

IDENTIFICATION OF RAPD MARKERS ASSOCIATED WITH CROWN FORM IN *CUPRESSUS SEMPERVIRENS* BY BULKED SEGREGANT ANALYSIS

A. L. Harfouche^{1,4}, F. A. Aravanopoulos^{2,*}, A. G. Doulis¹ & S. Xenopoulos³

¹⁾ Department of Environment and Renewable Resources, Mediterranean Agronomic Institute of Chania, P.O. Box 85, GR–73100 Chania, Greece (e-mail: adoulis@maich.gr).

²⁾ Laboratory of Forest Genetics and Tree Breeding, Department of Forestry and Natural Environment, P.O. Box 238, Aristotle University of Thessaloniki, GR–54006 Thessaloniki, Greece (e-mail: aravanop@for.auth.gr).

³⁾ Institute of Mediterranean Forest Ecosystems and Forest Product Technology, Terma Alkmanos, Ilisia, GR–11528 Athens, Greece.

Present address:

⁴⁾ Dipartimento di Protezione delle Piante, Università degli Studi della Tuscia, via S. Camillo de Lellis, I–01100 Viterbo, Italy (e-mail: aharfouc@unitus.it).

* To whom correspondence should be addressed

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ABSTRACT

Common cypress (*Cupressus sempervirens* L.) appears in two varieties: (a) *C. sempervirens* var. *pyramidalis* and (b) *C. sempervirens* var. *horizontalis* which present different crown forms. The phenotype of the former variety is characterized by columnar or narrowly conical habit, whereas the phenotype of the latter is characterized by broadly spreading branches. Bulk segregant analysis was used to identify random amplified polymorphic DNA (RAPD) markers associated with crown form. The two pools of individuals from an F₁ population segregating for crown form, and both parental clones were screened with 198 10-mer primers. Out of seven markers that exhibited joint segregation with the trait of interest, one RAPD marker (OPE-12₅₇₀) was found to be tightly linked to a genomic area associated with crown form. The marker was present in the “*horizontalis* form” bulk and parent and absent in the “*pyramidalis* form” bulk and parent. Further analysis of individual progeny clones, in this particular genetic background, showed that the mapping distance between OPE-12₅₇₀ and the above genomic area was 5.9cM. The tight linkage of this marker to a presumably major gene influencing crown form is an important first step towards the ultimate identification and cloning of such a gene. It may further permit early selection of forms at nursery or *in vitro* culture stage particularly in this family. It might also facilitate breeding programs and studies on the introgression of the two forms in natural populations.

Keywords: *Cupressus sempervirens*, crown form, bulked segregant analysis, RAPD markers, breeding programs.

INTRODUCTION

Crown form variability in cypress (*Cupressus sempervirens* L.; *Cupressaceae*) has been recognized since the time of Theophrastus (4th century BC) and yields two typical varieties: (a) variety *horizontalis* with broad spreading branches, broadly conical crown, and wide angles (approaching vertical) between branches and stem, and (b) variety *pyramidalis* with columnar or narrowly conical crown habit forming acute angles between branches and stem. Cypress natural populations are comprised by the *horizontalis* variety. The variety *pyramidalis*, a popular ornamental variety, is the most widely planted of all cypresses (JOHNSON 1974). Often *C. sempervirens* var. *pyramidalis* forms naturalized stands derived from a number of introduced individuals.

The two varieties are interfertile and can give progenies which, apart from the parental forms, present different intermediate types of crown structure (PANETSOS 1967). The two varieties are characterized by some particular morphological and geometrical features: relative branch length, branch rectitude or curvature, initial branch insertion angle, occurrence and importance of reiteration. As crown form and especially branch 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 in early selection of nursery or *in vitro* cultured individuals designated for reforestation and landscape architecture applications. The existence of the two crown varieties provides an excellent model framework into which genetic and molecular hypotheses concerning crown

form control could be assessed. Conclusions could be subsequently evaluated or extended to other forest or horticultural species in which plant form is of economic importance.

