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Testing the Conservation of *Quercus spp.* Microsatellites in the Cork Oak, *Q. suber* L.

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Summary

The transferability of microsatellite (SSR) loci from *Quercus* spp. to cork oak (*Quercus suber* L.) was investigated. Semi-automated analysis of fluorescently-labelled PCR fragments was used to test 24 primer sets developed for *Q. myrsinifolia* BLUME, *Q. petraea* (MATT.) LIEB. and *Q. robur* L. in 41 cork oak trees from four stands covering the main area of distribution of the species in Spain. Successful cross-species events occurred for 13 loci (54%). Two of them were monomorphic and another two appeared as multilocus. High levels of genetic variability were detected both for the number of alleles, 62 (7.5 per polymorphic locus, with a maximum number of 19 in locus ssrQpZAG110) and for the expected heterozygosity (mean H_E = 0.648). These results were much higher than those previously reported by other authors using allozyme loci. The usefulness of the SSR loci successfully amplified for studies on population genetics of cork oak is discussed.

Key words: Fagaceae, *Quercus suber,* cork oak, microsatellites.

Introduction

Cork oak (*Quercus suber* L., Fagaceae) is one of the most important evergreen oak species in the western part of the Mediterranean Basin. The geographic distribution of the

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species is divided into two main areas: one covers the Atlantic sides of the Iberian peninsula and Morocco, and the other includes some regions surrounding the Central Mediterranean Sea, such as Catalonia, Southern France and Italy.

Simple Sequence Repeats (SSRs) have become the tool of choice in a wide range of studies due to their clear advantages over other kinds of molecular markers: ubiquity, hypervariability, codominance of alleles, and the relatively simple polymerase chain reaction (PCR)-based screening methods. However, the need for prior knowledge of the flanking regions limits their usefulness. On the other hand, SSR primers developed for a particular species can be used to amplify homologous loci in related species, circumventing this major drawback (see LEFORT *et al.*, 1999 and PEAKALL *et al.*, 1998 for reviews). Primers for SSR loci have been developed for some species belonging to the genus *Quercus* such as *Q. macrocarpa* MICHX. (DOW and ASHLEY, 1996), *Q. myrsinifolia* BLUME (ISAGI and SUHANDONO, 1997), *Q. robur* L. (KAMPFER *et al.*, 1998), *Q. petraea* (MATT.) LIEB. (STEINKELLNER *et al.*, 1997a), and *Q. salicina* BLUME (T. KAWAHARA, unpublished data). The transferability of some of them to cork oak has been already proven (ISAGI and SUHANDONO, 1997; H. STEINKELLNER, pers. comm.). As we are involved in a survey on the genetic variability of *Q. suber* in Spain, we decided to test the transferability of some additional microsatellite markers and, together with those previously proven useful, test the nature and amplitude of genetic diversity of cork oak.

Material and Methods

Plant Material and DNA Extraction

Four stands in the main area of distribution of cork oak in Spain were sampled: Sierra Madrona (Ciudad Real province), Valdelosa (Salamanca), Torrelaguna (Madrid) and Cassá de la Selva (Gerona). In order to facilitate the process of DNA extraction, small branches from up to 12 mature, old individuals from each population were taken and placed to sprout in a greenhouse. About 200 mg of recently flushed leaves were ground in a pestle and mortar in the presence of liquid nitrogen, and genomic DNA was extracted using the Plant Dneasy Kit (Qiagen, GmbH) following the protocol supplied by the manufacturer.

PCR and Electrophoresis

Preliminary studies using the primer sets developed for *Q. myrsinifolia* and following the conditions stated by ISAGI and SUHANDONO (1997) did not give successful amplifications, except for locus QM33GA1. Therefore we modified some PCR factors, such as primer, $MgCl_2$, DNA polymerase and/or DNA template concentrations, and PCR program. The final conditions, considered to be the standard along the study, are the following: for a single locus, genomic DNA (25 ng) was amplified by PCR in a total volume of 25 µL containing 1x reaction buffer (75 mM Tris – HCl pH 9, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.001% BSA), 2.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 1 unit of Biotools DNA polymerase (Biotechnological and Medical Laboratories), and 0.5 µM of each primer. The studied loci are listed in *table 1*.

