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Nuclear Microsatellite Markers for the Identification of *Quercus ilex* L. and *Q. suber* L. hybrids

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Summary

We report the transfer of a set of nuclear microsatellite markers from *Quercus macrocarpa* Michx. and *Q. petraea* (Matts) Liebl. to *Q. ilex* L. and *Q. suber* L. as a useful tool for further genetic studies on these species. Their specific applicability for the praecox and doubtless identification of *Q. ilex* x *Q. suber* hybrids is also shown. Hybrids were obtained by controlled pollinations on *Quercus ilex* L. with pollen from *Quercus suber* L. trees. This is the first work in which nSSR have been used in *Q. ilex.*

Key words: Q. ilex, Q. suber, microsatellites, hybridisation.

Introduction

Cork oak (*Quercus suber* L.) and especially holm oak (*Quercus ilex* L.) are widely distributed along the Western Mediterranean region, where they share part of their distribution areas and often occur in mixed stands. From ecological point of view, *Q. ilex* L. is considered one of the most important tree species in this region. Its eurioic temperament enables successful settlement in a broad range of different habitats and climates. *Q. ilex* L. is reckoned as a climactic species in extended regions of the Iberian Peninsula, where its acorns are very esteemed for pork feeding. In contrast, cork oak is a more demanding species. It is less resistant to extreme temperatures and lives only in non-calcareous or decarbonated substrates. Cork production makes *Q. suber* L. one of the most important non-timber forest trees in Western Mediterranean region.

Hybridisation between these two species has been proposed as a feasible mechanism in their evolution, as suggested by cytoplasmic DNA studies (BELAHBIB *et al.,* 2001). Since several interspecific barriers have been described (BOAVIDA *et al.,* 2001), hybridisation is not likely a frequent event; however, it has negative effects from an economical point of view, as it implies a decrease in the quality of both cork and acorns, the most important products obtained from these species. Therefore, identification of hybrids in mixed stands could be of great interest, with both scientific and practical implications. At present, this identification is based on morphological criteria, not always reliable on young plants.

Despite ecological and economical interests, few studies have been carried out on the variability and population genetics of these species. Some recent works have focused on the chloroplast genome variation (LUMARET *et al.,* 2002; BELAHBIB *et al.,* 2001, and only few studies utilised nuclear molecular markers, mostly isozymes (JIMÉNEZ *et al.,* 1999; TOUMI and LUMARET, 1998; ELENA-ROSELLÓ and CABRERA, 1996). Isozymes are inexpensive, fast, technically not demanding and reproducible, with high transferability among species, so they are very often used as a first approach in the study of population genetics of a species. However, they are often difficult to score and interpret, and they usually reveal relatively low levels of polymorphism, so they may not be suitable for more detailed studies.

Microsatellites frequently show much higher levels of polymorphism than isozymes; they are codominant and usually considered as neutral markers, so they are suitable for a number of different research problems, including paternity analysis, studies on pollen dispersal, etc. In this paper we report the transfer of six nuclear microsatellites (nSSR) developed in other *Quercus* species to holm and cork oaks. We also present the specific applicability of the set of microsatellites for the praecox and doubtless identification of *Q. ilex* L. and *Q. suber* L. hybrids.

Material and Methods

Eleven holm oak and nine cork oak adult trees were used to test the transferability of nSSR from *Quercus macrocarpa* Michx. and *Q. petraea* (Matts.) Liebl.

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Pollen was collected separately from adult cork oak trees for controlled crosses. Isolation cloth bags were set around branches of the holm oak mother trees, and pollen was injected inside. Bags were used to preserve the pollen of the donor from being carried away by the wind and to avoid the entrance of pollen from the surrounding trees. Since autogamy is not thought to be frequent in holm oaks, male flowers were not removed from mother trees, nor emasculated. Acorns were collected at maturation and germinated in the nursery of the research centre.

DNA was extracted from leaves following the method described by DOYLE and DOYLE (1990).

