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ORIGINAL PAPER

Comparative study of microsatellite profiles of DNA from oil and leaves of two Tunisian olive cultivars

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Abstract The potential of microsatellites in traceability of Tunisian oils was assessed by comparison of the genetic profile for six SSR markers of DNA extracted from oil and leaves of the two Tunisian major olive cultivars: *Chemlali* and *Chetoui*. Different protocols of DNA extraction from oil were tested and compared and we found that the most reproducible protocol in terms of successful microsatellites amplification was the Qiagen QIAamp DNA Stool extraction Kit, which we slightly modified. After amplification and automatic genotyping of six SSRs, it has been shown that the pattern of the DNA purified from a monovarietal oil did not correspond exactly to the profile of the same cultivar leaves' DNA. In fact, we found evidence for the presence in the oil of alleles originating from the pollinators, which are present in the genome of the seed embryo. These alleles appear as minor peaks and could be easily distinguished from the mother tree's alleles, so they do not interfere with oil traceability.

Keywords Olive · Microsatellites · Oil · Pollinator · Traceability

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Introduction

Olive tree (*Olea europaea* L.) represents the most important oil producing crop in the Mediterranean basin. Olive tree is a diploid species ($2n = 46$) that is able to survive for a long time [1, 2], is outcrossing and sometimes self-incompatible which implies that seeds are produced by cross-pollination [3, 4].

The olive oil is known for its beneficial effects on health, such as ability to reduce blood pressure and low-density lipoprotein (LDL) cholesterol, as well as for its cancer prevention, antimicrobial and antioxidant virtues [5]. Moreover, the olive oil sector is a major component in the culture and socio-economy of many Mediterranean countries, including Tunisia. In fact, olive trees in Tunisia represent the third of the cultivated area and 50% of the different olive tree plantation is dominated by two major cultivars, *Chemlali* in the south and the centre and *Chetoui* in the north. These two cultivars represent 80% of the exported olive oil.

However, in the current economic context, where olive oil is concurred by others oils and by typical and Protected Designation of Origin oils, there is an urgent need for a better knowledge of Tunisian olive germplasms and their oils in order to develop typical oil products with high quality label. The European Union has developed several instruments (appellation) such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) which are all utilised for quality control of the olive oils. Hence, it is crucial to develop procedures of traceability that allows rapid an easy identification of cultivars and oils.

Chemical analyses are not enough to verify olive oil authenticity [6]. In fact, the chemical characterisation of

fatty acids, sterols, phenols and secondary metabolites composition does not allow exact identification of cultivars due to environmental effect on the phenotype and particularly on the chemical composition [7]. In addition, chemical analyses necessitate expensive and numerous tests [8]. Recently, it has been suggested that molecular markers such as RAPDs (random amplified polymorphic DNA) [7], AFLPs (amplified fragment length polymorphisms) [9] and SSR (simple sequence repeats) can be used to solve traceability and provenance issues of olive oil [10].

The extraction of DNA from olive oil is a hard task due to the low amount and the highly degraded DNA by nucleases present in olive oil [10–12]. Furthermore, residual polysaccharides and phenolic compounds are able to inhibit the activity of DNA polymerases and provide erratic (irregular) PCR amplifications [10]. In order to achieve the amplification from degraded DNA, it is necessary to use the most reliable molecular markers. At present, microsatellites (SSRs) are the most appropriate genetic markers used in olive cultivar characterisation and olive oil authentification because of their several advantages.

SSR markers are multiallelic, codominant, highly polymorphic, widely distributed along the plant genomes, easily amenable to PCR based analyses, have great reproducibility and are currently the most reliable DNA profiling techniques in forensic investigation [13].

In the present work, In order to assess the potential of SSRs in traceability of Tunisian oils, we have studied and compared the genetic profiles for six SSR markers in olive oil and leaves from the two major cultivars: *Chemlali* and *Chetoui.*

Materials and methods

Plant material

The two Tunisian olive tree cultivars of major commercial interest were used in this study. The cultivar *Chemlali* dominates the south of the country whereas *Chetoui* is present in most olive areas in the North of Tunisia. For each cultivar, two trees were used, and from each tree, DNA was extracted from young leaves and from olive oil.

Production of oil

Olive oil is produced by grinding 2.5 Kg stoned olives and extracting the oil by mechanical means. The procedure for monovariety oil production followed the standard methods used in oils factories, including milling, malaxation for 30 min at 25 °C, centrifugation at 2000*g* for 3 min and olive oil was obtained by natural decantation. Samples were stored at 4 °C until DNA extraction.