Forest trees have long generation times and are still in the earliest stages of domestication. They are typically highly heterogeneous and few extended pedigrees are available. Because of these limitations, there is a relatively greater potential for DNA markers to improve genetic analysis and to accelerate breeding in forest trees, compared to highly domesticated crops. However, DNA markers have not been extensively applied to practical tree improvement due to technical and theoretical limitations, such as high levels of heterozygosity and marker linkage equilibrium in populations (STRAUSS *et al.* 1992). Recent progress has shown that PCR based DNA markers can be widely used to map individual forest trees. Some quantitative trait loci (QTLs) have already been identified in forest trees for commercially important traits. Randomly amplified polymorphic DNA genetic markers (RAPDs) were used to rapidly identify markers linked to genes or genomic regions of interest by bulked segregant analysis (MICHELMORE *et al.* 1991). Using this method WILCOX *et al.* (1996) identified a dominant gene that confers resistance to fusiform rust disease in loblolly pine by genomic mapping. In eucalypts, RAPD markers were used for genetic mapping of QTLs controlling vegetative propagation (GRATTAPAGLIA *et al.* 1995), while in oaks the molecular differentiation between *Q. petraea* and *Q. robur* was evaluated with RAPD markers (MOREAU *et al.* 1994). In the present study we used bulked segregant analysis (BSA) of RAPD products to identify markers linked to tree form and branch habit gene(s) in cypress.

MATERIALS AND METHODS

Plant material

A full-sib family, resulting from a cross of a *C. sempervirens* var. *horizontalis* and a *C. sempervirens* var. *pyramidalis* individual, segregating in a 1:1 ratio ($\chi^2 = 0.891$, $p = 0.345$) of the parental forms was used. In particular the progeny of this cross consisted of 24 *horizontalis* and 31 *pyramidalis* individuals. The material was derived from an experimental plantation of the Institute of Mediterranean Forest Ecosystems and Forest Products Technology, near Patras, Greece. Fifty-five full-sib progeny as well as parental individuals were sampled for genetic analysis. This full-sib family was regarded as representing a pseudo-testcross population for BSA purposes. The age of the progeny (6-year

old) was considered as adequate for full expression of the tree form, which was scored according to the Bouroulet test (BOUROULET *et al.* 1993).

DNA extraction and quantification

Genomic DNA from the two parent trees and the 55 progeny individuals was isolated according to DOULIS *et al.* (2000). The protocol for isolation of DNA from cypress needles with the QIAGEN DNeasy Plant Mini Kit (Cat. 69104) was modified and optimized for 100 mg of starting material. Needles were ground under liquid nitrogen and a volume of 400 μ l of buffer AP1 (lysis buffer) and 4 μ l of RNase A stock solution (100 mg \cdot ml⁻¹) were added to the ground plant tissue, vortexed vigorously, incubated for 10 min at 65 °C and mixed for 2–3 times by tube inversions. A volume of 130 μ l of buffer AP2 (precipitation buffer) was added to the lysate, mixed, and incubated for 5 min on ice. The solution was centrifuged for 5 min at maximum speed using a Sorvall MC microcentrifuge. A volume of 570 μ l of the lysate was applied to the QIAshredder™ spin column and centrifuged for 2 min at maximum speed. The flow-through fraction of a volume of 430 μ l was transferred to a new Eppendorf tube, then 215 μ l of buffer AP3 (binding buffer) plus 430 μ l ethanol were added, and mixed. Of the above mixture 650 μ l were applied onto DNeasy mini spin column and centrifuged for 1 min at 8000 rpm. The procedure was repeated for the remaining 425 μ l. The DNeasy column was placed in a new collection tube, and a volume of 500 μ l of buffer AW (washing buffer) was added onto the column and centrifuged for 1 min at 8000 rpm. This step was carried out twice. The column was transferred to a new collection tube and spinned for 5 min under a vacuum, to ensure complete dryness of the column membrane. Lastly, two elutions with 100 μ l each of buffer AE (elution buffer), were made by using two different Eppendorf tubes, resulting in 200 μ l DNA stock solution.

