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Short Note: High Throughput Microsatellite Genotyping in Oak Species

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Abstract

Microsatellites are widely used markers for multiple purposes in oaks. We describe a complete procedure for cheap DNA extraction and fast microsatellites genotyping by multiplex PCR. 10 loci were selected to form two multiplex kits including three loci that show a high differentiation between *Quercus robur* and *Q. petraea*. The loci were tested in three oak species and show a high mean genetic diversity of 0.84. The cumulative exclusion probability for parentage analysis was 0.999977 for single parent and 1.0 for paternity. Finally, the relatively high differentiation coefficient (Gst = 0.04) will facilitate species assignment based on genotypes in oaks.

Key words: microsatellites; multiplex PCR; *Quercus*; genetic assignment; parentage analysis.

Oaks are common species in a large part of the world and are involved in an increasing number of genetic studies concerning especially population genetic structure and gene flow. New powerful methods for analysing multilocus genetics data allow inference about past demographic events, genetics assignment or detection of selection (CHIKHI and BRUFORD, 2005). We took advantage of technical laboratory progresses to increase availability of multilocus microsatellites data. Combining multiplex PCR and automated capillary sequencer speed up the genotyping compared to silver straining gel or gel plate automated sequencer. In the same time it decreases cost of genotyping and allows to reach high loci number analysis which is needed for high resolution studies in natural populations. In this short note, we present a protocol that allows fast DNA extraction and genotyping for 10 microsatellites in two multiplex PCR.

We used a modified protocol based on CTAB/dichloromethane (DUMOLIN et al., 1995) allowing DNA extraction in 96 wells plates format. This modification provides high quality DNA extraction from 192 individuals in one day at a low cost compared to commercial kits. One centimeter square of leave or three to five buds are cut into small pieces and put in a 1.2 mL microtube (Qiagen, Cat. No. 19560) with two 2 mm steel balls, a small amount of insoluble PVP and alumina, 100 µL of extraction buffer (CTAB 20 mg.mL^{-1}, EDTA (pH 8) 0.02M, Tris HCl (pH 8) 0.1M, NaCl 1.4M, soluble PVP 10 mg.mL^{-1}). Vegetable tissues and 300 µL of extraction buffer are ground in a Retsch-Mill at 30 vibrations per second for 1 min for each side. The microtubes are incubated at 55 °C for 1 hour in a vertical position with shaking. After cooling, 300 µL of dichloromethane is added and microtubes are shaken. After 20 minutes of centrifugation at 6200 rpm and 4 °C, the upperphase is transferred in new microtubes, 300 µL of cold isopropanol is added and after shaking the microtubes are placed at –20 °C for 1 hour in a vertical position. After a centrifugation step of 20 minutes at 6200 rpm and 4 °C, the supernatant is removed, 500 µL of ethanol is added and microtubes are shaken before centrifugation for 20 minutes at 6200 rpm and 4 °C. Supernatant is then removed and the pellet is dried in a vacuum system concentrator for 15 minutes at 55 °C. Finally the pellet is resuspended in 50 µL of pure water. To avoid lost of DNA pellet when removing the upperphase after each centrifugation step, microtubes are kept on ice.

We selected microsatellites loci that are highly differentiated between *Quercus robur* and *Q. petraea* (SCOTTI-SAINTAGNE et al., 2004; P. Goikoetxea, unpublished data). Before multiplexing, primers were checked for primer-primer interaction using the AutoDimer software (VALLONE and BUTLER, 2004). One set, called KIT1, comprises the five following loci QrZAG11, QrZAG39, QrZAG96, QrZAG112 and QpZAG110 and the second one, five others (KIT2): QrZAG5, QrZAG7, QrZAG20, QrZAG65 and QrZAG87 (KAMPFER et al., 1997; STEINKELLNER et al., 1997). On the 10 selected microsatellites, 7 are unlinked (*Table 1*). Each forward primer was marked with a fluorescent dye (MWG and Applied Biosystems) as shown in *Table 1*. PCR were carried out in a final volume of 15 µL including 5 µL of 1/50 to 1/200 diluted DNA, depending on DNA concentration

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Table 1. – Characteristics of the ten microsatellites for a sample of 90 oaks (30 trees by species), Aurignac, France.