Amplification of eight (GA)n nuclear microsatellite (nSSR) loci have been tested in this study; two of them, MSQ4 and MSQ13, were first described in *Q. macrocarpa* Michx. (DOW *et al.,* 1995) and six, QpZAG9, QpZAG15, QpZAG36, QpZAG46, QpZAG104, and QpZAG1/5, in *Q. petraea* (Matts.) Liebl. (STEINKELLNER *et al.,* 1997a). For their amplification we have used the corresponding primers designed by the mentioned authors. Transferability of QpZAG9, QpZAG15, QpZAG36, and QpZAG46 to cork oak has been previously reported (GOMEZ *et al.,* 2001; HORNERO *et al.,* 2001).

PCR amplifications were performed in a thermocycler (GeneAmp 9700, Perkin-Elmer) in final volume of 10 µl, with 5 ng of genomic DNA, 0.2 µM of each primer [forward primers labelled on the 5' end with IRD 800 (MWG-Biotech)], 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris/HCl pH 9, 2 mM $MgCl_2$ and 0.4 U of *Taq*-DNA polymerase (Ecogen). Standard amplification profile consisted on a first denaturalisation step at 94ºC for 5 minutes, followed by 30 amplification cycles (consisting on a denaturalisation step at 94 °C for 1 minute, an annealing step at 50 °C for 30 seconds and an elongation step at 72 °C for 1 minute), and a final elongation step at 72 °C for 10 minutes. For QpZAG36 amplification cycles consisted of three one-minute steps and 48ºC were used as annealing temperature.

To improve the specificity of the amplification obtained for QpZAG9 and QpZAG1/5, the following touchdown procedure was used: the first denaturing step (5 minutes at 94 °C) was followed by eleven cycles of 94 °C for 1 minute, 60 °C to 55 °C (decreasing 0.5 °C each cycle) for 30 seconds, and 1 minute at 72 °C; after that, 19 more amplification cycles with 55 °C as annealing temperature were performed with a final elongation step at 72 °C for 10 minutes. Final concentration of magnesium chloride was decreased to 1.5 M.

All PCR products were analysed on an automatic sequencer (Li-Cor 4200). Power of discrimination was calculated for each marker according to KLOOSTERMAN *et al.* (1993).

Results

In order to verify the applicability of the selected nSSRs to the current study, their amplification was tested on adult trees. Standard PCR reactions yielded products for most of the primer pairs, with similar sizes to those described in other oak species (DOW *et al.,* 1995; STEINKELLNER *et al.,* 1997b) (*Table 1*). On the contrary, no amplification was detected for QpZAG104, even at less restrictive conditions (Ta = 48 °C).

The amplification pattern for MSQ4, QpZAG15, and QpZAG36 in both species suggests the existence of another mutations such as indel mutations, along with the variation in the number of tandem repeats, typical of microsatellites.

QpZAG9 and QpZAG1/5 showed a very noticeable amplification pattern, with multiple bands. This pattern did not change in more restrictive PCR conditions, with an annealing step at 55 °C for 15 seconds or even using the touchdown procedure, although total yield decreased. The results obtained for QpZAG1/5 were too confusing in both cork and holm oaks, as previously reported for cork oak (HORNERO *et al.,* 2001), making impossible any further analysis. However, QpZAG9 primers amplified up to 4 fragments. This marker could still be informative, and it was included in the following analyses. These multiple amplifications could be attributed to a duplication of

Table 1. – Amplification pattern shown by the adult trees. For each locus both alleles are expressed as the size in base pairs of the fragments amplified. Note that for QpZAG9 each allele is represented by two amplified fragments. For each species the power of the genetic discrimination (PD) of each locus is provided.