In order to accelerate and facilitate the screening process, a touchdown procedure was followed: 5 min. at 94 °C as initial denaturing step, followed by ten cycles of 94 °C for 15 sec., 65 to 56 °C (decreasing 1°C each cycle) for 30 sec, and 72 °C for 30 sec., followed by 25 cycles of 94 °C for 15 sec., 55 °C for 30 sec., and 72 °C for 30 sec. A final extension step was carried out at 72 °C for 5 min. PCR reactions were performed in a MJ

Table 1. – Amplification of *Quercus myrsinifolia* (ISAGI and SUHANDONO, 1997), *Q. petraea* (STEINKELLNER *et al.,* 1997a) and *Q. robur* (KAMPFER *et al.,* 1998) SSR loci in cork oak, *Q. suber.* The published size range of PCR products (in bp) and the number of found alleles/studied trees are given for each locus in the source species. The size range and number of detected alleles in *Q. robur* is always referred to 41 individuals. The type of result (TR) per locus is given: polymorphic (P), monomorphic (M), unclear result (?), more than two PCR products (unsuccessful amplification, u.a.) and no amplification (n.a.). Expected (H_E) and observed (H_O) heterozygosity and fixation index (F_{is}) values are provided.

		Source species		*Quercus suber*					
Locus	Microsatellite motif	Size range	alleles/Trees	Size range	alleles	TR	H_E	H_O	F_{is}
			Q. myrsinifolia						
QM33GA1	$(GA)_{33}$	103†	8/NS			?			
QM58TGT	$(CAA)_{11}$	212†	4/NS	195-214	5	P	0.629	0.625	0.007
QM69-2M1	$(TGG)_6(CGG)(TGG)_2$	217†	4/NS	222	1	M	---	---	---
QM50-3M	$(CCT)_3(CCG)(CCT)_2(CCA)(CCT)2+(CCA)_7$	253†	4/NS	270-290	10	P	0.834	0.780	0.065
			Q. petraea						
ssrQpZAG16	$(AG)_{21}$	164-199	9/34			?			
ssrQpZAG9	$(AG)_{12}$	182-210	11/28	224-239	3	P	0.263	0.244	0.075
ssrQpZAG15	$(AG)_{23}$	108-152	11/45	120-136	4	P	0.545	0.512	0.061
ssrQpZAG36	$(AG)_{19}$	210-236	9/25	208-222	6	P	0.608	0.512	0.159
ssrQpZAG110	$(AG)_{15}$	206-262	7/40	218-260	19	P	0.907	0.927	-0.022
ssrQpZAG1/5	$(GT)_5(GA)_9$	160-190	7/33			?			
ssrQpZAG7	$(AG)_{13}(AAAG)_3$	140-160	8/20			u.a.			
ssrQpZAG46	$(AG)_{13}$	190-222	8/24			u.a.			
ssrQpZAG58	$(GA)_{34}$	150-210	12/35			u.a.			
ssrQpZAG108	$(AG)_{13}$	213-237	10/44			u.a.			
ssrQpZAG119	$(GA)_{24}$	64-98	10/35			u.a.			
			Q.robur						
ssrQrZAG7	$(TC)_{17}$	115-153	10/6	115-134	7	P	0.664	0.439	0.341*
ssrQrZAG11	$(TC)_{22}$	238-267	9/10	261-275	6	P	0.731	0.537	0.269*
ssrQZAG39	$(TC)_{34}$	109-146	8/6			n.a.			
ssrQrZAG44	$(GA)_{29}(TG)_8$	118-200	9/6			n.a.			
ssrQrZAG74	$(GA)_{23}$	112-200	8/6			n.a.			
ssrQrZAG96	$(TC)_{20}$	135-194	8/6	144	1	M	---	---	---
ssrQrZAG30	$(GA)_{26}$	172-248	10/5			n.a.			
ssrQrZAG87	$(TC)_{20}$	110-131	8/6			n.a.			
ssrQrZAG103	$(TC)_{15}$	99-163	6/6			n.a.			

† = length of sequenced allele.

NS = Not Stated.