Bulked segregant analysis

Two bulked DNA samples from eight *pyramidalis* and eight *horizontalis* progeny were constructed (MICHELMORE *et al.* 1991) from the segregating population, each bulk therefore representing the alternate phenotypic states of the loci controlling the selected trait against a random genetic background of several loci. Equal amounts of DNA from 8 individuals (200 ng DNA per individual) with identical form (*pyramidalis* or *horizontalis*) were mixed to create the respective bulks. One hundred ninety-eight decamer primers

(Operon Technologies, Alameda, California) were used to screen the parents and the two bulked DNA samples based on the method of WILLIAMS *et al.* (1990) with minor modifications. Initially, four PCR amplifications were performed with each primer using DNA from (1) the *horizontalis* bulk, (2) the *pyramidalis* bulk, (3) the *horizontalis* parent, and (4) the *pyramidalis* parent. The presence of a RAPD fragment in one bulk and the corresponding parent and its absence in the others provided evidence for a marker putatively linked to crown form.

RAPD assays

RAPD reactions were completed in a Gene Amp® PCR System 2400 (PERKIN-ELMER®) in PCR certified cycle plates TM-24 (Robbins Scientific). The thermal cycles used were: 1 cycle of 4min at 97.5 °C, followed by 45 cycles of 1min at 94 °C, 1min at 40 °C and 1min at 72 °C, and finally 1 cycle of 4min at 72 °C for the final extension. For a reaction volume of 25 µl, the following were used: 20 ng DNA template, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM Operon primer, 2.5µl 10× Stoffel buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl), 4 mM MgCl₂, 0.2 units of Stoffel fragment.

Scoring and analyzing RAPD data

Amplified PCR products (i.e., RAPD bands) were resolved using 1.8 % (w/v) agarose 1X TBE gel containing 0.1µg·ml⁻¹ ethidium bromide and electrophoresed under constant voltage (90 V) for 3.5 h in a horizontal gel electrophoresis system (20 × 25 cm gel mould) which can accommodate 60 samples simultaneously. A 100 bp DNA standard (GIBCO-BRL) was used to assign base pair size to individual RAPD bands. Photographs of each gel were taken in UV transilluminator using polaroid film (type 667). Gels were screened visually for the presence of polymorphic bands in the *horizontalis* bulk and *horizontalis* parent and their absence in the *pyramidalis* bulk and *pyramidalis* parent, or vice versa. For each primer yielding possible polymorphisms, duplicate PCR reactions were run to exclude false positives. In case of a confirmed result of the BSA further PCR reactions were performed using the DNA of the 24 F₁ *horizontalis* and the 31 F₁ *pyramidalis* individuals, in order to determine the linkage of the PCR product to the target genomic region.

The RAPD markers were named according to the convention used by PARAN *et al.* (1991). For example, the fourth primer in Operon Kit H yielding a RAPD

fragment with a size of 800 bp would be termed “OPH-04₈₀₀”.

Linkage analysis

Linkage was tested based on joint segregation and independent assortment using the χ^2 test. When linkage was detected maximum-likelihood estimation was used to calculate the recombination frequency and the associated standard error (ARAVANOPOULOS 1998). In the consideration of linkage we introduced two additional thresholds: (1) the recombination frequency to be $\theta < 0.25$, (2) the associated probability to be $p < 0.01$. Mapping distances were estimated by using the function suggested by KOSAMBI (1944), with a standard error according to OWEN (1950).

