



Linkage mapping of the Mediterranean cypress, *Cupressus sempervirens*, based on molecular and morphological markers

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ABSTRACT. Gene mapping for a *Cupressus* species is presented for the first time. Two linkage maps for the Mediterranean cypress (*Cupressus sempervirens*) varieties, *C. sempervirens* var. *horizontalis* and *C. sempervirens* var. *pyramidalis*, were constructed following the pseudo-testcross mapping strategy and employing RAPD, SCAR and morphological markers. A total of 427 loci (425 RAPDs,

two SCARs) representing parents and F_1 progeny were screened for polymorphism with 32 random decamer and two SCAR primers. A morphological marker defined as “crown form” was also included. Of 274 polymorphic loci, the 188 that presented Mendelian inheritance formed the mapping dataset. Of these loci, 30% were mapped into seven linkage groups for the *horizontalis* (maternal) and four linkage groups for the *pyramidalis* (paternal) map. The putative “crown form” locus was included in a linkage group of both maps. The *horizontalis* and the *pyramidalis* maps covered 160.1 and 144.5 cM, respectively, while genome length was estimated to be 1696 cM for the former variety and 1373 cM for the latter. The four RAPD markers most tightly linked to crown form were cloned and converted to SCARs. Each of the cloned RAPD markers yielded two to three different sequences behaving as co-migrating fragments. Two SCAR markers, SC-D05₄₃₂ and SC-D09₆₆₇, produced amplified bands of the expected sizes and maintained linkage with the appropriate phenotype, but to a lesser extent compared to their original RAPD counterparts. These linkage maps represent a first step towards the localization of QTLs and genes controlling crown form and other polygenic traits in cypress.

Key words: Cypress; Fastigate; Canopy form; Pseudo-test cross; SCAR; RAPD

INTRODUCTION

The Mediterranean cypress (*Cupressus sempervirens*) is a species of great interest in the Mediterranean region for various reasons. Its resilient nature makes it irreplaceable in arid and poor soils, its elegant shape makes it ideal for ornamental purposes, its wind-breaking capacities make it appropriate for agroforestry, and its high-quality wood makes it valuable for timber production. *Cupressus sempervirens* appears in two distinct forms: *C. sempervirens* var. *horizontalis* and *C. sempervirens* var. *pyramidalis*. The former variety forms natural stands, with the westernmost representatives being found on islands in the eastern Aegean Sea. The latter fastigate variety is thought to have arisen through a mutation of the former. Both varieties are naturalized within the species' current distribution. The two varieties present different developmental patterns, representing essentially different architectural models that are thought to have a genetic basis (Sterck, 2005). In *pyramidalis* individuals, lateral branches are smaller, and the crown is narrower. These are features that may improve wood quality and stand densities in afforestations (Hertel and Kohlstock, 1994; Harfouche et al., 2000; Gerendiain et al., 2008). Branching angle and size differences and the concomitant crown dimorphism are characteristics of considerable economic importance, as can be seen in the two varieties of common cypress. Cypress trees have long generation times; this hampers classical breeding programs based on crossing and recurrent selection. However, cypress germplasm is still in the early stages of domestication and exhibits a large amount of genetic variability, which is a posi-

tive characteristic for selection and genetic gains. Because of these features, employing marker-assisted selection would be useful in breeding programs. Seedlings can be pre-selected in the nursery or during *in vitro* culture stages for traits of importance before trees mature. Marker-assisted selection could result in significant temporal or financial gains related to cypress cultivation.

The random amplified polymorphic DNA (RAPD) marker technique generates large numbers of polymorphic molecular markers for genetic mapping and genomic fingerprinting applications (Harfouche et al., 2000). RAPD markers have demonstrated their utility in establishing genomic maps in conifers, particularly in pines (Gomez et al., 2002). Furthermore, the conversion of genetic markers to sequence-characterized amplified regions (SCARs) through the development of longer specific primers from RAPD sequences drastically improves the reproducibility and reliability of polymerase chain reaction (PCR) assays. SCAR markers are sequence specific and produce individual PCR fragments. The SCAR approach has been used successfully to generate variety-specific markers (e.g., in *Olea europaea*; Busconi et al., 2006) for mapping genes affecting plant architecture in peas (Rameau et al., 1998), for sex determination (e.g., in *Pistacia vera*; Yakubov et al., 2005) and to study postglacial recolonization routes (e.g., for *Picea abies*; Scotti et al., 2000). Despite the advent of a new generation of molecular genetic and genomic approaches, linkage maps are widely recognized as essential tools for genetic research and for breeding in many species (Brondani et al., 2006). Linkage maps can be used in molecular breeding for studying genes that control the expression of polygenic quantitative trait loci (QTLs) and for identifying genomic regions underlying QTLs using map-based cloning approaches (Peters et al., 2003). Very little is known about the molecular, genomic and chromosomal organization of the *Cupressus* genus (haploid chromosome number, $N = 12$), and there is no information about the size and length of the cypress genome. Moreover, no physical or genetic maps have been constructed for cypress species, despite the development of tools, such as RAPD (Harfouche et al., 2000) and simple sequence repeat (SSR; Sebastiani et al., 2005; Xu et al., 2008) markers and gene cloning (Pedron et al., 2009). Linkage studies on cypress were initiated in an attempt to identify genetic markers associated with tree crown form (Harfouche et al., 2000). Seven RAPD markers were identified, forming a single linkage group. A genomic region for crown form might be located in an interval within this group (Harfouche et al., 2000). The identification of one such RAPD marker (OPE-12₅₇₀), positioned at a distance of 5.9 cM from the genomic region containing the major gene presumably influencing crown form, was regarded as a first step towards the ultimate identification of important candidate genes for this trait (Harfouche et al., 2000).

We carried out linkage mapping of *C. sempervirens* using molecular and morphological markers.

