic DNA was used in the amplification reaction, which contained 2.0 mmol/L $MgCl_2$, 0.1 mmol/L dNTP, 0.4 µmol/L of the primer, 2.5 U of Taq polymerase (GibcoBRL, USA), and 1 mg/ml bovine serum albumin (BSA) in PCR buffer (10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl), with a final reaction volume of 25 µl. The amplification reaction was performed in a GeneAmp PCR 2400 thermocycler (Perkin Elmer, USA) programmed for 5 min of pre-denaturation at 94 °C, followed by 45 cycles with a denaturation at 92 °C for 1 min and a hybridization for 5 min, and next, 45 cycles with a denaturation at 92 °C for 1 min, an annealing at 36 °C for 1.5 min, and an extension at 72 °C for 1 min. The final extension was done at 72 °C for 5 min. In order to verify the reproductibility of RAPD reaction, 2~3 DNA samples independent of each clone were assayed with selected primers that, for RAPD analysis, were assayed in at least 3 independent PCR reactions. The amplification products were detected by electrophoresis in 1.2% agarose gels (v/v) in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA; pH 8), and stained with ethidium bromide.

Statistic analysis

With the primers selected, the bands obtained for each clone were identified and ordered in accordance with their molecular weight, the one with the largest size being the first. The band pattern obtained was translated into a binary matrix of 0 s and 1 s, where 0 corresponded to the absence and 1 to the presence of this fragment size. The degree of similarity between the clones was obtained by the Jaccard (1908) index, which considers the number of shared bands over the total number of the bands compared. The similarity was estimated based on the following formula:

$$J=a/(a+b+c),$$

where J is Jaccard index; a is the number of bands that

are common to the two compared genotypes; *b* is the number of bands present in the 1st genotype and absent in the 2nd one; *c* is the number of bands absent in the 1st genotype and present in the 2nd one. The sum of *a*, *b*, and *c* will give the total number of the characters measured. The dendrogram was constructed by a cluster analysis using the unweighted pair group method using arithmetic averages (UPGMA) with bootstrap values over 1000 replicates using the packages FreeTree (Manly, 1991; Pavlicek *et al*., 1999).

RESULTS

RAPD analysis

To identify primers that detect polymorphism, 60 primers corresponding to the BF, BG, and BH series (Operon Technologies, Alameda, CA, USA) were screened using genomic DNA from the clones (Table 2). From the set of primers assayed, five were selected, which gave a reproducible positive reaction and polymorphic bands. The sequence of the primers selected and the number of bands amplified are shown in Table 3. With these primers, a total of 252 bands was obtained, 49 of which were sufficiently polymorphic to allow the differentiation of the clones under study. Depending on the primer, 6~16 polymorphic bands were obtained with an average of 9.8.

Fig.2 shows, as an example, the RAPD markers obtained with primer BF14 in the PCR reaction. The size of each polymorphic fragment amplified with the five primers and for all the clones studied is given in Table 4.

The amplicons obtained with the different primers were distributed between 350 and 2300 bp. All the clones exhibited at least one polymorphic band with each primer, e.g., PLUS B with BF07, although in the majority of the cases the primers amplified between 4~9 polymorphic bands. Some bands were common to majority of clones, such as the 2300-bp and 14000-bp fragments obtained with BF13, which were common to 8 clones from all assayed clones.

The results show that the RAPD markers, polymorphic bands, obtained with the primers selected produced a single pattern of bands for each clone assayed, permitting their identification.

Table 2 Properties of the evaluated primers of *Sequoia sempervirens* (D. Don) Endl. in Chile