	Locus	Primer Sequence	LG	[Primer] (µM)	Dye	Allele Number	Size Range (pb)	*Q. robur*			*Q. petraea*			*Q. pubescens*			EP(sp)	EP(p)	EP(pp)	Gst
								Ho	He	Pval	Ho	He	Pval	Ho	He	Pval				
KIT1	QrZAG11	CCTTGAACTCGAAGGTGTCCTT	10	0.350	HEX	16	242-286	0.75	0.88	0.265	0.79	0.84	0.095	0.74	0.76	0.562	0.67	0.80	0.94	0.082
		GTAGGTCAAAACCATTGGTTGACT																		
	QrZAG39	CACCGCTGGAATTTTAAGGGA	5	0.025	NED	17	103-139	0.86	0.85	0.728	0.67	0.92	0.003	0.83	0.93	0.309	0.71	0.83	0.95	0.015
		GACCTAAGCCAAAGTGTGGGC																		
	QrZAG96	CCCAGTCACATCCACTACTGTCC	10	0.120	HEX	19	137-179	0.37	0.37	0.377	0.83	0.85	0.100	0.81	0.91	0.536	0.56	0.72	0.90	0.157
		GGTTGGGAAAAGGAGATCAGA																		
	QrZAG112	TTCTTGCTTTGGTGCGCG	12	0.040	FAM	15	72-106	0.74	0.66	0.931	0.47	0.61	0.005	0.81	0.85	0.216	0.36	0.55	0.77	0.042
		GTGGTCAGAGACTCGGTAAGTATTC																		
	QpZAG110	GGAGGCTTCCTTCAACCTACT	8	0.300	FAM	18	193-235	0.71	0.66	0.807	0.83	0.85	0.348	0.92	0.86	0.976	0.49	0.67	0.86	0.017
		GATCTCTTGTGTGCTGTATTT																		
	KIT1 all loci			1.670		85		0.68	0.68		0.70	0.81		0.82	0.86		0.986	0.998	0.999	0.063
KIT2	QrZAG5b	TGAAGAGTAAGACCATTCACATCA	6	0.400	HEX	22	217-263	1.00	0.91	0.997	0.89	0.92	0.574	0.87	0.94	0.075	0.76	0.87	0.97	0.018
		GTATGTGAGTGTTTGTGGTTTGG																		
	QrZAG7	CAACTTGGTGTTCGGATCAA	2	0.120	FAM	21	109-152	0.92	0.91	0.364	0.79	0.94	0.064	0.93	0.92	0.748	0.77	0.87	0.97	0.018
		GTGCATTTCTTTTATAGCATTCAC																		
	QrZAG20	CCATTAAAAGAAGCAGTATTTTGT	1	0.150	HEX	17	155-195	0.78	0.78	0.472	0.90	0.88	0.807	0.83	0.90	0.509	0.56	0.72	0.89	0.010
		GCAACACTCAGCCTATATCTAGAA																		
	QrZAG65	CAGTGGTGTCAACTCCTCCCAG	10	0.500	FAM	27	249-306	0.79	0.94	0.030	0.75	0.96	0.001	0.71	0.94	0.000	0.81	0.89	0.98	0.001
		GTCAGGTGACCATTCAAACCTAGAA																		
	QrZAG87	TCCCACCACTTTGGTCTCTCA	2	0.025	NED	18	101-141	0.81	0.85	0.231	0.73	0.88	0.053	0.78	0.81	0.506	0.64	0.78	0.93	0.051
		GTTGTCAGCAGTGGGATGGGTA																		
	KIT2 all loci			2.390		105		0.86	0.88		0.81	0.92		0.82	0.90		0.998	0.999	1.000	0.019

Legend – LG: linkage group, Ho: observed heterozygozity, He: expected heterozygozity, EP(sp): single parent exclusion probability, EP(p): paternity exclusion probability, EP(pp): parent pai exclusion probability, Gst: differentiation coefficient.