MATERIAL AND METHODS

Plant material

The mapping population consisted of 55 F_1 individuals derived from a cross between *C. sempervirens* var. *horizontalis* (female parent) and *C. sempervirens* var. *pyrami-*

dalis (male parent). This is a perpetual mapping population located at the “Lapa” experimental plantation of the Institute of Mediterranean Forest Ecosystems and Forest Products Technology, which is located near Patras, Greece. For mapping purposes, the progeny of this cross was considered to be a pseudo-testcross population, as it segregated for the two phenotypes in a 1:1 ratio (Harfouche et al., 2000). The cypress crown form (horizontal or pyramidal) was also included in subsequent analyses as a binary morphological marker (“form” locus; Harfouche et al., 2000).

DNA extraction

Total genomic DNA was extracted as described by Doulis et al. (2000) from needle samples of both parents and progeny using the DNeasy™ Plant Mini Kit (QIAGEN). The DNA yield and purity of each sample were determined using a computerized diode array spectrophotometer.

RAPD markers

Thirty-two 10-base oligonucleotide primers of arbitrary sequence (17 from Operon Technologies and 15 from Pharmacia Biotech) were used for RAPD analysis (Table 1), according to the method described by Harfouche et al. (2000). Images of gels were screened visually, and fragments, whether polymorphic or not, were counted and their size in base pairs was determined using an MS Excel spreadsheet. The repeatability and stability of the results were checked twice using either the same or different extractions. Only bands of equal thickness across the whole progeny were selected for scoring to minimize noise in the dataset due to the potential presence of different homoplastic fragments under a uniquely scored RAPD fragment. Two independent individuals were always used to carry out scoring, and any differences were re-checked and reconciled. Some DNA bands showing polymorphism were omitted from the mapping analysis based on their faint appearance. Hence, three separate datasets were generated: one associated with the horizontal parent, one associated with the pyramidal parent and one including markers present in both parents that segregated in the progeny.

SCAR marker development

SCAR markers were developed by cloning and full-length sequencing from both directions of the previously identified RAPD marker bands and then by designing new SCAR primers from the two ends of the cloned fragment. The RAPD bands to be cloned were identified on gel slabs and were then excised and extracted using the QIAquick Gel Extraction Kit (QIAGEN). Fragments were subsequently cloned into PCR 2.1®-TOPO plasmids (INVITROGEN), following manufacturer instructions. Sequencing of the cloned RAPD fragments was carried from both ends to obtain the entire consensus sequence. The Vector NTI software was used to align and identify RAPD-PCR inserts, as well as to design pairs of oligonucleotide primers at both ends of the cloned sequences. Based on the consensus sequence of four to six independent clones for each of the cloned RAPD product, pairs of SCAR primers were designed and tested. The first primer pair

consisted of the 10 bases of the RAPD primer and was completed by the following 3' base sequence to design a 19-25 mer primer. SCAR amplifications were performed in a Peltier Thermal Gradient 200 Cyclor (MJ Research), following the same conditions that were used for RAPD amplifications, with two modifications: a) the amount of each SCAR primer used was 0.1 μ M, and b) the annealing temperature was increased to accommodate the longer SCAR primers (Table 4). The exact annealing temperature for each SCAR primer pair was determined following gradient analysis according to individual primer length and GC content. Subsequently, maintenance of SCAR polymorphism (with respect to RAPD counterparts) was assessed between the two mapping parents. Once consistency was found between the SCAR and the corresponding RAPD marker, SCAR linkage was assessed by employing the 55 progeny individuals following electrophoretic separation of SCAR products on 1.8% agarose gels and staining with ethidium bromide. Each SCAR marker was named using the primer name and the fragment size in base pairs; for example, SCAR marker SC-D05₄₃₂ corresponds to an SCAR fragment originally amplified as an RAPD sequence by Operon primer OPD5 with an amplified SCAR size of 432 bp.

Analysis of sequence data

The DNA sequences of SCAR markers that maintained linkage to their original RAPD counterparts, specifically Cs_SC-D5_432 and Cs_SC-D9_667, were submitted to GenBank at the National Center for Biotechnology Information (NCBI). Two additional RAPD markers (Cs_OPE12_550 and Cs_OPG04_e_348) that were most closely linked to the crown form locus but had lost their proper polymorphism upon conversion into SCAR markers were also submitted to GenBank in their original RAPD sequence. Subsequently, for the sequences of the two SCAR markers maintaining proper polymorphism, homology searches were performed within GenBank's nucleotide collection "nr/nt database" using the BLAST (Basic Local Alignment Search Tool) application and the Blast algorithm (Altschul et al., 1997).

Linkage analysis and map construction

Prior to linkage analysis, segregation distortion was tested as departure from the 1:1 (testcross markers) or 3:1 (intercross markers) expected ratios, using the chi-square test ($P < 0.05$). Any distorted markers were eliminated from the dataset. Kuang et al. (1999) showed that recombination frequency is not affected by the segregation distortion caused by a viability gene or sampling error. Nevertheless, distorted markers were eliminated based on low marker coverage of the genome and low number of progeny. Linkage analysis was carried out using MAPMAKER/EXP Version 3.0 (Lincoln et al., 1993). The "0" and "1" codes from the binary 1/0 matrices were converted to "H" (heterozygote) and "A" (homozygote), allowing MAPMAKER to compute logarithm of the odds (LOD) scores for backcross data and to identify linkages. An LOD score of 3.0 and a maximum recombination fraction θ of 0.25 were set as linkage thresholds for grouping markers. Map distances in cM were calculated using the Kosambi mapping function.