Primer	PS 5′–3′	Primer	PS 5′–3′	Primer	PS 5′–3′
OPBG-01	GTGGCTCTCC	OPBH-01	CCGACTCTGG	OPBF-01	GGAGCTGACT
OPBG-02	GGAAAGCCCA	OPBH-02	GTAAGCCGAG	OPBF-02	GACACACTCC
OPBG-03	GTGCCACTTC	OPBH-03	GGAGCAGCAA	OPBF-03	TCCCTTGACC
OPBG-04	GTTCCCGACA	OPBH-04	ACCTGCCAAC	OPBF-04	GACAGGTTGG
OPBG-05	CAAGCCGTGA	OPBH-05	GTAGGTCGCA	OPBF-05	CACCCCGAAA
OPBG-06	GTGGATCGTC	OPBH-06	TCGTGGCACA	OPBF-06	TCCACGGGCA
OPBG-07	CAGAGGTTCC	OPBH-07	TGTACGGCAC	OPBF-07	CACCATCGTG
OPBG-08	GACCAGAGGT	OPBH-08	ACGGAGGCAG	OPBF-08	CCTGGGTCCA
OPBG-09	GGCTCTGGGT	OPBH-09	GTCTTCCGTC	OPBF-09	ACCCAGGTTG
OPBG-10	GGGATAAGGG	OPBH-10	GTGTGCCTGG	OPBF-10	GTGACCAGAG
OPBG-11	ACGGCAATGG	OPBH-11	AGCCCAAAGG	OPBF-11	GACGACCGCA
OPBG-12	CCCGAGAAAC	OPBH-12	TCGCCTTGTC	OPBF-12	CTTCGCTGTC
OPBG-13	GGTTGGGCCA	OPBH-13	AGTTGGGCAG	OPBF-13	CCGCCGGTAA
OPBG-14	GACCAGCCCA	OPBH-14	ACCGTGGGTG	OPBF-14	CCGCGTTGAG
OPBG-15	ACGGGAGAGA	OPBH-15	GAGAACGCTG	OPBF-15	ACGCGAACCT
OPBG-16	TGCTTGGGTG	OPBH-16	CTGCGGGTTC	OPBF-16	AGGGTCCGTG
OPBG-17	TCCGGGACTC	OPBH-17	CTCTTACGGG	OPBF-17	CAAGCTCGTG
OPBG-18	TGGCGCTGGT	OPBH-18	GACGCTTGTC	OPBF-18	AGCCAAGGAC
OPBG-19	GGTCTCGCTC	OPBH-19	GTCGTGCGGA	OPBF-19	TTCCCGCACT
OPBG-20	TGGTACCTGG	OPBH-20	CACCGACATC	OPBF-20	ACCCTGAGGA

PS 5′–3′: primer sequence 5′–3′

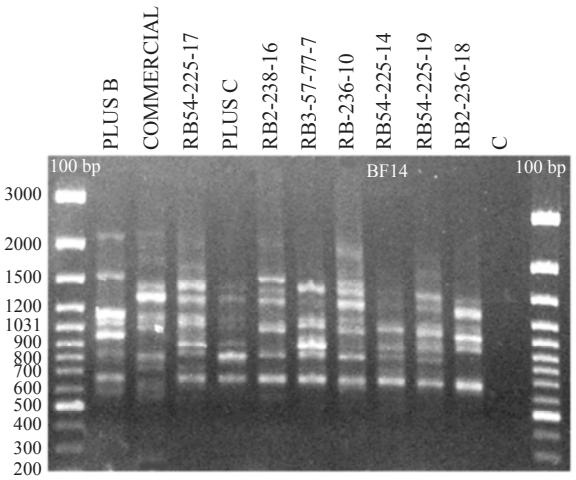

Fig.2 Comparison of RAPD profiles obtained with genomic DNAs from 10 *S. sempervirens* clones with primer BF14. The amplification products obtained in the PCR reaction were separated by electrophoresis in 1.2% agarose gels and stained with ethidium bromide. Each clone is identified with its code, the lane "C" corresponds to the negative control without DNA and lane "100 bp" to the molecular size marker

Genetic similarity and clustering of genotypes

RAPD marker differences among the pooled DNA samples were analyzed to infer genetic relationships among populations (Table 5). The similarity coefficients among clones varied from maximum 0.719 between RB-236-10 and RB2-238-16 clones to minimum 0.306 between PLUS C and PLUS B clones, with the average of 0.502, indicating the high level of genetic variation that exists in the *Sequoia* clones

Table 3 Properties of primers selected of *Sequoia sempervirens* (D. Don) Endl. in Chile

Primer	PS 5′–3′	n_1	n_2
BG 04	GTTCCCGACA	38	8
BF 07	CACCATCGTG	27	6
BF 12	CTTCGCTGTC	71	10
BF 13	CCGCCGGTAA	64	16
BF 14	CCGCGTTGAG	52	9
Overall		252	49