and quality. For routine analysis, we advise to test amplification with several DNA dilution to find the one producing a complete amplification in all individuals. PCR reaction buffer contains 1.2X buffer (Invitrogen), 2mM $MgCl_2$, 0.2mM dNTPs, 0.025 unit of *Taq* polymerase (Invitrogen) for one individual and each primer at different concentrations (*Table 1*) to increase homogeneity of amplification intensity. Even if primer concentrations produced a balanced signal with several DNA extractions series, it can be empirically adjusted to homogenize loci amplification by increasing the primer concentration of weaker loci and at the same time decreasing the primer concentration of stronger loci. The amplification is performed with a MJResearch DNA Engine Tetrad2 (Biorad) thermocycler in ABgene PCR plates (AB-800) covered with adhesive sealing sheets (AB-0558) to avoid evaporation during PCR. The cycling condition consists of a denaturation step of 3 min at 94 °C, following by 30 cycles of 30 s at 94 °C, 30s at 50 °C, 90 s at 65 °C and a final extension of 15 min at 65 °C. We found that elongation at 65 °C reduces stutter phenomena and at the same time increases PCR efficiency during multiplex PCR of microsatellites (HENEGARIU et al., 1997).

After amplification, 8 µL of PCR products were mixed with 3 µL of bromophenol blue and migrated on 3% agarose gels for 20 minutes at 100V to check for a correct amplification of loci. PCR products were diluted sixfold in pure water and 2µL were combined with 10 µL of diluted size standards ET-400Rox (Amersham Biosciences). Separation of fragments and alleles detection was carried out on a MegaBACE 1000 96 capillaries automated sequencer (Amersham Biosciences). Injection parameters were 60 s at 3 kV and the run was performed during 75 min at 10 kV and a constant temperature of 45 °C. Data were then scored with the Fragment Profiler v1.2 software (Amersham Biosciences).

The genotyping procedure was tested in several oak species. It gave consistent results for *Q. robur*, *Q. petraea*, *Q. pubescens*, *Q. pyrenaica* (European white oaks species) and also for *Q. laurina*/*Q. affinis* (Mexican red oaks) and *Q. resinosa* (Mexican white oaks). In *Q. suber* and *Q. ilex*, only few loci were successfully amplified. This shows the transferability limit of microsatellites due to divergence of these species belonging respectively to *Cerris* and *Ilex* groups. Loci and multiplex PCR are therefore easily transferable in other white and red oak species.

Both microsatellites multiplex have been tested in 90 oaks of three species: *Q. robur*, *Q. petraea* and *Q. pubescens* from Aurignac (Haute Garonne, France). Allele number, observed and expected heterozygosities and P value for Hardy-Weinberg exact test (Genepop, RAYMOND and ROUSSET, 1995) are given in *Table 1*. Genetic diversity is high with a mean of 0.78 for KIT1 and 0.90 for KIT2. QrZAG65 shows significant heterozygote deficit for all species and some other loci show a significant heterozygote deficit or excess in one of the species.

To test for potential null alleles we genotyped one pedigree (30 offsprings) obtained by controlled crosses of

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two trees (data not shown). All loci followed Mendelian inheritance and none of them, including QrZAG65, showed missing allele. So, we believe that QrZAG65 results indicate that panmixia is not expected in a sample of such a limited size because of the regional sampling and hybridization between species.

The 10 loci could be used for species assignment because they differentiate well between *Q. robur* and *Q. petraea* (Gst = 0.028) but also *Q. petraea* and *Q. pubescens* (Gst = 0.019) and *Q. robur* and *Q. pubescens* (Gst = 0.045). Moreover, KIT1 contains QrZAG96, QrZAG112 and QrZAG11, the three highest differentiating microsatellites between *Q. robur* and *Q. petraea* (SCOTTI-SAINTAGNE et al., 2004). These loci are located close to QTLs involve in leaf morphology that differentiate the two species (SAINTAGNE et al., 2004).

To measure the efficiency of the loci for parentage analysis, we computed exclusion probabilities for single parent (EP(sp)), paternity (EP(p): mother known) and parent pair (EP(pp)) using FaMoz (GERBER et al., 2003). The two sets of microsatellites show high exclusion probabilities which could be slightly overestimated due to partial linkage between some of the loci (*Table 1*). However, considering such high probabilities, these loci are highly advisable for contemporary gene flow analyses. KIT2 is composed of loci showing high allelic richness and is routinely used for controlled crosses checking in the lab. The DNA extraction procedure and the multiplex PCR described here allow a fast and convenient genotyping for 10 microsatellites in oaks for a wide variety of applications.

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