*) = $P<0.05$

Research PTC-100 or in a Perkin Elmer 9600 thermocycler. Successful amplification was checked by means of agarose (2%, w/v) gel electrophoresis and ethidium bromide staining detected by UV light. An amplification was considered to be successful when 1 to 2 defined, sharp bands in the expected size range were detected on agarose gels.

One of the primers ("upper") corresponding to the sets of the loci successfully amplified were 5' labelled with one of the following fluorescent dyes: 6-FAM, TET or HEX (PE Biosystems). Using the touchdown program described above, QM50-3M (FAM), ssrQpZAG9 (TET) and ssrQpZAG15 (HEX) were multiplexed in a single reaction containing 0.3 µM, 0.3 µM, and 0.5 µM of each primer set, respectively. Another PCR multiplex was carried out with ssrQpZAG16 (FAM), ssrQpZAG36 (TET) and ssrQpZAG110 (HEX); the final molarity of each primer set in the reaction mix was adjusted to 0.4 µM, 0.2 µM and 0.2 µM, respectively. In this case, the PCR program used consisted of a preliminary denaturation step at 94 °C for 5 min., followed by 30 cycles at 94°C for 15 sec., 50°C for 30 sec. and, 72°C for 30 sec. No final extension step was carried out.

PCR products generated by the remaining primer sets were mixed in trios, taking into account the expected fragment sizes and the fluorescent labelling used.

After PCR amplification, 3 µL of water-diluted PCR product were mixed with 0.5 µL of GeneScan 350 internal lane size standard labelled with TAMRA (PE Biosystems) and 12 µL of formamide. The mixture was incubated at 95°C for 5 min. and placed on ice until used. The PCR products were visualized following separation by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (PE Biosystems). Fragment sizes were automatically calculated to two decimal places using the Local Southern Method option of the GeneScan v. 3.1 software (PE Biosystems).

All the experiments were performed at least twice.

Data Analysis

Fragment sizes were scored in a spreadsheet and allele binning was performed with average values following GHOSH *et al.* (1997). GenePop version 3.2 (RAYMOND and ROUSSET, 2000) was used to calculate descriptive statistics (observed heterozygosity -H_O-, expected heterozygosity -H_E- and WEIR and COCKERHAM (1984)' fixation index -F_{is}- values).

Results and Discussion

Transferability of SSR Markers

Our first objective was to verify a successful amplification of the chosen primer sets in cork oak. According to the established criterion (see Material and Methods), 13 out of 24 (54%) primer sets yielded successful amplification and, therefore, considered to be fluorescently labelled: QM33GA1, QM58TGT, QM69-2M1, QM50-3M, ssrQpZAG16, ssrQpZAG9, ssrQpZAG15, ssrQpZAG36, ssrQpZAG110, ssrQpZAG1/5, ssrQrZAG7, ssrQrZAG11 and ssrQrZAG96 (*Table 1*). Five primer sets (21%) gave unsuccessful amplifications (more than two products) and lack of amplification, detected simply by the absence of any PCR product, was observed for the remaining primer sets (*Table 1*). There was a noteworthy difference between the discarded loci from *Q. petraea* and *Q. robur*: while with the former some products could be detected on agarose gels (unsuccessful amplification), the latter failed to amplify any product (no amplification) (*Table 1*). The additional efforts that were done in order to optimize or achieve the amplification of the unsuccessful loci from *Q. petraea* or *Q. robur* failed. As an example, the fluorescent pattern corresponding to the ten microsatellite loci finally considered, obtained from an individual tree is shown in *figure 1.*

After the analysis in the capillary electrophoresis apparatus, the electrophoretograms corresponding to QM33GA1, ssrQpZAG16 and ssrQpZAG1/5 loci showed a pattern different