PS 5′–3′: primer sequence 5′–3′; n_1: number of amplified bands; n_2: number of polymorphic bands

Table 4 Size of amplified fragments in base pairs with different primers used*

Clone	Molecular weight (bp)	
	BF07	BF13
PLUS B	650	2300-1900-1700-1400-1300-1100-950-850-650
COMMERCIAL	1500-950-650	2300-1400-1200-950-850-700-650
RB54-225-17	1500-1200-950	2300-1400-1300-1100-950-850-750-700-650
PLUS C	1500-1200-950	2300-1400-1200-850-750-450
RB2-238-16	1500-1200-950-500	2300-1400-1300-1200-1100-950-850
RB3-57-77-7	1500-700-650	2300-1700-1400-1200
RB-236-10	1500-950	2300-1900-1500-1200-950-850
RB54-225-14	1500-950	2300-1700-1400-1200-1100-750
RB54-225-19	1500-1200-950	1200-1100-750-650-550
RB2-236-18	1500-950-700	2300-1400-1200-850-350

Clone	Molecular weight (bp)		
	BF12	BF14	BG04
PLUS B	1400-1300-1200-1031-800-700	1500-1100-1031-950-850-700	1300-1200
COMMERCIAL	1600-1400-1300-1200-1100-950-800-700	1500-1300-1031-800	950-850
RB54-225-17	1600-1300-1200-1100-1031-800-700	1500-1300-1100-1031-900-850-700	1300-1200-850
PLUS C	1600-1400-1300-1200-800-700	1300-900-850-700	1200-950-850-550
RB2-238-16	1600-1400-1300-1200-950-800-700	1500-1300-1031-850-700	1300-1200-950-850-550
RB3-57-77-7	1300-1100-800-700-600	1500-1100-900-850-700	1300-1200-950
RB-236-10	1600-1400-1300-1200-950-800-700	1500-1300-1100-1031-850-700	1300-1200-950-850-550
RB54-225-14	1600-1400-1300-1200-1100-1031-950-800-700	1031-900-850-700	1300-1200-950-850
RB54-225-19	1600-1400-1300-1200-1100-1031-800-700	1500-1300-1031-900-850-700	1300-1200-950-850-550
RB2-236-18	1600-1400-1300-1200-1100-950-800-700	1300-1031-950-900-700	1700-1200-850-700-450

*Molecular weights are ordered decreasedly

Table 5 Gene similarity matrix for pairs of combinations of clones of *Sequoia sempervirens* (D. Don) Endl. in Chile

Clone*	1	2	3	4	5	6	7	8	9	10
1	1									
2	0.371	1								
3	0.559	0.514	1							
4	0.306	0.469	0.529	1						
5	0.486	0.576	0.629	0.645	1					
6	0.419	0.333	0.400	0.387	0.371	1				
7	0.457	0.594	0.556	0.563	0.719	0.424	1			
8	0.441	0.485	0.588	0.600	0.606	0.500	0.576	1		
9	0.378	0.457	0.647	0.613	0.618	0.382	0.588	0.677	1	
10	0.316	0.515	0.447	0.531	0.500	0.353	0.514	0.545	0.432	1

*Clone: 1: PLUS B; 2: COMMERCIAL; 3: RB54-225-17; 4: PLUS C; 5: RB2-238-16; 6: RB3-57-77-7; 7: RB-236-10; 8: RB54-225-14; 9: RB54-225-19; 10: RB2-236-18

selected. Likewise, the similarity coefficients within the most frequent clones used in Chile ranged from 0.306 to 0.559 and 0.333 to 0.594 for PLUS B and COMMERCIAL clones, respectively. Taking the similarity matrix, a dendrogram was set up by means of a UPGMA cluster analysis (Fig.3). The reliability of the dendrogram was determined by bootstrap analysis over 1000 replicates. The results of the cluster analysis show that RAPD profiles obtained from RB-236-10 and RB2-238-16 clones had the highest similarity degree (close to 0.72) forming a cluster with a high bootstrap value (70%). In addition, this cluster appeared related to PLUS C clone, although the association was not supported by the bootstrap value. Another cluster was formed by genotypes RB54-225-19, RB54-225-14, and RB54-225-17, which had a similarity degree of over 0.6 but a bootstrap value of lower than 50%.