RESULTS AND DISCUSSION

Bulked segregant analysis for screening candidate markers

Previous attempts to dissect the genetic control of crown form in cypress (PANETSOS 1967; BASSIOTIS 1985) suggested possible hypotheses (PANETSOS 1967), but were not conclusive. In the present study, BSA was used as a rapid way to identify markers linked to a genomic region associated with crown form in the common cypress. Of the 198 random decamer primers used in the bulked segregant analysis, 196 (99%) revealed amplified DNA products in the tested family, while the other 2 failed to amplify. The primers amplified a total of 1632 fragments of scorable quality, producing an average of 8.1 scorable fragments per primer (Fig. 1). During this survey we identified 403 polymorphic fragments that distinguished the *horizontalis* from the *pyramidalis* parent. Nevertheless, only 3 were specific for the *horizontalis* parent and the *horizontalis* bulk, and 5 for the *pyramidalis* parent and the *pyramidalis* bulk, yielding a total of eight putative markers.

Confirmation of linkage for putative markers

The presence of a fragment in one bulk and corresponding parent and absence in the other bulk and parent is an indication for a putatively linked locus, as shown for fragment OPE-12₅₇₀ (Fig. 2). The primers that produced phenotype-specific RAPD fragments are presented in Table 1. The distribution of the eight polymorphic fragments within each bulk was determined following amplifications of individual genotypes (Table 2).