Q. suber L.	MSQ13	MSQ4	QpZAG9	QpZAG15	QpZAG36	QpZAG46
Qs 1	218	200	224 / 219	126	220	192
	218	198	224 / 219	123	218	188
Qs 2	218	200	224 / 219	126	213	192
	218	200	224 / 219	122	211	190
Qs 3	218	200	224 / 219	126	219	188
	218	198	224 / 219	123	215	188
Qs 4	218	200	224 / 219	126	211	189
	218	200	224 / 219	123	209	187
Qs 5	218	214	224 / 219	123	213	192
	218	200	224 / 219	123	211	190
Qs 6	218	200	239 / 234	123	213	188
	218	200	239 / 234	123	211	188
Qs 7	218	214	224 / 219	126	213	190
	218	200	224 / 219	123	213	190
Qs 8	218	200	224 / 219	126	213	190
	218	200	224 / 219	123	211	188
Qs 9	218	200	224 / 219	123	223	188
	218	200	224 / 219	122	211	188
PD	0.000	0.593	0.198	0.617	0.741	0.790
Q. ilex L.	MSQ13	MSQ4	QpZAG9	QpZAG15	QpZAG36	QpZAG46
Qi 1	205	198	249 / 239	128	217	180
	203	196	243 / 233	126	203	180
Qi 2	207	196	243 / 233	130	207	182
	201	196	243 / 233	128	205	182
Qi 3	211	198	248 / 233	135	205	182
	209	196	245 / 235	135	203	180
Qi 4	207	196	249 / 239	133	205	186
	207	196	243 / 233	118	203	180
Qi 5	203	196	243 / 233	145	217	180
	199	196	243 / 233	120	217	180
Qi 6	205	196	248 / 233	133	217	182
	203	196	243 / 233	115	205	182
Qi 7	207	196	249 / 239	128	219	180
	203	196	243 / 233	118	203	180
Qi 8	203	198	249 / 239	118	207	200
	201	196	243 / 233	118	207	200
Qi 9	207	196	243 / 233	134	203	182
	205	196	243 / 233	132	203	182
Qi 10	205	198	254 / 239	137	203	180
	199	196	248 / 233	118	203	180
Qi 11	203	196	249 / 239	133	203	182
	199	196	243 / 233	126	203	180
PD	0.876	0.463	0.694	0.909	0.843	0.744

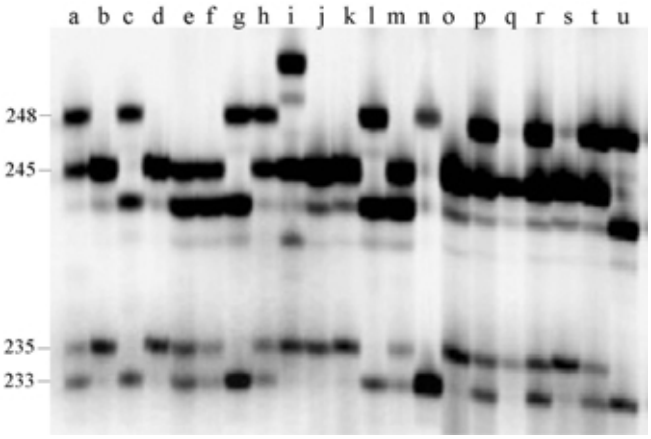

Figure 1. – Segregation of QpZAG9 alleles. *a:* mother tree Qi 3, *b-u:* half-sib family, obtained from acorns from Qi 3. Note the cosegregation of the 248 and 233 bp fragments and of the 245 and 235 bp ones.

the SSR in the genome, but, for QpZAG9, the analysis of the segregation of these bands in the offspring (*Figure 1*) allowed us to conclude that the primers amplify two fragments per allele, at a single locus. The difference in the size of both bands is not the same for all the trees. Even though 10 bp separate the two bands in the majority of the holm oaks studied (5 bp in cork oaks), we have also detected an allele with bands spaced by 15 bp, as shown for trees Qi 3, Qi 6 and Qi 10 (*Table 1*). Therefore, it may be useful to include both bands in the analysis of this locus, at least in holm oak.

Once suitability of markers to distinguish *Q. ilex* and *Q. suber* had been verified, we undertook the identification of hybrids among the seedlings obtained in the controlled pollinations. *Table 2* shows the interspecific crosses performed. The analysis of the PCR products amplified with the six usable primer pairs allowed the doubtless identification of three hybrids (*Figure 2*), and led us to consider the other seedlings as coming from contaminations in the pollinations. The overall success in the crosses was rather low, which is in concordance with the interspecific barriers described by BOAVIDA *et al.* (2001).