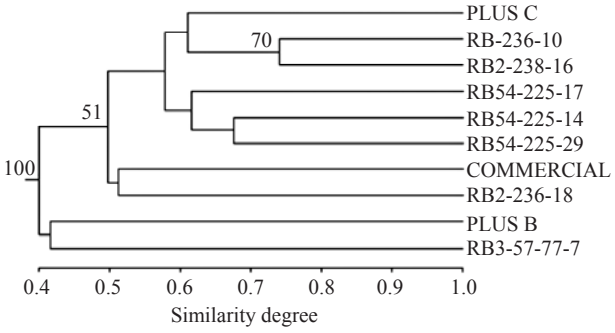

Fig.3 Dendrogram obtained by UPGMA cluster analysis, showing the genetic similarity among the *S. sempervirens* clones used in this study. Bootstrap values for nodes are presented only for those clusters of samples that occurred more than 50% of the time over 1000 replicates

Both clusters formed a separated group from the rest of the clones with a bootstrap value of 51%. Although the clones assayed derived the localities geographically closed, they exhibited enough variability, detected by RAPD marker, to its molecular identification. With the data available, it is not possible to correlate the genetic variability observed with the geographical origin of the clones.

DISCUSSION

Diverse comparative studies have been conducted to assess the genetic variability and population relationships in *Sequoia sempervirens* (Rogers, 1999; 2000; Bruno and Brinegar, 2004; Rogers and Westfall, 2007). Specifically, genetic relationships between old-growth populations have shown a considerable fine-scale spatial genetic structure (Rogers and Westfall, 2007). However, the delimitation of clone variability still is an important problem in forest species (Hansen, 2008).

In this study, RAPD markers were applied. The value of RAPD analysis for efficient germplasm management in plants is already known (Krutovskii *et al*., 1998; Nkongolo *et al*., 2002; Allnutt *et al*., 2003). The use of RAPD markers for phylogeny has been questioned mainly because of the possible lack of homology of comigrating bands (Harris, 1999). Nevertheless, RAPD data have been very helpful for the inference of genetic relationships among closely related species and subspecific taxa (Furman *et al*., 1997; Nkongolo *et al*., 2002).

Apparently, the present work constitutes the first

application of RAPD markers for the study of relationships among *Sequoia* clones in Chile. The RAPD profiles were easy to reproduce by use of a standardized protocol, and consistency was shown in producing identical bands.

Genetic differences among clones were successfully identified with the DNA pooling strategy, and an average of 9.8 polymorphic markers was obtained per primer, which is consistent with the number of markers identified per primer in plants (5~20). Our results show the morphological similarities observed between *Sequoia* populations, which is consistent with previous report (Millar *et al*., 1985; Anekonda *et al*., 1993; 1994). The 49 polymorphic markers obtained in this study represent 19.4% of the total number of bands amplified. It has been possible to find five primers from the Operon Technologies that provided polymorphic bands for *Sequoia* in a RAPD analysis (BG04, BF07, BF12, BF13, and BF14). These molecular markers permit the characterization and detection of the differences and/or similarities between the clones evaluated. We have found that reliable RAPD data can be generated by repeating each amplification reaction three times and using conservative criteria of band selection.

Similarity index and clustering analysis reveal a high degree of differentiation between *Sequoia* clones; however, a larger number of clones are necessary to determine genetic structure. The cluster analysis showed that RAPD markers permitted the characterization of the clones assayed and the quantification of similarities between them (Rogers, 2000). The RAPD grouping of the clones per similarity degree seems to be related to their geographical origin. Namely, the clones RB-236-10 and RB2-238-16 formed a cluster, which had a higher similarity degree between them and was supported by a high bootstrap value. Another cluster was formed by the clones RB54-225-19, RB54-225-14, and RB54-225-17. Both clusters came from Humboldt County and from localities close by. Additionally, two clones (PLUS B and RB-236-10) are clustered more externally in the dendrogram with high bootstrap value, but with a low similarity degree between them. One explanation for this dissimilarity could be that although both clones derived from Humboldt, they are derived from places farther apart (Table 1). Since the clones studied derived from collection of Humboldt County (California, USA) with the same geographical origin, a high similarity between them would be expected; however, the values of similarity obtained suggest that they have enough genetic variability detectable with RAPD markers. However, the small number of clones assayed and their uncertain genetic relationship to one another do the "resolving power" of the RAPD method unknown on this trial, which should be taken into account for future applications (Rogers, 1999).