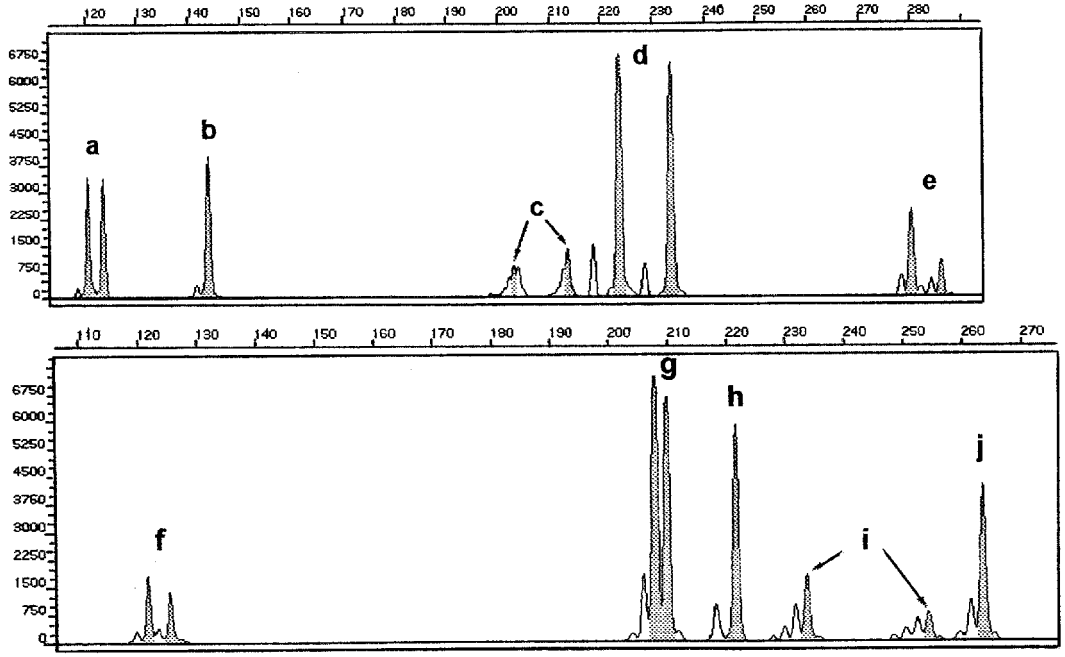

Fig. 1. – Amplification of ten microsatellite loci in a cork oak (*Q. suber*) tree. For each locus (bold designated), alleles are marked in grey. Upper panel: a) ssrQpZAG15, b) ssrQrZAG96, c) QM58TGT, d) ssrQpZAG9, e) QM50-3M. Lower panel: f) ssrQrZAG7, g) ssrQpZAG36, h) QM69-2M1, i) ssrQpZAG110, j) ssrQrZAG11. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units.

from that expected for a diploid (2n = 24) species such as *Q. suber* (*Fig. 2*). The more likely explanation for this result is a duplication of one genomic region. Another possible cause is that the primer set can hybridise simultaneously to the flanking sequences of other, different loci; although more stringent PCR conditions were assayed with these loci, the pattern remained constant, however. An additional difficulty to interpret the electrophoretograms corresponding to these loci was the so called plus-A phenomenon (CLARK, 1988), especially when the putative alleles were in such a narrow size range (*Fig. 2*). Due to the character of this work, made as a preliminary survey, these loci were not considered further. Additional research will be needed in order to explain the true nature of this result.

The analysis of locus QM58TGT also showed the plus-A phenomenon (*Fig. 1c*); the fact that the alleles were so different in size within each tested tree avoided any uncertainty in genotyping the samples. Considering the loci developed for *Q. petraea*, we have confirmed the transferability to cork oak of ssrQpZAG9, ssrQpZAG15, ssrQpZAG16, ssrQpZAG36 and ssrQpZAG110 (H. STEINKELLNER, pers. com.). Under our criteria, the remaining loci failed to be transferred. Noteworthy, this result was unexpected since ssrQpZAG7, ssrQpZAG46, ssrQpZAG58, ssrQpZAG108 and ssrQpZAG119 were successfully amplified in *Q. cerris* (STEINKELLNER *et al.*, 1997b), a taxon very closely related to cork oak (TUTIN *et al.*, 1993). On the other hand, only three (ssrQrZAG7, ssrQrZAG11 and ssrQrZAG96) of the selected nine SSR loci developed for *Q. robur* (KAMPFER *et al.*, 1998) could be amplified in *Q. suber* (*Table 1*).