Genetic linkage maps constructed, using the two-way pseudo-testcross strategy, are genotype specific, in principle. Thus, two separate linkage maps (maternal and pa-

ternal) were constructed using the MAPMAKER software. To investigate the coupling-repulsion phase of the gametes, a dataset with both alternative marker configurations was created. The order of non-distorted markers within each linkage group was approximated using the “compare” and “ripple” (LOD >2) commands to evaluate all three-locus permutations of the framework order. The markers that could not be ordered with equal confidence were placed as accessory markers and localized to the closest framework marker using the “near” command. The error detection function of MAPMAKER was employed to check potential genotyping errors in the framework markers. Unlinked markers were tested at an LOD = 2.0 and at a distance threshold of 40 cM. Those that met the above criteria were placed as accessory markers using the “near” command. The JOINMAP (Van Ooijen and Voorrips, 2001) computer program, version 3.0, was employed to investigate possible intercross marker linkages and to explore the possibility of joining the parental maps. This software was also used to verify the quality of the segregation data and marker order within each linkage group. The results obtained via MAPMAKER and JOINMAP software packages were compared. The marker distribution, marker density and genetic length of both parental maps were estimated and evaluated.

Estimation of genome size and genome length

The number of base pairs of the haploid genome was estimated based on haploid genome mass, Avogadro's number and the average molecular weight of a base pair. The mean of the two *C. sempervirens* 2C DNA mass values available was used. The first value (22.74 pg/2C) was produced following Feulgen microdensitometry (Ohri and Khoshoo, 1986), while the second (19.34 pg/2C) used a flow cytometric method (Pichot C, personal communication).

Genome length was estimated from partial linkage data using the method of moments (Hulbert et al., 1988), which assumes absence of interference and chromosomes of equal length, as modified in method “3” described by Chakravarti et al. (1991): $G = (2MX) / K$, where M = number of informative meioses, Z = threshold value for LOD, K = number of locus pairs, where LOD > Z and X = maximum observed map distance corresponding to the LOD threshold Z. Pairwise combinations of framework map loci at LOD = 3.0 were employed. The number of markers needed to cover a particular genome was estimated from: $k = [-n \ln(1 - P)] / [1 - d / L]^2$, where P = proportion of the genome that needed to be covered, d = specified distance between the markers and L = average chromosome length (Fjellstrom and Parfitt, 1994).

RESULTS

Segregation analysis of RAPD loci

The 32 random decamer primers screened against the cypress parents and progeny amplified a total of 425 RAPD loci that were stable and repeatable; of these, 274 were polymorphic (64.8%). The number of polymorphic bands per primer ranged between 3 and 15 (Table 1), and their size ranged from 178 to 1752 bp. The parental heterozygosity was 23.3% for *C. sempervirens* var. *horizontalis* and 23.5% for *C. sempervirens* var. *pyramidalis*. The markers were classified into two different groups on the basis of the segregation

model (Table 2): 1) loci in a test-cross configuration that were heterozygous in one parent and homozygous null in the other, which included 201 (72% of the total) testcross markers (100 for the maternal data and 101 for the paternal data), and 2) loci in an intercross configuration that were heterozygous in both parents and segregated in a 3:1 ratio, for which 77 markers (28% of the total) were identified (Table 2). When these markers were tested for a 1:1 or 3:1 segregation ratio, 188 markers (68%) were found to show the expected segregation ($\alpha = 0.05$) and 90 markers (32%) showed segregation distortion (Table 2).

Table 1. Random primers, their sequences and detected polymorphism.

Primer	Sequence (5'→3')	Total number of bands	Polymorphic bands	Percentage of polymorphism (%)
OPA-9	GGGTAACGCC	12	10	93.3
OPA-17	GACCGCTTGT	14	9	64.3
OPB-19	ACCCCGAAG	15	4	26.7
OPC-03	GGGGTCTTT	16	7	43.8
OPC-17	TTCCCCCAG	8	6	75.0
OPD-05	TGAGCGGACA	12	11	91.6
OPD-09	CTCTGGAGAC	15	13	86.7
OPE-12	TTATCGCCCC	9	8	88.9
OPF-14	TGCTGCAGGT	9	7	77.8
OPG-04	AGCGTGTCTG	17	15	88.2
OPG-06	GTGCCTAACC	11	7	63.6
OPG-13	CTCTCCGCCA	10	7	70.0
OPH-07	CTGCATCGTG	10	9	90.0
OPI-05	TGTTCCACGG	15	10	66.7
OPJ-07	CCTCTCGACA	15	9	60.0
OPJ-08	CATACCGTGG	5	3	60.0
OPJ-09	TGAGCCTCAC	12	6	50.0
A-12	ATCGCACACT	18	7	38.9
AG-01	AGGTCACTGA	17	7	41.2
AH-01	CACTTCCGCT	15	11	73.3
AH-02	AGAACCGAGG	14	7	50.0
AH-09	AGAACCGAGG	17	12	70.6
AH-11	TCCAACGGCT	17	15	88.2
AH-12	TCCGCTGAGA	14	12	85.7
AH-16	CAAGGTGGGT	14	7	50.0
AH-19	GGCAGTTCTC	15	10	66.7
AH-29	TGGTACTGA	18	12	66.7
AH-30	TGGTACTGT	14	7	50.0
AI-05	GTCGTAGCGG	15	7	46.7
AI-15	GACACAGCCC	11	7	63.6
CD-12	TCCGAGTCTG	7	4	57.1
N-10	ACAACCTGGGG	14	8	57.1
Total		32	425	274

RAPD = random amplified polymorphic DNA; SCAR = sequence-characterized amplified regions.

Table 2. Number and segregation types of markers used for cypress map construction.

Marker type	Marker segregation model			Total
	Testcross (segregation 1:1) female	Testcross (segregation 1:1) male	Intercross (segregation 3:1)	
RAPD	70	68	46	184
SCAR	-	2	-	2
Morphology	1	1	-	2
Distorted	29 (29.0%)	30 (29.7%)	31 (40.3%)	90 (32.4%)
Total segregating	100	101	77	278
Monomorphic				151 (35.2%)
Total				429

RAPD = random amplified polymorphic DNA; SCAR = sequence-characterized amplified regions.