We conclude that RAPD variation is useful in establishing genetic relationships between *Sequoia* clones. In the future, the inclusion of more clones in the analysis with RAPD markers and the complementing of molecular data with other techniques will improve the resolution of genetic relationships and the potential use in *Sequoia* plantations in Chile. The techniques used in the present study seem to be useful for organization and conservation of *Sequoia* clones collections. PCR-RAPD technique may provide useful information on the level of polymorphism and diversity in *Sequoia*, showing their utility in the characterization of germplasm accessions. The establishment of genetic relatedness and molecular characterization of clone collection in Chile is fundamental as an informational basis for breeding and plantations programs. The results obtained from this study would be useful for better management and identification of clones and also for avoiding mislabelling of the genotypes studied.

References

Ahuja, M.R., Neale, D.B., 2002. Origins of polyploidy in coast redwood (*Sequoia sempervirens* (D. Don) Endl.) and relationship of coast redwood to other genera of Taxodiaceae. *Silvae Genetica*, **51**:93-100.

Albert, F.F., 1908. Algunas observaciones sobre las especies forestales en la sección de aguas y bosques. Santiago, Chile, p.52 (in Spanish).

Allnutt, T.R., Newton, A., Premoli, A., Lara, A., 2003. Genetic variation in the threatened South American conifer *Pilgerodendron uviferum* (Cupressaceae), detected using RAPD markers. *Biological Conservation*, **114**(2): 245-253.

Anekonda, T.S., Criddle, R.S., Libby, W.J., Hansen, L.D., 1993. Spatial and temporal relationships between growth traits and metabolic heat rates in coast redwood. *Canadian Journal of Forest Research*, **23**(9):1793-1798. [doi:10.1139/x93-227]

Anekonda, T.S., Criddle, R.S., Libby, W.J., 1994. Calorimetric evidence for site-adapted biosynthetic metabolism in coast redwood (*Sequoia sempervirens*). *Canadian Journal*

of Forest Research, **24**(2):380-389. [doi:10.1139/x94-051]

Bruno, C., Brinegar, C., 2004. Microsatellite markers in coast redwood (*Sequoia sempervirens*). *Molecular Ecology Notes*, **4**(3):482-484. [doi:10.1111/j.1471-8286.2004.00720.x]

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. *Focus*, **12**:13-15.

Furman, B.J., Grattapaglia, D., Dvorak, W.S., O′Malley, D.M., 1997. Analysis of genetic relationships of Central American and Mexican pines using RAPD markers that distinguish species. *Molecular Ecology*, **6**(4):321-331. [doi:10.1046/j.1365-294X.1997.00183.x]

Grotkopp, E., Rejmanek, M., Sanderson, M., Rost, T., 2004. Evolution of genome size in pines (Pinus) and its life-history correlates: supertree analyses. *Evolution*, **58**(8):1705-1729. [doi:10.1554/03-545]

Hall, G.D., Langenheim, J.H., 1987. Geographic variation in leaf monoterpenes of *Sequoia sempervirens*. *Biochemical Systematics and Ecology*, **15**(1):31-43. [doi:10.1016/0305-1978(87)90077-9]

Hansen, O.K., 2008. Mating patterns, genetic composition and diversity levels in two seed orchards with few clones—impact on planting crop. *Forest Ecology and Management*, **256**(5):1167-1177. [doi:10.1016/j.foreco.2008.06.032]

Harris, S.A., 1999. RAPDs in Systematicsda Useful Methodology? *In*: Hollingsworth, P.M., Batesman, R.M., Gornall, R.J. (Eds.), Molecular Systematics and Plant Evolution. Taylor and Francis, London, p.211-228.

Jaccard, P., 1908. Nouvelles recherches sur la distribution florale. *Bulletin Société Vaudoise des Sciences Naturelles*, **44**:223-270 (in French).