Amplification in a target species only means that the flanking sequences are more or less preserved. Since size homoplasy has been reported (DOYLE *et al.*, 1998; PEAKALL *et al.*, 1998), the mere coincidence between the size of the fragment in the target species and the sequenced one in the primer source species should not be considered as a clue of homology. However, the presence of stutter peaks, a typical feature of SSR markers, in all loci (*Fig. 1*) led us to assume as homologous the amplified fragments in cork oak. Moreover, STEINKELLNER *et al.* (1997b) verified the homology of the fragments in a broad range of Fagaceae species by sequencing and, therefore this fact reinforces our assumption. The sizes of amplified fragments in cork oak coincided with the expected ones moreover. Another piece of evidence was that, generally, the alleles in each locus differed in the expected number of base pairs according to the length of its core motif. This fact should indicate that the polymorphism found was mainly due to changes in the number of repeats. On the other hand, however, some loci, as ssrQpZAG9 and ssrQpZAG110 which are perfect dinucleotide microsatellites, showed single base-pair differences between alleles. This can be explained assuming that other mutation mechanisms, including insertion/deletion events in the flanking sequences, have operated (HUANG *et al.*, 1998; PEAKALL *et al.*, 1998).

SSR Polymorphism

We have started a survey of the polymorphism of SSR loci in *Quercus suber*. A comparison with the primer source species (*Q. myrsinifolia*, *Q. petraea* and *Q. robur*) in terms of per locus size range, number of detected alleles/number of tested trees is shown in *table 1*. For cork oak, expected (H_E) and observed (H_O) heterozygosity and F_{is} values per locus are also given.

We have detected 62 alleles among 41 individuals. The average number of alleles per locus and polymorphic loci were 6.2 and 7.5, respectively. Despite the relatively low number of trees studied, this allelic diversity was higher than that previously reported for cork oak using other markers. ELENA-ROSSELLÓ and CABRERA (1996) found 30 alleles at 13 isozyme loci in 168