Conversion of RAPD markers to SCARs

Four of the mapped RAPD markers (Table 3, column 1) that were most closely linked to the genomic region associated with form were selected, successfully cloned and sequenced. The termini of all cloned RAPD fragments consisted of their respective 10-mer primer sequences (data not shown). In three of the four selected markers for which there was a single observed RAPD fragment, more than one amplified (homoplasic) sequence was identified (Table 3, column 2). Specifically, two different sequences were identified under the RAPD markers OPD-05₄₄₅ and OPD-09₆₄₀, three under OPG-04₃₃₀ and one under OPE-12₅₇₀. Four to six different independent clones were sequenced per cloned fragment (Table 3, column 3). Subsequently, SCAR primers were designed (Table 3, column 5) and used to assess the consistency of SCAR polymorphism with the original RAPD band (Table 3, column 9). The tested and retained annealing temperatures of the SCAR primers are shown in Table 3, column 8. Employing parental DNA, only two SCAR markers (SC-D05₄₃₂ and SC-D09₆₆₇) exhibited behavior identical to their RAPD counterparts (i.e., maintained proper polymorphism). Their GenBank accession numbers are GU183685 and GU183686 (Table 3, column 10). Linkage of these two SCAR markers to the form locus was subsequently assessed employing the entire progeny. Figure 1 shows such a linkage assessment of SCAR marker SC-D09₆₆₇. Both of the SCAR markers maintaining proper polymorphism were in coupling phase with the allele associated with the pyramidal form, as were their RAPD counterparts (data not shown). The efficiency of converting a multi-locus RAPD marker to a single locus SCAR marker can be assessed by comparing Figure 1 to Figures 1, 3 and 4 presented by Harfouche et al. (2000). For marker SC-D05₄₃₂, the recombination frequency θ between the form locus and the marker increased from 0.236 (RAPD) to 0.345 (SCAR). Similarly, for marker SC-D09₆₆₇, θ increased from 0.135 (RAPD) to 0.340 (SCAR).

Overall, two of the four initially selected RAPD markers were successfully converted into SCAR markers, albeit with a diminution of their linkage to the locus of interest. For the remaining two SCAR markers, proper polymorphism was not detected, and they were not pursued further.

Analysis of SCAR sequence data

The BLAST results revealed that both SCAR marker sequences showed homology with known plant nucleotide sequences at different sequence similarity levels (Table 4). Fragment SC-D05₄₃₂ was highly homologous to two different stretches of DNA of 61 and 62 bp residing in the *Populus trichocarpa* sequencing clone POP094-K03, which are 4789 bp apart. The similarities between the overall SCAR fragment query and the two *P. trichocarpa* subjects were 80 and 77%, respectively. Nevertheless, to avoid redundancy, only one of the two fragments is tallied in Table 4.

Map construction

A total of 142 non-distorted testcross loci, 71 from the female parent and 71 from the male parent (including the crown form locus), were tested for linkage analysis using the

Table 3. Different RAPD marker sequences, primers designed, product sizes, annealing temperatures, and polymorphism of derived SCAR markers.

Initial RAPD marker nomenclature	Different sequences identified	Number of different clones sequenced	SCAR primer nomenclature (top row; forward, bottom; reverse)	SCAR primer sequences (5'→3')	Design ^b	SCAR product size (bp)	Range of annealing temperatures tested ^c employing gradient analysis and (retained; °C)	SCAR polymorphism maintenance ^d	SCAR marker name	GenBank Accession number	
OPD-05 ₄₄₅	I	1	SC-D5-I-F	GAGCGACATCAAGAGAAAAGAATG	24	Extended	54.4-70 (65.8)	Yes	SC-D05 ₄₃₂	GU183685	
			SC-D5-I-R	TGAGCGGACAGAAATAAAAAC	21	Extended					
			SC-D5-II-F	TGAGCGGACAATTGTGTAC	20	Extended	458	54.4-70	No		
OPD-09 ₆₄₀	I	1	SC-D9-I-F	CTCTGGAGACGATCAGAAAAG	22	Extended	56-71.7 (65.5)	Yes	SC-D09 ₆₄₇	GU183686	
			SC-D9-I-R	AGACCAATAGGCTGTGGCATC	21	Extended					
			SC-D9-II-F	TGGAGACCAATAGGCTGTATG	19	Extended	640	56-71.1	No		
OPE-12 ₅₇₀	I	4	SC-D9-II-R	CCTTGACCAATCAACAAGTAG	23	Internal	42-71.1	No		GU183687	
			SC-E12-L-F	TCGCCCTTGGTATATAGCAG	23	Extended	493				
			SC-E12-L-R	TGCAAAAAGATCACCCAAAA	22	Internal					
OPG-04 ₃₃₀	I	2	SC-G4-I-F	AGCGTCTGGGAAATA	17	Extended	56-71.1	No			
			SC-G4-I-R	TGTTTAAACAATGTCAAGATTAGTT	24	Extended					
			SC-G4-II-F	TGCTGTTTATTATTATTAAAGA	25	Extended	307	60.5-70.8	No		
	III	1	SC-G4-II-R	GTTGGTTTCCAAAAGAG	17	Internal					
			SC-G4-III-F	AGCGTCTTGACCACCTTAAC	21	Extended	344	56-71.1	No		GU183688
			SC-G4-III-R	TGCTGAGGAGGGTTTATGTG	21	Extended					

^aSequences in bold are the same as those of the corresponding RAPD primer; ^bInternal SCAR primers are constructed from sequences internal to the RAPD band and contain either a partial (extended - in bold) sequence or no sequence (internal) in common to the corresponding RAPD primers; ^cThe temperatures shown are those allowed by the PTC-200 gradient cycler; ^dSCAR marker polymorphism was tested against the two parental genotypes for agreement with corresponding RAPD markers. RAPD = random amplified polymorphic DNA; SCAR = sequence-characterized amplified regions.

