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SIMULTANEOUS MUTATION SCANNING AND GENOTYPING OF OLIVE GERMPLASM BY HIGH-RESOLUTION DNA MELTING ANALYSIS

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New methods to monitor DNA melting by fluorescence have recently become popular in conjunction with Real-Time PCR. High-Resolution DNA Melting Analysis (HRMA), with saturation fluorescent DNA dyes for either mutation scanning of PCR products or genotyping with unlabeled probes, has been reported for human genes involved in many genetic diseases. HRMA for gene scanning relies primarily on the shape of the melting transition of the PCR products, and very little on melting temperature. In this work we report: a) the HRMA application in plants, for scanning and genotyping the *Olea europaea* germplasm; b) the simultaneous scanning and genotyping PCR products without probe in the same reaction, that has never been described before. All the SNPs found with HRMA technique were confirmed by sequence analyses and tetra primer ARMS-PCR techniques.

As no functional gene markers have been analysed in olive, an other result reached by this work was to identify SNP markers on coding sequences correlated with traits important for the ecology of the species and able to show polymorphism in loci under natural selection, such as phytochrome A (*phyA*) and glycosyl transferase-4-like gene (*ltg4*).

On every fragment of *phyA* and *ltg4* gene, isolated from cv "Canino", we have designed two sets of oligos able to produce two amplicons of 308 bp and 800 bp, respectively, to scan for the presence of single polymorphic mutations. PCRs were performed in 10-µl volumes Light Cycler (Roche Applied Science) with programmed transition of 20°C/s. Into the amplification mixture, made in capillary tubes, DNA templates, primers, dNTPs, bovine serum albumine, Taq Polymerase, appropriate buffer and LCGreen^{TM} DNA dye (Idaho Technology) were added. PCR cycling varied with the target. After amplification, the samples were heated in the Light Cycler to 94°C and cooled to 40°C at the programmed rate of 20°C/s. The capillary tubes were then transferred to the High-Resolution Melting instrument (HR-1; Idaho Technology), and melting curves were obtained by heating from 70 to 95°C at 0.3°C/s. High-resolution melting data were analysed with HR-1 software by normalization, derivate and difference of fluorescence of the melting temperature. The presence of many heterozygous allelic mutations was observed, as well as mutations for substitution in *ltg4* and *phyA* genes between and inside Canino and Nocellara Etnea cultivars. Mutations were confirmed and localized by sequence analysis.

Very close to the single mutations we designed specific primers to genotype 38 cultivars from Italy and Croatia by HRMA and confirmed by tetra primer ARMS-PCR. In these experiments we showed that HRMA has a very high sensitivity both for screening procedures, for detecting

mutations and heterozygous single-nucleotide polymorphisms within a PCR product up to 800 bp. Using the shape of the curve melting transition we were able to identify single heterozygous mutations, close- and far-localized double heterozygous mutations, and multiple mutations, in *phyA* and *ltg4* gene. Moreover, in all analysed cases, HRMA of melting transition also allowed us genotyping without a probe.

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