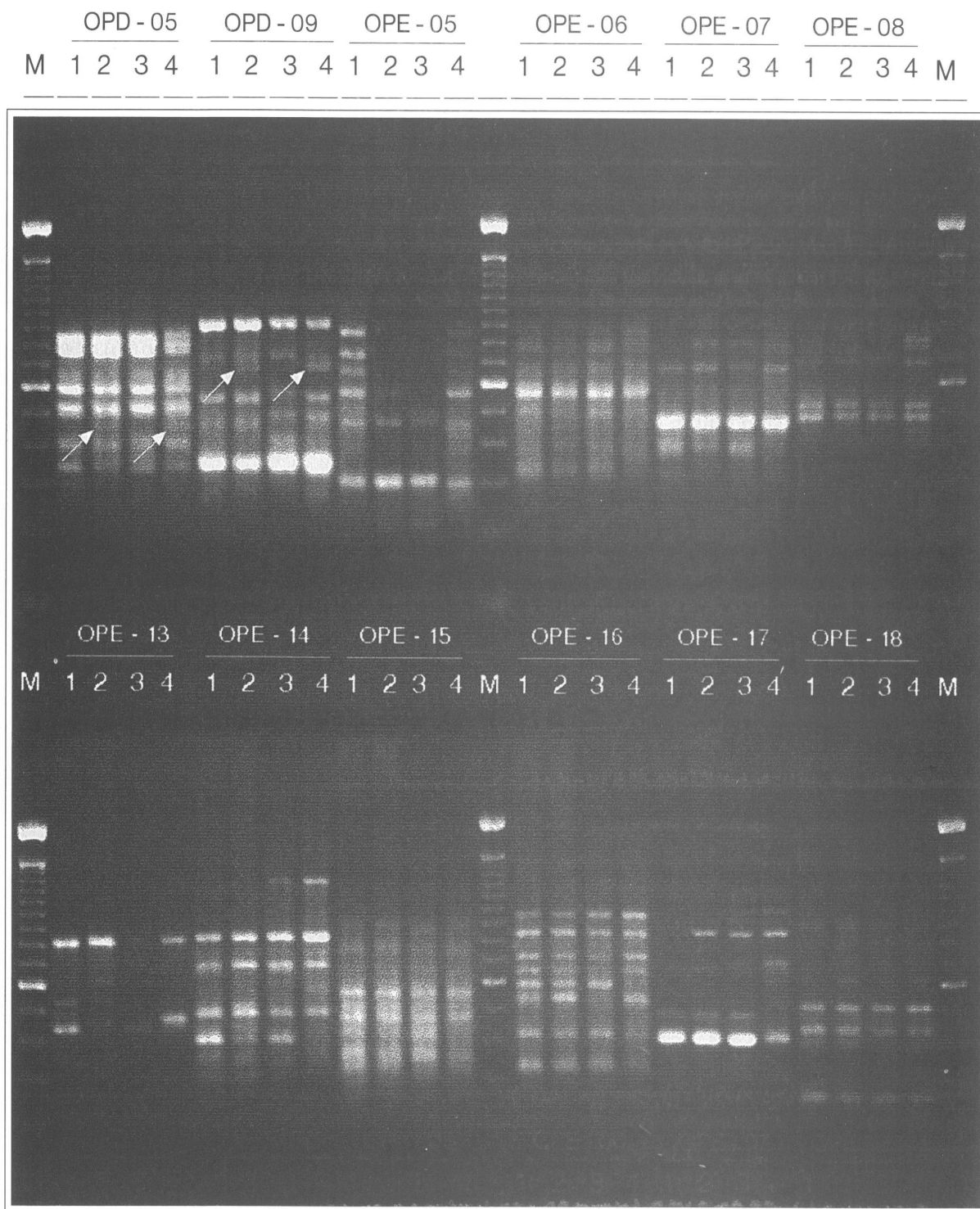


Figure 1. Bulk and parent samples of DNA from *horizontalis* and *pyramidalis* individuals used to screen 198 RAPD primers. Amplification products from 12 primers are shown. Any difference between the *horizontalis* bulk, *horizontalis* parent, and the *pyramidalis* bulk, *pyramidalis* parent identifies a putatively linked marker, such as with OPD-05 and OPD-09 which are indicated by arrows. Lane M; 100bp DNA ladder, 1 *horizontalis* bulk, 2 *pyramidalis* bulk, 3 *horizontalis* parent, 4 *pyramidalis* parent.

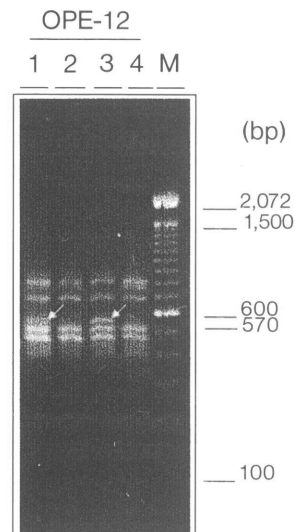


Figure 2. Amplification pattern using RAPD primer to identify the OPE-12₅₇₀ marker linked to crown form. Lane M; 100bp DNA ladder, 1 *horizontalis* bulk, 2 *pyramidalis* bulk, 3 *horizontalis* parent, 4 *pyramidalis* parent.

Linkage analysis

Estimates of recombination fractions between the genomic region responsible for crown form (major QTL or single gene) and the eight putative markers are shown in Table 3. Out of the eight putative markers, seven were found to present some degree of linkage to crown form according to the χ^2 test. Results for primer OPG-13₁₂₃₀ indicated absence of linkage since the associated p-value ($p = 0.056$) was slightly above the nominal level of significance. Only three markers met our threshold criteria. Primer, OPE-12, produced a 570bp fragment which is unique in the *horizontalis* bulk, and *horizontalis* parent as well as in 22 *horizontalis* individuals that amplified (Fig. 3). This marker produced only three recombinants out of 29 in the *pyramidalis* individuals that amplified (Fig. 4), indicating a fairly tight linkage between the marker and crown form. The mapping distance between OPE-12₅₇₀ and the genomic region controlling crown form was 5.9cM

Table 1. RAPD markers associated with crown form as indicated by bulked segregant analysis.

Putative markers		
Operon Primer	Size (bp)	Sequence of primer
OPC-17	800	5'-TTCCCCCAG-3'
OPC-17	750	5'-TTCCCCCAG-3'
OPD-05	445	5'-TGAGCGGACA-3'
OPD-09	740	5'-CTCTGGAGAC-3'
OPD-09	640	5'-CTCTGGAGAC-3'
OPE-12	570	5'-TTATCGCCCC-3'
OPG-13	1230	5'-CTCTCCGCCA-3'
OPH-07	360	5'-CTGCATCGTG-3'

(Table 3). Linkage of the other two markers (OPD-09₆₄₀, OPD-05₄₄₅) appeared to be of medium strength (mapping distances 13.8 cM and 25.6 cM respectively). Based on the relative mapping distances among genetic markers (ARAVANOPOULOS *et al.*, manuscript in preparation) it can be deduced that the genomic region controlling crown form is flanked by markers OPE-12₅₇₀ and OPD-09₆₄₀, while marker OPD-05₄₄₅ is located further at a distance of about 20 cM, from the OPE-12₅₇₀ marker.