Table 4. Database sequences identical to fragments of the SCAR markers.

Marker sequence range	Homologous sequence origin and identity	Alignment	Identities
SC-D05 ₄₃₂			
176 to 207	<i>Populus trichocarpa</i> predicted protein	GGGTTTAGGGTTAGGTATTTTCATTATGTTGT GGGTTTAGGGTTAGGTATTTTTTGAIGTTGT TTGGTGACGTAGCCFAGG--GATTGGTGAACCAATGTTAAATCTTGGTGCCCTTGTTGCATT-TT	29/32 (90%)
306 to 389	<i>Pinus pinaster</i> SSR	GTGTGATCTAT---TATTTTTT TTTGTGACGTAGCCCAAAATTTGGTTGGTGAACCAACCGTTAAATTTGTTGTCCTCGTGTGCATTA TTTATTTGTTGTGATAIGTTTT-	66/88 (75%)
309 to 368	<i>Populus trichocarpa</i>	GTGGACGTAGCCCTAGGGATTGGTGAACCAATGTTAAATCTTGGTGCCTTGTTGCATT-TT GTGGACGTAGCCCAA---ATTGGTGAACCAACGTTAAATCTTG-IGTCTTATTTCTCATT-	49/61 (80%)
361 to 416	<i>Vitis vinifera</i> whole-genome shotgun sequence	TTCATTTTGTGTGATCTATATTTTTTTTATTTGGTTAATTTGCAATGATGTTT TTCATTTTGTTHAATTTTTAGTTTTTTTTT-TGGTTTTAATGTATTAAATTT	45/56 (80%)
SC-D09 ₆₆₇			
473 to 501	<i>Pinus pinaster</i> SSR	GGTGGAGATGGAGGTGGA--AGGAGTAGAAG GGTGGAGATGGAGGTGGAGTAGGAGTAGAAG	29/31 (93%)
176 to 207	<i>Vitis vinifera</i> whole-genome shotgun sequence	AICTCTTCTCTTTAGTTTGTCTCT AICTCTTCTCTTTAGTTTGTCTCT	26/28 (92%)
340 to 367	<i>Vitis vinifera</i> whole-genome shotgun sequence	AICTCTTCTCTTTAGTTTGTCTCT AICTCTTCTCTTTAGTTTGTCTCT	26/28 (92%)
337 to 385	<i>Arabidopsis thaliana</i> glyceme-rich protein	TGATCTCTTCTCTTTAGTTTGTCTCTTCTTACTAICTAICTAGCT TGTATGTCGTTTCTCTTTAGTTTGTCTCTTTGAAATGATGTAICT	40/51 (78%)

SSR = simple sequence repeat.

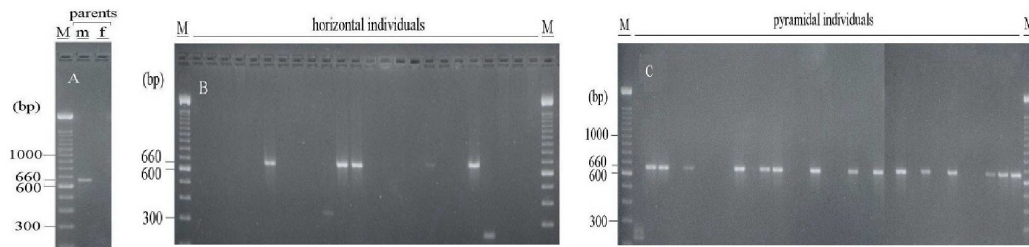


Figure 1. Segregation of the 667-bp SCAR marker SC-D09₆₆₇ among the parents and 55 F₁ individuals of the *Cupressus sempervirens* var. *horizontalis* x *C. sempervirens* var. *pyramidalis* cross using specific SC-D9-I oligonucleotides. **A.** Parents. m = male, f = female. **B.** Horizontal individuals (N = 24). **C.** Pyramidal individuals (N = 31). M = molecular weight marker.

MAPMAKER software. Fifty-six markers (39%) were mapped, while the other 86 markers remained unlinked. A total of 17 originally unlinked markers were found to be linked at an LOD = 2.0 and at a 40 cM threshold distance, and they were added as accessory markers beside their closest framework marker. The alternative marker configuration did not lead to the detection of any additional markers linked in repulsion phase. Two separate maternal and paternal linkage maps were constructed.

Cupressus sempervirens var. *horizontalis* (maternal) map

From a total of 71 loci investigated, 29 loci (41%) were included in the current map, covering a length of 160.1 cM (Figure 2A). The framework loci were mapped into seven linkage groups ranging in size from 5.5 to 28.1 cM (average length of a linkage group = 22.9 cM). The mean distance between the markers was 10.67 cM, and there were 4.1 markers per linkage group on average. A number of markers originating from the bulked segregant analysis (BSA) approach (Harfouche et al., 2000) linked with the form locus were found in the maternal map. They were placed as accessory markers, along with the markers introduced in the map at an LOD = 2.0, near the crown form locus.

Cupressus sempervirens var. *pyramidalis* (paternal) map

Seventy-one loci were used to establish the paternal map. Approximately 38% (27 loci) were located on the framework map. This map was structured into four linkage groups and covers a total length of 144.5 cM (Figure 2B). The size of the linkage groups varied between 21.2 and 55.7 cM, and the average length of a linkage group was 38.1 cM. The average distance between two framework markers was 12.04 cM. A value of 6.8 markers per linkage group reflected marker distribution.