Table 2. – Interspecific *Q. ilex* x *Q. suber* crosses performed in this study. For each cross, number of seedlings and number of acorns obtained are shown.

m\p	Qs 5	Qs 6	Qs 7	Qs 8	Qs 9
Qi 1	0/0	0/0			
Qi 2			0/1	2/2	
Qi 3				5/7	1/1
Qi 4	1/1				7/10

Discussion

Nuclear microsatellites, due to their biparental codominant inheritance, and very high reproducibility are very suitable molecular markers for different studies in population genetics. On the other hand, microsatellites are very conserved within certain genera. This is the case for the genus *Quercus*, which allows transferability of microsatellites from one species to another (BARRENECHE *et al.,* 1998; ISAGI and SUHANDONO, 1997; STEINKELLNER *et al.,* 1997b). In this work we report the transfer of two nSSR markers developed in *Q. macrocarpa* Michx. (Subgenus *Lepidobalanus*) and four others developed in *Q. petraea* (Matts.) Liebl. (Subgenus *Lepidobalanus*), to two other oak species, namely *Q. ilex* L. (Subgenus *Sclerophyllodrys*) and *Q. suber* L. (Subgenus *Cerris*). One of these markers, (QpZAG9 from *Q. petraea*) shows a very noticeable pattern in cork and holm oaks, amplifying two bands per allele, even in very restrictive conditions. The secondary band was earlier detected in cork oak by HORNERO *et al.* (2001), using another system for