Genetic analysis of tree-form traits and identification of RAPD markers

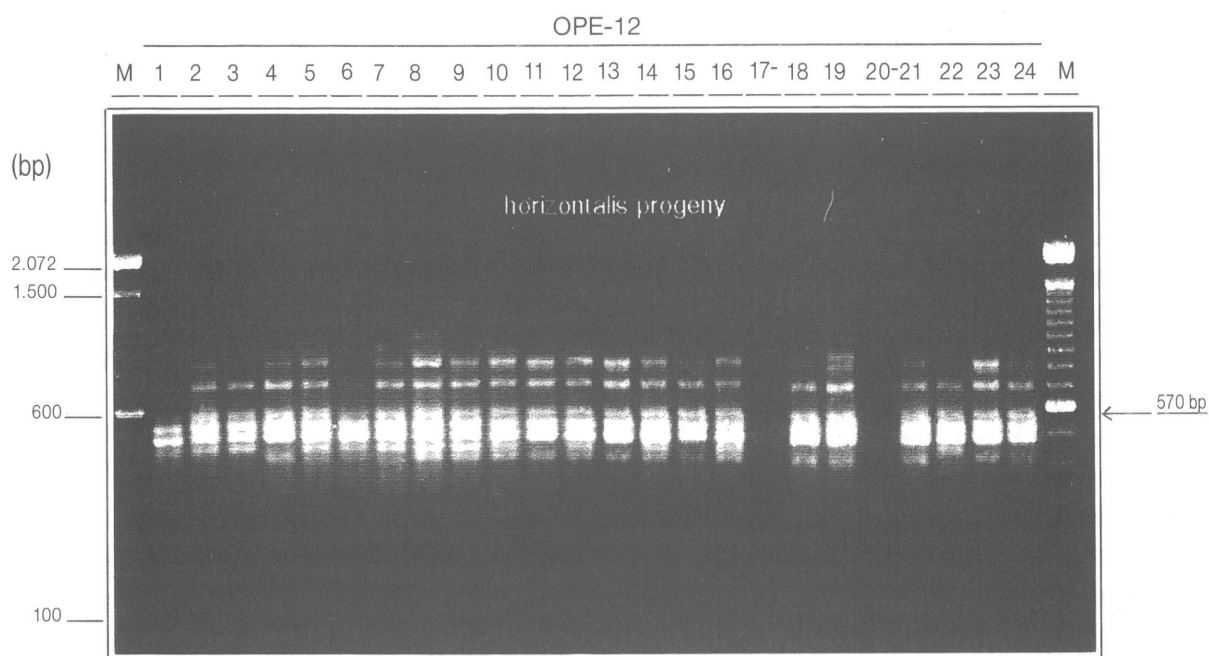
One of the most useful aspects of mapping quantitative traits in woody plants may be the ability to dissect the genetic components of complex quantitative traits (STRAUSS *et al.* 1992; O'MALLEY 1996). Correlated traits often have QTLs that map to similar genomic locations (PATERSON *et al.* 1991; XIAO *et al.* 1995). This apparent clustering of QTL alleles can be due either to tight linkage between QTLs or to a single QTL with pleiotropic effects. Differentiating between pleiotropy or QTL clustering is difficult because crossovers between tightly linked genes will be rare, and mapping

Table 2. Co-segregation analysis of *pyramidalis* and *horizontalis* crown form traits and genetic markers.