Two marker pairs, markers AH020508 and AH020482 and markers AI150640 and AI150606, appeared only as double-banded phenotypes on the RAPD gels. This result may indicate an earlier duplication event in the respective chromosome regions, as such extremely tight linkage is rather unlikely. For this reason, one of the loci was removed from each of the pairs at random in the dataset. Most of the accessory markers of the first linkage group were very closely linked to the form locus. These markers were placed near the form locus as acces-

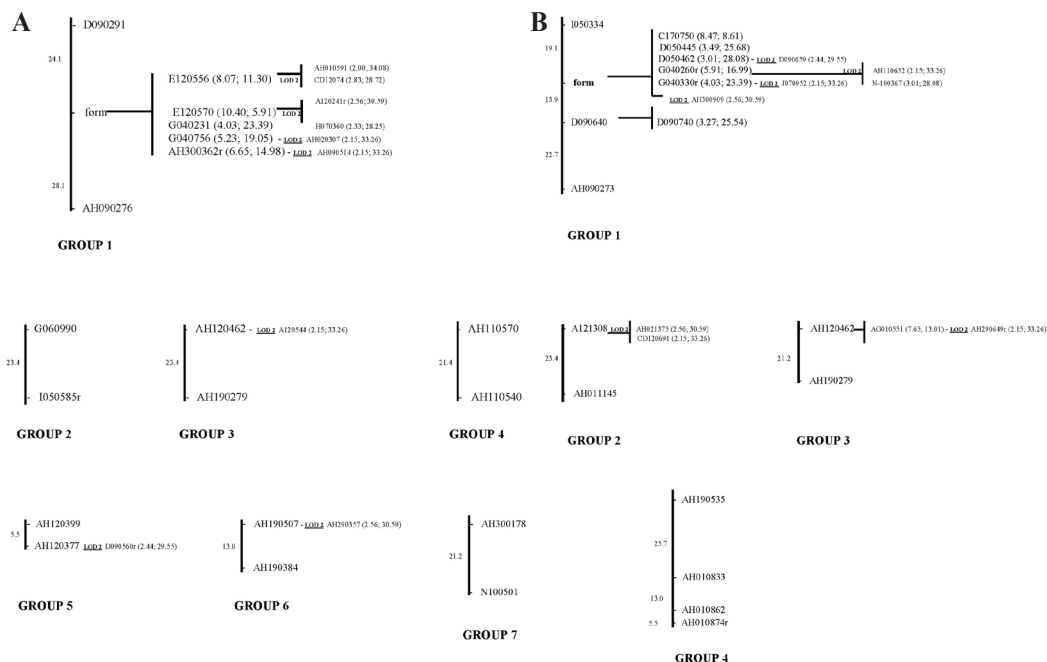


Figure 2. Maps of *A. Cupressus sempervirens* var. *horizontalis* (maternal) and *B. Cupressus sempervirens* var. *pyramidalis* (paternal) constructed employing LOD 3 and $\theta = 0.25$. The names of framework markers are indicated on the right of the linkage groups and are designated by primer identity followed by the molecular weight (bp) of the fragment. Accessory markers were located beside their closest framework markers (at the LOD score and the distance specified in parentheses nearby). Marker names ending with “r” are in reverse linkage phase. The genetic distances in cM determined using Kosambi’s mapping function are indicated on the left side of the linkage groups.

sory markers because linkage analysis showed that they could not be ordered on the map with equal confidence using the “compare” and “ripple” commands of MAPMAKER.

The total mapped lengths obtained for the maternal and the paternal maps, as well as the number of linkage groups established using MAPMAKER and JOINMAP software packages are presented in Table 5. In both cases, an LOD score of 3.0 and the Kosambi mapping function were employed. A comparison between the maps showed that they were very similar in structure. Few differences existed, which mainly involved variations in linkage group length.

Forty-six non-distorted markers in inter-cross configuration were tested for synteny between the parental maps using JOINMAP version 3.0. No syntenic regions could be identified between the maternal and paternal maps. Evidently, there is still a lack of adequate information between both the test-cross and inter-cross marker pairs (lack of syntenic markers). Merging the male and female maps will probably be possible when more inter-cross markers are identified and introduced into the dataset.

Estimation of cypress genome size and length

Based on the average values of the common cypress DNA mass, the haploid genome size was estimated to be approximately 10.39×10^9 bp. Genome length was estimated to be

Table 5. Total mapped lengths and number of linkage groups (LG) obtained (Kosambi mapping function) for maternal and paternal maps using two different software packages.

Maps	MAPMAKER	JOINMAP	Average
Maternal	160.1 cM (4 LG)	131.0 cM (7 LG)	146.5 cM
Paternal	144.5 cM (5 LG)	172.0 cM (5 LG)	158.3 cM
Average	152.3 cM	151.5 cM	

1696 cM for *C. sempervirens* var. *horizontalis* and 1373 cM for *C. sempervirens* var. *pyramidalis*, with an average value of 1534 cM. Based on the total genome length mapped, it can be deduced that the maps cover 9.5% of the *C. sempervirens* var. *horizontalis* genome and 10.6% of the *C. sempervirens* var. *pyramidalis* genome. Average chromosome length was estimated to be approximately 140 cM for *C. sempervirens* var. *horizontalis* and 115 cM for *C. sempervirens* var. *pyramidalis*. To obtain a genome coverage of one marker in every 10 cM, 263 markers are needed for *C. sempervirens* var. *horizontalis*, while 215 markers are required for *C. sempervirens* var. *pyramidalis*. Based on the values of genome size and length, every cM corresponds to approximately 6100 kbp in *C. sempervirens* var. *horizontalis* and 7600 kbp in *C. sempervirens* var. *pyramidalis*. The physical/genetic size ratio was 6.13 Mb/cM for *C. sempervirens* var. *horizontalis* and 7.57 Mb/cM for *C. sempervirens* var. *pyramidalis*.