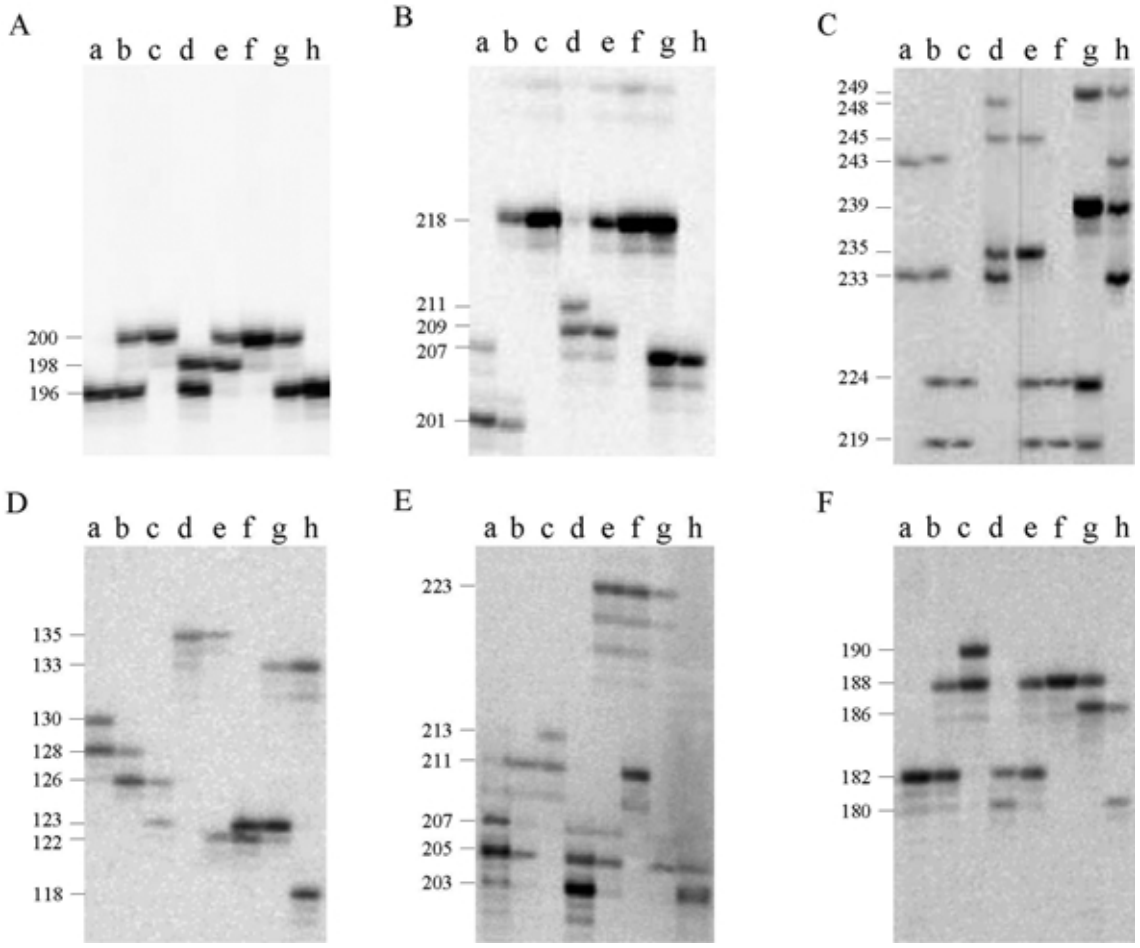

Figure 2. – Amplification pattern shown by the parent trees and the hybrids for *A:* MSQ4, *B:* MSQ13, and *C:* QpZAG9, *D:* QpZAG15, *E:* QpZAG36 and *F:* QpZAG46 as detected by the automatic sequencer (Li-Cor 4200). Each hybrid is flanked by its parent trees. In each panel, *a:* mother tree Qi 2, *b:* hybrid 1 (Qi 2 x Qs 8), *c:* pollen donor Qs 8, *d:* mother tree Qi 3, *e:* hybrid 2 (Qi 3 x Qs 9), *f:* pollen donor Qs 9, *g:* hybrid 3 (Qi 4 x Qs 9), *h:* mother tree Qi 4. Size of the amplified fragments are expressed in base pairs.

the detection of PCR products (ABI PRISM 310 Genetic Analyzer). According to the segregation of bands (*Figure 1*), this multiplicity of amplified fragments is probably due to the presence of two targets for at least one of the primers within the same locus, differing the fragments in an almost fixed number of bases (5 bp for cork oaks and 10 bp for holm oaks), rather than to a duplication of the SSR in the genome. Such multiplicity of targets is evident in the case of cork oak: nucleotides 9 to 15 (both included) of the forward primer, are repeated with just a single discrepancy in positions 14 to 20, exactly at the 3' end of the primer (5'GCAATTACAGGCTAGGCTGG). Sequencing of these regions would certainly clarify this point. However, we have found alleles whose bands differed by an atypical number of nucleotides, so both fragments can be useful in certain cases to distinguish alleles and might be taken into consideration for the analyses.

Several studies reported the presence of chloroplast DNA haplotypes from holm oak in populations of cork oak, and vice versa. Capture of unexpected chloroplast haplotypes by hybridisation (introgression) has been proposed as possible explanation, but it is still a controversial point, and more studies on the reproduction and hybridisation patterns of these species are needed (LUMARET *et al.,* 2002; BELAHBIB *et al.,* 2001; JIMÉNEZ *et al.,* 1999). The nSSR loci MSQ4, MSQ13, QpZAG9, QpZAG15, QpZAG36 and QpZAG46 allow the identification of hybrids of cork and holm oak through direct paternity analysis, which however, requires identification of the genotypes of parental trees. Size range for QpZAG15 overlaps in both species and the size differences observed for MSQ4, QpZAG36 or QpZAG46 are not large enough for the doubtless discrimination between both species. Nevertheless, the differences in the other two loci (QpZAG9 and MSQ13) and particularly, the specific pattern observed for QpZAG9 allow such discrimination and the identification of hybrids avoiding detailed paternity analyses.

In conclusion, this work reports the transfer of a set of nuclear microsatellites to cork and holm oaks, as a reliable tool that will enable further studies on the reproduction patterns of both species, including the rapid and doubtless identification of hybrids. The knowledge of these patterns will give us insights in the historical evolution of their populations and will also be useful for genetic conservation and forestry management purposes.

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