Crown form	Putative markers															
	OPC-17 ₈₀₀	OPC-17 ₇₅₀	OPD-05 ₄₄₅	OPD-09 ₇₄₀	OPD-09 ₆₄₀	OPE-12 ₅₇₀	OPG-13 ₁₂₃₀	OPH-07 ₃₆₀								
	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent
<i>Pyramidalis</i>	10	15	21	4	20	11	17	12	23	6	3	26	3	26	7	22
<i>Horizontalis</i>	0	24	11	13	2	22	5	18	1	22	22	0	7	15	15	8

Table 3. Linkage analysis of the eight putative markers identified by bulked segregant analysis.

Item	Putative markers							
Parameter estimates	OPC-17 ₈₀₀	OPC-17 ₇₅₀	OPD-05 ₄₄₅	OPD-09 ₇₄₀	OPD-09 ₆₄₀	OPE-12 ₅₇₀	OPG-13 ₁₂₃₀	OPH-07 ₃₆₀
χ^2 test (degrees of freedom in parentheses)	12.061 (1)	7.872 (1)	17.791 (1)	7.148 (1)	29.003 (1)	40.237 (1)	3.659 (1)	8.868 (1)
Associated probability (<i>p</i>)	0.001	0.005	<0.001	0.008	<0.001	<0.001	0.056	0.003
Recombination frequency (θ)	0.306	0.306	0.236	0.327	0.135	0.059	0.353	0.288
Standard error (SE)	0.066	0.066	0.057	0.065	0.047	0.033	0.067	0.063
Mapping distance in cM(D)	35.6	35.6	25.6	39.1	13.8	5.9	—	32.9
Associated standard error (<i>s_D</i>)	10.5	10.5	7.4	11.4	5.1	3.3	—	9.4

**Figure 3.** Representative individuals of the F_1 progeny (*horizontalis* progeny), scored for marker OPE-12₅₇₀ (indicated by arrow); —: individuals that failed to amplify. Lane M; 100bp DNA ladder, Lanes 1–24 *horizontalis* individuals.

population sizes are often insufficient for their detection. However, when the mode of action of the concurrent QTLs is similar, pleiotropy seems to be the most likely explanation. LAWSON *et al.* (1995) found that QTL studies may assist in the understanding of the relationship between plant growth and architecture traits in apples. In this case, a QTL for branching habit in apple was associated with initial vegetative growth, thus, suggesting pleiotropic effects of a single locus. In *Populus*, BRADSHAW & STETTLER (1995) found QTLs for diameter growth to be closely linked to QTLs for branch traits, providing evidence of single QTLs underlying these multiple traits. It seems likely that the branch habit and tree form traits in *Populus* are the

result of a single QTL with pleiotropic effects. Additionally, LEHNER *et al.* (1995) have proved that the pendula phenotype of Norway spruce is controlled by a single dominant gene.

The results of our research indicate that under the particular genetic background studied, crown form should, to a large extent, be controlled by a distinct genomic region. A genomic map of *Cupressus sempervirens* (ARAVANOPOULOS *et al.*, manuscript in preparation) shows that the marker set associated with crown form, constitutes a single linkage group and therefore a QTL for crown form might be placed in an interval within this group. Nevertheless, the trait of interest could be regarded either as monogenic, or polygenic. In