DNA extraction

DNA was extracted separately from leaves using the CTAB protocol followed by two purification steps. Young leaves were frozen and powdered under liquid nitrogen using a mortar and pestle, total DNA was extracted from leaves of two *Chemlali* and two *Chetoui* cultivars using the CTAB methods described by [14] in which DNA was precipitated in 2.5 volumes of ice-cold absolute ethanol and the pellet was resuspended in 150 µL TE buffer.

The DNA was extracted from oil using different extraction procedures and Kits (Table 1). The performance of these protocols was assessed by success of PCR amplification for all SSR primers.

DNA was quantified by Hoechst H33258 dye incorporation detected by spectrofluorometer (Tecan GENIOS Plus) and dilution series of lamda DNA (D150A Promega) were used as calibration standard. Genomic DNA was undiluted in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) at −20 °C.

Microsatellite markers

Six microsatellites (SSRs) markers were used in this study. Two markers (DCA1, DCA3) from the primer set designed of Sefc et al. [15], three markers (GAPU59, GAPU71A, GAPU71B) from Carriero et al. [16] and one marker (UDO12) from Cipriani et al. [17] were selected for their high polymorphism in many olive cultivars [18] and in Tunisian cultivars [14].

PCRs and capillary sequencer

PCRs were performed in a 15 µL volume consisting of 10 ng of olive oil genomic DNA or 20 ng of young leaves genomic DNA, 2 mM $MgCl_2$, 0.05 mM of each dNTP, 0.1 µM of forward primer (forward primer was labelled with FAM fluorescent dye), 0.4 µM of reverse primer, 0.5 unit of Go Taq (Go Taq Flexi DNA polymerase, Promega), 1.buffer Go Taq; performing PCR amplifications on a thermal cycler verity (Applied Biosystems, 96 well) for:

Table 1 Protocols used for DNA extraction from oil

DNA extraction procedures	Microsatellite amplification	References
Protocol using added water	No	
Wizard kit	Yes (but inconsistent)	http://www.promega.com
CTAB	No	[7]
Qiagen QIAamp DNA stool	Yes	[10]

Table 2 Genotypic profiles for six SSR markers and two olives cultivars

Cultivar-organ	DCA1*	DCA3	GAPU59	GAPU71A	GAPU71B	UDO12
Chemlali1_leave	230/234	247/255	208/212	212/214	120/140	155/157
Chemlali1_oil	230/234	247/255 **259**	208/212 **218 + 220 + 222**	212/214	120/140 **126**	155/157
Chemlali2_oil	230/234 **222 + 242**	247/255 **261**	208/212 **218 + 222**	212/214	120/140	155/157 **166**
Chetoui1_leaves	222/222	247/255	212/214	210/228	117/122	157/166
Chetoui2_leaves	222/222	247/255	212/214	210/228	117/122	157/166
Chetoui1_oil	222/222	247/255	212/214	210/228	117/122 **140**	157/166
Chetoui2_oil	222/222	247/255 **261 + 263**	212/214 **218 + 222**	210/228	117/122 **140**	157/166
Allele range in pb	222–242	247–263	208–222	210–228	117–140	155–166

*Alleles present in oil but not in leaves are indicated in bold

DCA1, DCA3, GAPU59, GAPU71B primers at: 95 °C for 5 min for 1 cycle, 95 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s for 35 cycles, then 72 °C for 10 min and for GAPU71A and UDO12 primers at: 95 °C for 5 min for 1 cycle, 95 °C for 30 s, 57 °C for 45 s, 72 °C for 45 s for 35 cycles, then 72 °C for 10 min.

Five microliters of PCR products was mixed with 0.3 µL of (marqueur 420pb) and 14.7 µL deionised H_2O, centrifuged at 2000 rpm for 1 min, denaturated at 94 °C for 3 min, cooled in ice and analysed on a (3130XL Genetic analyser of Applied Biosystems) capillary sequencer.

Data analysis

The alleles detected for each microsatellite were recorded into a data matrix of presence (1) and absence (0) of bands (each allele representing a band). Since most olive cultivars are auto-incompatible (pollen could not germinate on an ovary from the same tree) the DNA extracted from oil contains alleles of the tree (fruit pulp somatic tissues) as well as alleles of the seed embryo which may contain exogenous alleles from the pollinator. This means that three alleles or more (different pollinators for the same tree) can be found in olive oil.

We tried to track the origin of alleles from pollinators by browsing the genotypes of others Tunisian cultivars studied for the same markers from the previous studies by Taamalli et al. [19] and Rekik et al. [14].