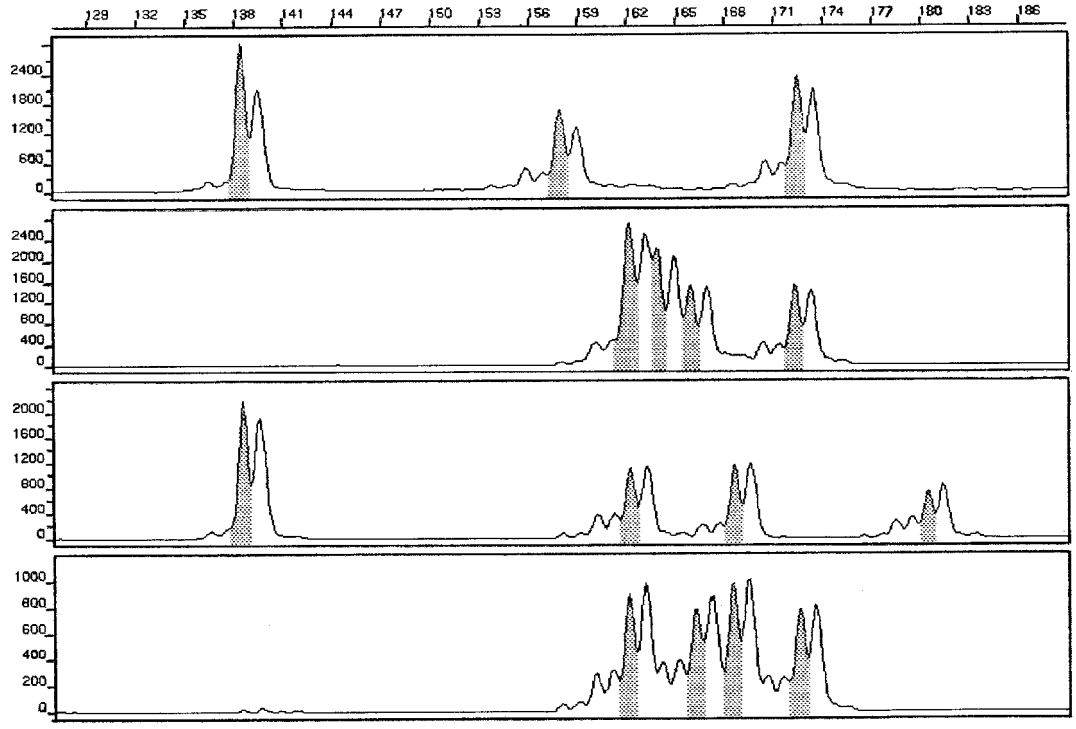

Fig. 2. – Amplification of microsatellite ssrQpZAG16 in four cork oak (*Quercus suber*) trees. The electrophoretograms show unexpected SSR patterns for *Q. suber*, a diploid species: two loci have been apparently amplified. Peaks represent fluorescence intensities of dye-labelled DNA fragments. Each putative allele (grey filled) is followed by a "plus A" fragment. The scale at the top indicates product size in nucleotides. The scale on the left indicates fluorescence intensity measured in relative fluorescence units.

trees, while TOUMI and LUMARET (1998) reported 32 alleles at 11 allozyme loci in more than 1,000 individuals, averaging 2.31 and 2.91 alleles per polymorphic locus, respectively. Thus, compared with isozymes, the SSR markers used in this work were much more informative. The observed SSR levels of polymorphism and allelic diversity in the cork oak were, however, lower that those previously reported for other *Quercus* species. STREIFF *et al.* (1998) detected 15 to 32 alleles per locus in a mixed forest of *Q. petraea* and *Q. robur* (111 alleles; 18.5 average number of alleles per locus), and DOW and ASHLEY (1996), 13 to 20 alleles per locus in a natural population of *Q. macrocarpa*. Conversely, TANAKA *et al.* (1999) reported similar values to those presented here for *Fagus crenata* (79 alleles; 3 to 21 alleles per locus; 9.8 mean number of alleles per locus) and *F. japonica* (77 alleles; 3 to 18 alleles; 8.6 average number of alleles per locus).

The observed heterozygosity (H_O) ranged from 0.244 with ssrQpZAG9 to 0.927 with ssrQpZAG110 (*Table 1*). The mean H_O value, 0.572, doubled that previously reported for cork oak by ELENA-ROSSELLÓ and CABRERA (1996) and TOUMI and LUMARET (1998), 0.262 and 0.283, respectively. However, the genetic diversity found in cork oak was lower than that reported for other *Quercus* species (STEINKELLNER *et al.*, 1997a; STREIFF *et al.*, 1998). This lower polymorphism was even more evident if the H_O values showed by the four polymorphic microsatellite loci developed for *Q. petraea* and successfully transferred to cork oak were compared: only ssrQpZAG110 showed a similar H_O value to that found in the primer source species (STEINKELLNER *et al.*, 1997a; STREIFF *et al.*, 1998). This reduction in the level of polymorphism in the target species is often considered as a general trend and has been reported in the animal kingdom (PRIMMER *et al.*, 1996; ELLEGREN *et al.*, 1997), in plants (AWADALLA and RITLAND, 1997; VAN TREUREN *et al.*, 1997) and also in the genus *Quercus* (STEINKELLNER *et al.*, 1997b). Other studies in plants, however, have not confirmed this tendency (DAYANANDAN *et al.*, 1997; PERRY and BOUSQUET, 1998). In an example phylogenetically close to cork oak, TANAKA *et al.* (1999) have shown that the genetic diversity in *Fagus japonica* was even higher than that found for the primer source species, *F. crenata.* Clearly, additional research on the cross-species amplification of homologous SSR loci is necessary to ascertain this issue.

Two out of the eight polymorphic loci, ssrQrZAG7 and ssrQrZAG11, showed a significant (P<0.05) deviation from HARDY-WEINBERG expectations. In both cases, a deficiency of heterozygotes is apparent (*Table 1*). Feasible explanations are that they are not neutrally selected or, more probably, that they are linked to genes not neutral for selection. An alternative explanation for these deficits is the presence of null alleles. Such non-amplifying alleles were detected for other loci in the same study from which ssrQrZAG7 and ssrQrZAG11 were selected (KAMPFER *et al.*, 1998). We did not find a single tree lacking amplification for these loci but, from the low number of studied trees, this was the expected result and, hence, the occurrence of null alleles can not be discarded. The existence of null alleles should be verified by segregation analysis and therefore, until the homozygosity at these loci is confirmed, precautions should be taken in population studies including these loci.