DISCUSSION

Marker polymorphism and segregation

In the course of this study, 276 polymorphic loci were obtained from 32 RAPD and two SCAR primers, with an average number of 8.4 RAPD loci per primer. When the polymorphic markers were tested for independent segregation, most presented the expected Mendelian ratios. Nevertheless, 32% of the loci presented segregation distortion. Polymorphisms that showed significant departures from Mendelian expectations have been reported for all types of markers (RFLP, RAPD, SCAR, AFLP, microsatellite, isoenzyme) used in crops and forest trees (Aravanopoulos et al., 1993; Kuang et al., 1999), but the causes of this phenomenon are not well understood. The percentage of loci showing segregation distortion is highly variable in forest trees: 69% in *Cryptomeria japonica* (Nikaido et al., 1999), 20% in *Quercus robur* (Barreneche et al., 1998), 18% in *Pseudotsuga menziesii* (Krutovskii et al., 1998), 14% in *Populus* (Bradshaw et al., 1994), and 15% in *Salix exigua* (Aravanopoulos et al., 1993). Compared to these data, the distortion frequency observed in our study appears to be higher than expected by chance alone, suggesting that biological or technical factors also influence these ratios (Krutovski et al., 1998). Segregation distortion in conifers has frequently been attributed to a large number of recessive embryonic lethal genes, which usually maintain high genetic loads (Mukai et al., 1995). Other reasons for such distortion include chromosome loss (Bradshaw et al., 1994), RAPD marker size homoplasy (Studer et al., 2006) and genotyping and scoring errors (Plomion et al., 1995). Additionally, segregation distortion may be due to linkage to other loci that can apparently distort segregation due to some form of pre-zygotic or post-zygotic selection (Kuang et al., 1999; Gomez et al., 2002). The latter explanation may represent the case in this particular cross, which involved the typical form of a species and a

naturalized variety that presumably arose by mutation and was subsequently established by artificial selection. However, a specific experimental design and complementary analyses will be necessary to investigate this hypothesis.

Linkage analysis and marker ordering were performed, considering only non-distorted markers. Bailey (1961) showed that linkage investigation can be safely performed when one locus participating in the joint segregation analysis presents significant segregation distortion. However, he suggested that when both loci are distorted, spurious linkages may be created, and thus, joint analysis should be avoided. Recent studies indicate that distorted loci can often be mapped and that recombination frequency is thought not to be affected by segregation distortion (Kuang et al., 1999). Nevertheless, the inclusion of such loci can weaken map structure (Krutovski et al., 1998), particularly when progeny size is rather small, and distorted loci were excluded from our dataset for this reason.

SCAR markers

Size differences between the initial RAPD markers and their corresponding SCAR markers can be accounted for on the basis of exact sizing (upon sequencing), on different amplified fragments underlying the same apparent RAPD marker (homoplastic fragments) or on alternate SCAR primer positions with respect to the cloned RAPD fragment (Table 3, columns 1 versus 7).

The two highly homologous sequences of 61 and 62 bp found in *P. trichocarpa* extend beyond the RAPD primer annealing positions and can be inferred to be forward repeats. If these repeats are confirmed in *P. trichocarpa*, assessing their presence in the cypress genome may be attempted. Additionally, both SCAR fragments contained sequences homologous to regions involving SSR of *Pinus pinaster*, indicating a possible link between inverted repeats (as those revealed by RAPD amplifications) and SSR regions in genomes, which might point towards a mechanism of SSR formation.

Genetic maps

A total of 71 loci from the male parent and 71 loci from the female parent were investigated for linkage analysis. The cypress tree genome is organized into 12 pairs of chromosomes; therefore, 12 linkage groups are expected. Seven linkage groups were obtained for the maternal map and four for the paternal map, which reflects low marker coverage. The length of the maps, which were 144.5 cM for the male parent and 160.1 cM for the female parent, showed that approximately 10% of the estimated genome length was covered on average. The number of polymorphic loci needed to obtain typical map coverage of one marker in every 10 cM (some 200-300 markers) is within reach, given the number of polymorphic loci per primer and the heterozygosity levels of the parents.

Thirty-nine (21%) polymorphic markers were mapped with an LOD score of 3.0 and a recombination fraction of 0.25. These values proved to provide adequate threshold criteria for map construction, taking into account the maintenance of a low number of unlinked markers and the distances between the framework markers. Most of the accessory markers of the first linkage group were very closely linked to the form locus. This was expected because these markers were produced by the bulked segregant analysis approach (Harfouche et al., 2000).

The remaining 149 polymorphic markers could not be mapped with the above thresholds to any of the current linkage groups. Possible explanations for the high number of unlinked markers could be the relatively low number of markers used for constructing the maps and the small size of the available mapping population. The two SCAR markers derived from RAPD markers were not incorporated into a linkage group in the present maps. A number of potential explanations may underlie this finding, such as RAPD to SCAR conversion problems, including problems related to the small population size, improper segregation after the conversion of RAPD into SCAR markers (increased θ), or low coverage of the map (Weng et al., 1998).

An intermediate evaluation of the linkage groups based on 58 polymorphic loci also resulted in a large number of unlinked markers. Inclusion of additional markers had little effect on the number of loci that remained unlinked. After a reduction of the LOD score to 2.5, the number of unlinked markers decreased slightly, and they were considered to not provide any additional information about the cypress genome coverage (results not shown). Similar results were reported for the Douglas fir, in which analysis of 50 additional markers did not greatly reduce the number of unlinked markers (Jermstad et al., 1998), and in lettuce (Kesseli et al., 1994). Linkage of the 149 originally unlinked markers was investigated further using an LOD score of 2.0 and a maximum distance of 40 cM. Seventeen markers (11%) were joined to the existing linkage groups as accessory markers; hence, the total number of mapped markers reached 56 (30% of the total). The accessory markers might be integrated into the linkage groups as framework markers when additional codominant (SCAR, SSR, SNP) markers become available.