Results and discussion

DNA extraction

Various methods of DNA extraction from olive oil were compared (Table 1). The quality and the yield of DNA extracted from virgin olive oil were low and the PCR amplifications were inconsistent with all methods tested. Nevertheless, the most reproducible results were obtained when the template DNA was recovered from the oil using the QIAamp DNA stool extraction Kit (Qiagen). For this reason, DNA was extracted from 200 µL for each olive oil sample via the QIAamp DNA stool (Qiagen) by the use of the protocol for pathogen detection [10] with minor modifications. In the first step of the protocol, 200 µL of olive oil was lysed in buffer ASL by incubating the homogenate at 80 °C. In the final step of the protocol, due to the low amount of DNA extracted from the olive oil, only 50 µL of the elution buffer (TE) provided by the kit was used for each sample. Different PCR product concentrations were used, but only diluted DNA samples, gave the best SSR amplification profile.

The amount of DNA extracted from virgin olive oil was quantified by spectrofluorometer assay and the quantities are very low ranging from 5 to 10 ng/µL.

Microsatellites markers and genotyping of DNA from olive oil and young leaves

Cultivar identification

The amplification was successful for all SSR markers. A total of 27 alleles were found with an average of four alleles per locus ranging from three for UDO12 to six for GAPU59 (Table 2). There were 16 alleles found in leave samples and 11 alleles present only in oil.

No differences were observed between the two cultivars *Chetoui* and *Chemlali* for marker DCA3, but different profiles were found for the other markers. DCA3 has also the lowest number of alleles showing its low potential for traceability. On the contrary, marker GAPU71A was able, alone, to differentiate these two olive varieties because they have no shared alleles.

This result is consistent with the findings of Rekik et al. [14] who found that marker GAPU71A showed a high heterozygosity in a set of 20 Tunisian cultivars. However, difference in genotypes found in our study and theirs was observed for markers GAPU59, GAPU71B and UDO12.