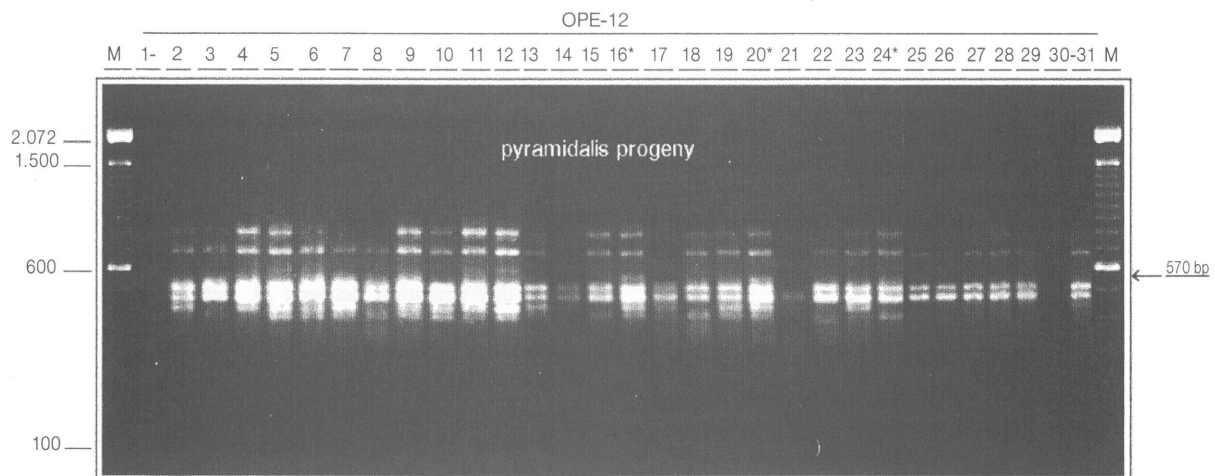


Figure 4. Representative individuals of the F_1 progeny (*pyramidalis* progeny), scored for marker OPE-12₅₇₀; *: recombinants; – individuals that failed to amplify. Lane M; 100bp DNA ladder, Lanes 1-31 *pyramidalis* individuals

the former case the pertinent gene should present multiallelism and/or pleiotropic effects, given the complex results of half-sib (PANETSOS 1967) or full-sib family tests (BASSIOTIS 1985). In the latter case the presence of a major gene, in the genomic region identified, should be assumed. The hypothesis of multi-locus involvement (assuming the existence of one major QTL), can be in accordance with the observation of intermediate crown forms in the areas where the two varieties come in contact. Nevertheless, its verification requires fine mapping of F_2 or backcross families.

The identification of RAPD markers has been made possible through the use of BSA, a method particularly suited to forest trees due to the lack of refined classical genetic tools. Such an approach has been realized in sugar pine (*Pinus lambertiana*) for resistance to blister rust, (DEVEY *et al.* 1995), in loblolly pine for resistance to fusiform rust (WILCOX *et al.* 1996), in poplars (*Populus sp.*) for resistance to leaf rust Kleb (VILLAR *et al.* 1996), for sex determination in the basket willow *Salix viminalis* (ALSTROM-RAPAPORT *et al.* 1998). In fruit trees, the BSA approach has been applied on the resistance to *Citrus tristeza* virus (CHENG *et al.* 1994), to fruit skin color in apple (CHENG *et al.* 1996), and for the identification of markers linked to self-incompatibility alleles in *Corylus avellana* L. (POMPER *et al.* 1998).

RAPD amplification revealed significant genetic variability among the cypress genotypes. BSA identified markers linked to crown form gene(s) and the screening of more primers should identify more closely linked markers, assuming a random distribution of loci detected as RAPD markers and sufficient polymorphism in the target region. Since mapping distance

between flanking markers is about 20 cM, it is far more practical to fine map this region than to attempt genome walking.

Marker OPE-12₅₇₀ should be validated against other genetic backgrounds and then employed in the early and highly efficient selection of *pyramidalis* or *horizontalis* individuals for subsequent breeding programs at very early stages of development, such as in the growth chamber, tissue culture, greenhouse or nursery. In the future more molecular markers with tighter linkages need to be identified so that combinations of these markers can be used, after verification, to screen progenies for crown form more effectively than by employing a single marker. It is hoped that the discovery of markers linked to crown form genes would eventually present the opportunity of cloning the gene(s) involved in this process. The importance of this goal lies in the opportunity to manipulate genetically a trait of major economic importance, since in *pyramidalis* individuals the size of the lateral branches is small and the crown narrower, two features that may improve wood quality and stand densities for afforestation. The isolation, study and potential transfer of this gene to other species where some degree of synteny with cypress can be postulated, may be regarded as an important long-term research goal.

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