The number of linked markers detected per linkage group ranged between 2 and 15. The differences between the expected and observed values may indicate a non-random distribution of RAPD markers in the cypress genome. Indeed, a portion of the original sampling of markers was not distributed randomly by definition, as these loci were selected after the application of the BSA approach (Harfouche et al., 2000). Therefore, a large number of markers may be expected to cluster around this region. Finding a non-random distribution of the markers could have several causes, such as recombination suppression around centromeres, large monomorphic segments of the parental genomes for the markers tested, hot-spots of recombination that may introduce genetic gaps, and genomic regions that may lack accessible markers (Kesseli et al., 1994). A map that is denser than the present one and that covers all chromosomes will allow testing these hypotheses. The maximum distance between adjacent markers was 25.7 cM for the paternal map and 28.1 cM for the maternal map. Additional markers could fill in these gaps in future studies. Liu (1998) described an ideal map as having a high level of confidence in the estimated locus order, with markers being evenly distributed on the map with sufficient density (at least one marker in each 5 cM segment).

The two parental maps obtained using the MAPMAKER software were compared to the maps obtained with the JOINMAP software, and their similarity was confirmed. The maps presented similar structure and the number of the mapped loci was almost the same. There were minor variations among the maps with respect to their lengths and the number of linkage groups obtained. The discrepancy between map lengths obtained with MAPMAKER and JOINMAP can be regarded as a consequence of the different algorithms employed in the calculation of map length. Furthermore, possible deviations in the levels of true interference in the two parental genomes compared to the interference assumed by the mapping function employed may contribute to the observed differences. Qi et al. (1996) and Sewell et al. (1999), who also used the Kosambi mapping function, reported the same phenomenon.

One of the objectives of our study was to provide better map coverage for the genomic area associated with crown form and to construct a framework for future genetic studies, particularly for localizing QTLs, genes and mutations controlling tree architecture in *C. sempervirens*. Harfouche et al. (2000) found seven markers linked to a genomic region associated with crown form. Increased mapping coverage was achieved in the present study. The pertinent linkage group included 13 markers in the *horizontalis* (maternal) map and 14 in the *pyramidalis* (paternal) map. Slight mapping distance differences were also observed to be related to the use of different software algorithms in the two cases and to increased marker coverage in the present analysis. However, no new marker was mapped closer to the crown form locus, and the nearest marker remains at 5.9 cM, as reported by Harfouche et al. (2000) and verified in our study. Particular morphological and geometrical features that may be controlled by a number of genes characterize the two varieties (*horizontalis* and *pyramidalis*) of the cypress tree: relative branch length, branch rectitude or curvature, initial branch insertion angle, and the occurrence and importance of reiteration. Similarly, Norway spruce narrow crowned phenotypes (*Picea abies* f. *pendula*) present decreased fiber length and stem volume, but higher wood density and reduced sensitivity to competition under dense spacing conditions (Gerendiain et al., 2008). Cypress crown form may be related to apical dominance and apical control and, at the physiological level, to gradients and fluxes of signaling hormones, such as auxin (Sterck, 2005), and transcriptional regulators may also play a role (Doebley and Lukens, 1998). QTLs for tree architecture have been mapped in *Malus domestica* (Segura et al., 2009) and related plant architecture QTLs have been mapped in a number of crop species (e.g., Li et al., 2008; Song and Zhang, 2009). As crown form and, especially, branch diameter and angle are significant determinants of technical wood quality and quantity, the establishment of molecular markers linked to form is of paramount practical importance. Such markers would be useful for the early selection of nursery or *in vitro* cultured individuals designated for reforestation and landscape architecture applications (Harfouche et al., 2000).

Genome size and length

The estimates of genome length for *C. sempervirens* var. *horizontalis* reported above (about 1700 cM) place cypress within the range of values reported for conifers, although with a lower than average value. After reviewing the relevant literature, for an LOD = 3.0 and the Kosambi mapping function, we arrived at a value of approximately 2000 cM for the average genome length in conifers. However, the genome length of *C. sempervirens* var. *pyramidalis* was found to be shorter than this value (about 1370 cM), indicating a reduced recombination frequency, possibly associated with the origin of the *pyramidalis* variety, which is a naturally derived mutation. The physical/genetic size ratio was 6.13 Mb/cM for *C. sempervirens* var. *horizontalis* and 7.57 Mb/cM for *C. sempervirens* var. *pyramidalis*. These values are much higher than the average of 0.46 Mb/cM calculated for angiosperms from data reported by Chagné et al. (2002) but lower than a corresponding value of 13.07 Mb/cM calculated for pines from data reported by the same authors. The observed relationship between genome length and genome size also indicates that even if further mapping efforts reduce the distance between a genetic marker and the crown form locus to 0.5 cM, there will still be approximately 3000 kbp (maternal map) between the marker and the trait, which represents a considerable obstacle for detailed molecular studies, such as involving positional cloning. In general, the estimated high physical/genetic size ratio makes the candidate gene approach more appealing for identifying QTLs and developing diagnostics for plus-tree selection in cypress. As future maps will

provide better coverage, the estimates of genome length will become more precise, and the findings presented above will be subjected to a more robust evaluation.

In general, it has been demonstrated that RAPD markers can be an efficient first step towards establishing a genetic map for previously unstudied species. Testing 198 random decamer primers in two bulk DNA samples, Harfouche et al. (2000) found that 196 (99%) primers were associated with amplification of DNA products. The random decamers used revealed a high number of polymorphic bands per primer and considerable variability in fragment size.

The next step in cypress genomic research will be to bridge the parental maps and increase mapping coverage. Building a consensus map is a long-term goal. A consensus map will be of notable value in identifying QTLs of interest, not only those involved in tree architecture, but also QTLs involved in the resistance of cypress to the fungus *Seiridium* spp, among others. The development of larger mapping families is an important prerequisite for such advances. Detailed molecular linkage maps are expected to contribute to our understanding of the genome structure, function and evolution of this Cupressaceae species, especially in conjunction with other Cupressaceae families (Ahuja, 2009). Additionally, they will provide a powerful tool for marker-assisted breeding in cypress trees, bypassing traditional phenotype-based selection methods.

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