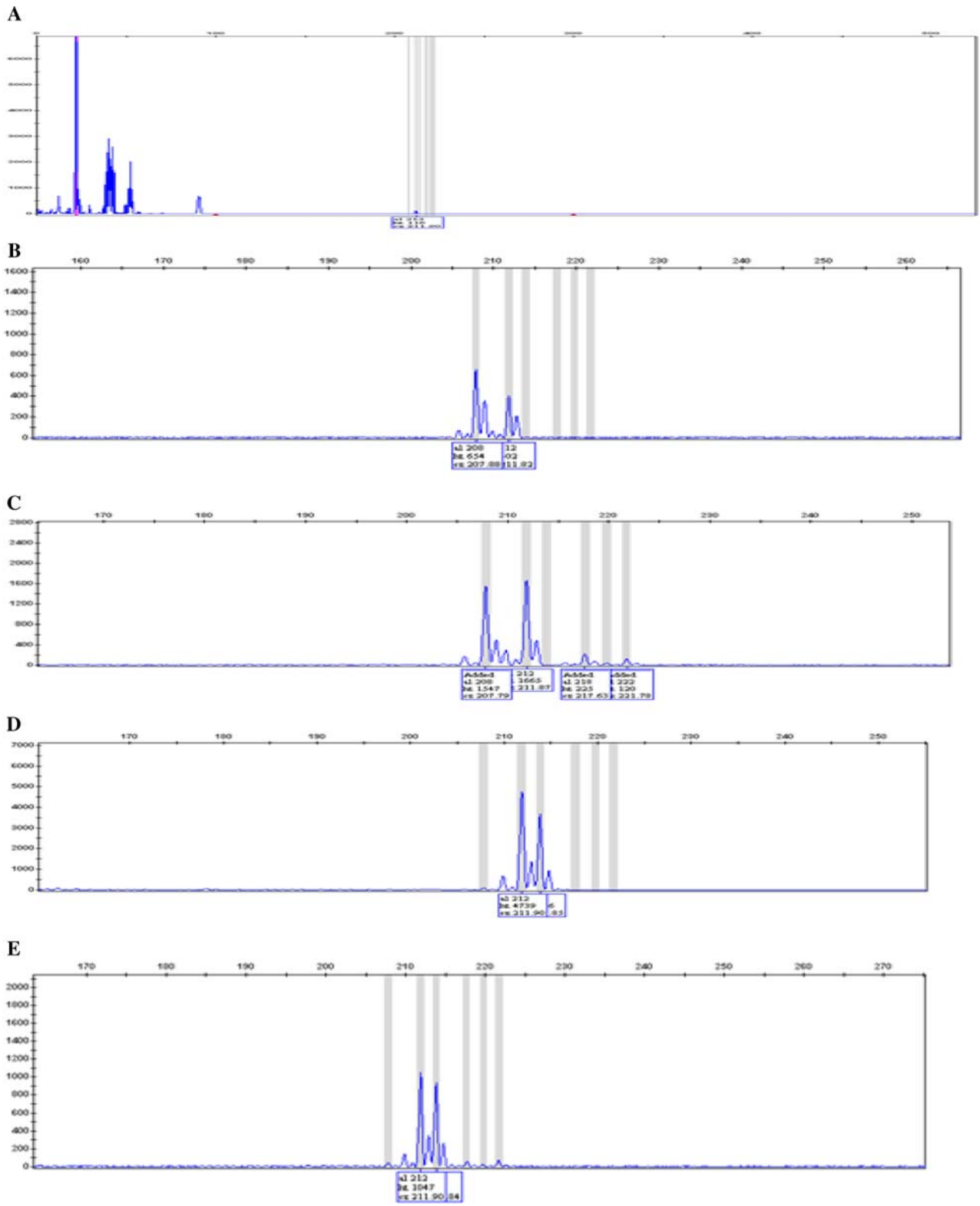

Fig. 1 Capillary electrophoresis electropherogram showing the amplification of the microsatellite GAPU59: **a** white sample, **b** DNA extracted from *Chemlali* leaves, **c** DNA extracted from *Chemlali* oil, **d** DNA extracted from *Chetoui* leaves, **e** DNA extracted from *Chetoui* oil. Peaks correspond to SSR alleles. The minor peaks in graph **c** show the presence correspond to embryo's alleles. The profile represents the region between 140 and 180 bp. In the *x*-axis is reported the size of DNA fragments, while the *y*-axis refers to the fluorescence intensity of the signal

These differences can be explained by the presence of null alleles (non amplification of some alleles resulting in a homozygous genotype) in the study of Rekik et al. [14] due to the low sensitivity of the technique used.

Oil genotypes

When compared with the profiles of DNA obtained from genomic DNA (extracted from leaves) genotypes of oil sample showed notable differences for some markers and the SSR profile contains a high number of peaks (Fig. 1). The low intensity peaks exist exclusively in oil sample whereas high intensity peaks are shared by oil and leaves' samples. So, it is clear that amplification produced additional alleles in the profile of DNA that was recovered from the oil. These alleles very likely originate from the paternal contribution (pollinator) and are present in the embryo (seed).

This demonstrates that embryo genome is released in the oil during the extraction process although the amount of the embryo's DNA is very low compared to mother's DNA (flesh part of fruit) which shows the high sensitivity of our genotyping technique. The size of the peaks corresponding to paternal alleles is at most one-fifth of the peaks of mother's alleles making them easily distinguishable in DNA from oil samples.

It is interesting to note that, for some markers, oil sample have exactly the genotype of the mother which indicates either self-pollination or pollination by another tree of the same clone. However, for other markers we found, in the same oil sample, up to three alleles originating from the pollinator (Table 2). This is particularly seen for marker GAPU59, where for two oil samples from the two cultivars, we found the same alleles (218 and 222) coming from the pollinator. In fact, allele 218 was found to be the most prevalent allele in twenty olive Tunisia cultivars (present in 50% of cultivars) but the origin of allele 222 is unclear since it was not found in any of these cultivars [14].

It is also worth noting that alleles from *Chemlali* varieties (present in the centre and in the south of the country) were detected in the oil samples from *Chetoui* (cultivated in the north) for marker GAPU71B and the inverse was seen for marker UDO12. This shows that these two cultivars can cross-pollinate each others and that olive pollen can travel over long distances. This was already pointed out by Ribeiro et al. [20] who found evidence that olive pollen can travel over 200 km.

The diverse genetic composition of embryos did not invalidate the molecular analysis of cultivars used for olive oil productions because the DNA profiles from oils show very low peaks corresponding to pollinators' alleles when compared with the major peaks corresponding to mother tree's alleles. Therefore, it is possible, not only to track the origin of the commercial oil obtained by crushing whole fruits but also to identify, in some circumstances, the pollinator. Consequently, the interpretation of DNA profiles obtained from DNA recovered from oil and using the microsatellite markers are sufficient to verify olive oil authenticity and provenances issues and also to defend the consumer from fraud. Our results contrasts with those of Doveri et al. [2] who suggested that the presence of paternal DNA from the embryos in oil would make traceability of olive oil very hard in practice.

Another critical step is the quality of DNA which plays a very important role in the success and reproducibility of SSR amplification. From our study we found that DNA extraction from olive oil was the most critical step in the success of the amplification of microsatellite markers.

Conclusion

In conclusion, we demonstrated that five among the six SSR markers investigated, show different genetic profiles in the two major Tunisian cultivars when studied for their genome as well as for their oils. For some markers we were able to identify alleles of the pollinators in oil samples and distinguish them from tree somatic tissues' alleles. So, these markers might be used with confidence for oil traceability. However, the most difficult task is that only small amounts of DNA can be extracted from olive oil and the reproducibility of the amplification markers depend on the quality of the recovered DNA.

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