Krutovskii, K.V., Vollmer, S., Sorensen, F.C., Adams, W., Knapp, S., Strauss, S., 1998. RAPD genome maps of Douglas-fir. *The Journal of Heredity*, **89**(3):197-205. [doi:10.1093/jhered/89.3.197]

Kuser, J.E., 1996. Redwood as an Exotic. Conference on Coast Redwood Forest Ecology and Management. Humbolt State University, Arcata, California, the United States, p.55-59.

Kuser, J., Bailley, A., Franclet, A., Libby, W., Martin, J., Rydelius, J., Schoenike, R., Vagle, N., 1995. Early results of a rangewide provenance test of *Sequoia sempervirens*. *Forest Genetic Resources*, **23**:21-25.

Manly, B., 1991. Randomization, Bootstrap and Monte Carlo Methods in Biology, 1st Ed. Chapman and Hall, London, p.300.

Millar, C.I., Dunlap, J.M., Walker, N.K., 1985. Analysis of Growth and Specific Gravity in a 20-Year-Old Provenance Test of *Sequoia sempervirens*. Rep. No. 59. Department of Agriculture, California Agriculture and Experiment Station, Forest Service, Berkeley, CA, USA, Vol. 59, p.1-6.

Moran, G.F., Bell, C., 1987. The origin and genetic diversity of *Pinus radiata* in Australia. *Theoretical and Applied Genetics*, **73**(4):616-622. [doi:10.1007/BF00289203]

Nkongolo, K.K., Michael, P., Gratton, W.S., 2002. Identification and characterization of RAPD markers inferring genetic relationships among pine species. *Genome*, **45**(1):51-58. [doi:10.1139/g01-121]

Noss, R.F. (Ed.), 2000. The Redwood Forest. History, Ecology, and Conservation of the Coast Redwoods. Island Press, p.335.

Osses, A.T., 2004. El estado de las plantaciones de *Sequoia sempervirens* (D. Don) Endl. y su relación con las características de los suelos para localidades de la VII, IX y IX región. Memoria. Ing. For., Fac. Cs. Forestales. Universidad de Chile, p.215 (in Spanish).

Pavlicek, A., Hrda, S., Flegr, J., 1999. FreeTree—Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. *Folia Biologica (Praha)*, **45**:97-99.

Rogers, D.L., 1997. Inheritance of allozymes from seed tissues of the hexaploid gymnosperm, *Sequoia sempervirens* (D. Don) Endl. (coast redwood). *Heredity*, **78**(2):166-175. [doi:10.1038/hdy.1997.24]

Rogers, D.L., 1999. Allozyme polymorphisms discriminate among coast redwood (*Sequoia sempervirens*) siblings. *Journal of Heredity*, **90**(3):429-433. [doi:10.1093/jhered/90.3.429]

Rogers, D.L., 2000. Genotypic diversity and clone size in old-growth populations of coast redwood (*Sequoia sempervirens*). *Canadian Journal of Botany*, **78**(11):1408-1419. [doi:10.1139/cjb-78-11-1408]

Rogers, D.L., Westfall, R.D., 2007. Spatial Genetic Patterns in Four Old-growth Populations of Coast Redwood. *In*: Standiford, R.B., Giusti, G.A., Valachovic, Y., Zielinski, W.J., Furniss, M.J. (Eds.), Proceedings of the Redwood Region Forest Science Symposium: What does the Future Hold? Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, Albany, CA, p.59-63.

Syring, J., Willyard, A., Cronn, R., Liston, A., 2005. Evolutionary relationships among *Pinus* (Pinaceae) subsections inferred from multiple low-copy nuclear loci. *American Journal of Botany*, **92**(12):2086-2100. [doi:10.3732/ajb.92.12.2086]

Toral, M., González, L.Y., Garfias, R., 2005. Secuoya: nueva opción, productos y mercados para el sur de Chile. Universidad de Chile, p.201 (in Spanish).

Tsumina, T., Yoshimaru, K., Tomaru, N., Ohba, K., 1995. Molecular phylogeny of conifer using RFLP analysis of PCR-amplifed specific chloroplast genes. *Theoretical and Applied Genetics*, **91**:1222-1236.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**(22):6531-6535. [doi:10.1093/nar/18.22.6531]