In summary, we have reported the transferability to *Q. suber* of some SSR markers developed for three species belonging to two different subgenera of *Quercus: Q. myrsinifolia* is assigned to subgenus Cyclobalanopsis (KITAMURA and MURATA, 1979) and *Q. petraea* and *Q. robur* belong to subgenus Lepidobalanus, section Robur (TUTIN *et al.*, 1993); the cork oak, on the other hand, belongs to section Cerris of this subgenus (TUTIN *et al.*, 1993). This fact, the conservation of microsatellite markers among related species, permitted us to initiate a study of genetic diversity of *Q. suber* without the costs and efforts needed to develop such markers in this species.

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Evaluating Efficacy of Early Testing for Stem Growth in Coastal Douglas-fir[1])

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Abstract

In a test to evaluate the ability to predict stem growth of families in the field from nursery performance (i.e., early testing), 67 open-pollinated families and 66 full-sib families of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* MIRB. (FRANCO)) were sown in two nursery conditions, each replicated as separate experiments: two bareroot nursery trials established in successive years in the same nursery, and two container-sown greenhouse trials sown in different greenhouses in the same year. First-year heights in the seedling trials were compared to mean stem volumes of the same open-pollinated families in eight 15-year-old field progeny tests and the same-full-sib families in eleven 12-year-old tests.

Family mean nursery-field correlations (r_{xy}) were similar for all four seedling trials for both open-pollinated (OP) and full-sib (FS) families, and generally ranged between 0.30 and 0.40. Although low, it is shown that nursery-field correlations of this magnitude can be quite useful in tree improvement programs. For example, based on the data in this study, it is estimated that a single-stage of family selection for first year seedling height would be about 50% as effective in improving 15-year volume as direct selection for this trait in field tests. Early testing, however, is probably of more practical significance as a tool for culling families prior to out-planting field tests in two-stage selection schemes. It is estimated that 25% of the OP families in this study could have been culled in an early test (first-stage selection), with gain in 15-year volume after subsequent field testing and selection of the remaining families (second-stage selection) being nearly the same as if all families had been field tested. Thus, early testing is an effective tool for reducing the size and cost of field progeny tests without sacrificing genetic gain.

Key words: Pseudotsuga menziesii, early selection, two-stage selection, genetic parameters, nursery-field correlations, genotype X environment interaction.

Introduction

Because of the long rotation lengths typically used in forestry, early evaluation of genotypes is often an important component of tree improvement programs (e.g., LAMBETH *et al.*, 1982; LAMBETH, 1983; TALBERT and LAMBETH, 1984; LOWE and VAN BUIJTENEN, 1989; CARTER *et al.*, 1990; BRIDGWATER and MCKEAND 1997). Early evaluation can be used in a number of ways, but in this paper, we refer exclusively to *early testing* defined by LAMBETH, (1983) as the "process whereby trees are selected after being grown at close spacing in a greenhouse, growth chamber or nursery for one or two years." Early testing can be used to select superior progeny, families, or parents, based on progeny performance.

Early testing may have one of two primary applications in tree improvement programs:

1) *Early single-stage selection* – Superior genotypes are identified based on seedling performance in order to shorten generation intervals and increase genetic gain per unit of time.

2) *Multiple-stage selection* – Screening at the seedling stage is used to identify and cull poor-performing genotypes prior to the establishment of field tests. Final selections are based on one or more additional stages of evaluation in the field. Early testing makes it possible to reduce the size of field tests, thereby increasing their statistical precision and reducing their cost.

Tree breeders may be reluctant to rely on early testing alone because some traits cannot be scored at the seedling stage. Thus, multiple-stage selection may be the most valuable application of early testing. Multiple-stage selection for disease resistance for example, is used routinely (e.g. PHELPS, 1977; WACKINSHAW *et al.*, 1980). There are fewer examples of multi-stage selection being used for growth (LOWE and VAN BUIJTENEN, 1989) although this would be highly desirable if it can be done effectively.

In this paper, we first review the factors influencing the efficacy of early testing in tree improvement programs, then evaluate the potential of early testing for stem growth in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* MIRB. (FRANCO)). In our analyses, we assume that the purpose of

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