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Phylogenetic analysis of selected olive genotypes by ISSR markers

Филогенетичен анализ на отбрани маслинови сортове чрез ISSR маркери

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ABSTRACT

The genetic relationships among six different olive (*Olea europaea* L.) genotypes were determined through ten ISSR primers by obtaining informative profiles using five of them. The studied cultivars 'Chondrolia Chalkidikis', 'Karidolia,' 'Prodromos,' 'Kalamon,' 'Koroneiki,' and 'Leucocarpa' are widespread and grown in Chalkidiki and Northern Greece. By analysis of the amplification, a total of 34 bands were observed, and it was noticed that 26 of them were polymorphic, showing the high efficiency and reliability of the markers used. The highest number of ISSR bands detected for primer E4 was 10 (8 polymorphic). The calculated percentage of polymorphism was 76.5. A cluster analysis was performed based on the molecular characterization results, and a dendrogram was constructed using average linkage. The current genotyping presents distinctive profiles useful for proving olive planting material's cultivar and clonal identity.

Keywords: *Olea europaea* L., diversity, genetic markers, identification

РЕЗЮМЕ

Генетичните връзки между шест различни генотипа маслина (*Olea europaea* L.) бяха определени чрез използване на десет ISSR праймера като са получени информативни профили за пет от тях. Изследваните сортове 'Хондролия Халкидикис', 'Каридолия', 'Продромос', 'Каламон', 'Коронейки' и 'Бяла маслина', са широко разпространени и се отглеждат в Халкидики и Северна Гърция. При анализ на резултатите от амплификацията са наблюдавани общо 34 фрагмента и се установява, че 26 от тях са полиморфни, което показва високата ефективност и надеждност на използваните маркери. Най-много ISSR фрагменти бяха установени за праймер E4 - 10 (8 полиморфни). Изчисленият процент на полиморфизъм е 76.5. Въз основа на резултатите от молекулярната характеристика беше извършен клъстерен анализ и конструирана дендрограма, използвайки средна връзка. Настоящото генотипиране представя отличителни профили, полезни за доказване на сортова и клонова идентичност на произведен посадъчен материал от маслина.

Ключови думи: *Olea europaea* L., разнообразие, генетични маркери, идентификация

INTRODUCTION

Olive (*Olea europaea* L.) is a critical culture with a specific economic and cultural value in Mediterranean countries. Wild olives (*Olea europaea oleaster* L.) have existed for thousands of years, domesticated in the Middle East for about 6000 years (Hosseini Gheydari and Tahernezhad, 2019).

More than 2600 olive varieties belonging to the species *Olea europaea* L. have been described using morphological analysis (Rugini and Lavee, 1992). According to Bartolini et al. (2005), more than 1200 varieties are cultivated in orchards, and roughly 4200 genotypes have been recorded in 79 international and national collections situated in 24 countries. Such a large number of olive varieties causes a considerable problem in the management of the germplasm and collections, as well as on the origin and authenticity of olive oils due to uncertainty in the correct name of the olive variety (Cipriani et al., 2002). Previously, cultivar identification was based only on the morphological characterization of the leaf, fruit and stone, and agronomic features, which is problematic, especially in the early development stages.

Molecular markers have numerous advantages over conventional phenotype-based alternatives. Molecular markers represent a specific DNA segment whose nucleotide sequence is polymorphic with different organisms (Kahl, 2004). In recent years, molecular markers have been successfully used to investigate the gene pool of olives, to identify differences between varieties, as well as the olive oil they produce.

Inter Simple Sequence Repeats (ISSR) are based on fragment amplification (200-2000 bp) between backward oriented, closely spaced microsatellites. ISSRs do not have the microsatellite markers' primer specificity, as they do not need nucleotide sequence information for primer synthesis. ISSR markers have been developed to address some of the disadvantages associated with RAPD (low reproducibility), AFLP (high cost), and the need to know border sequences to design primers for SSR polymorphism (Zietkiewicz et al., 1994; Terzopoulos et al., 2005). Alone or in combination with other marker systems, they have been widely used to analyze clonal variation and genetic diversity in olive varieties (Gemas et al., 2004; Terzopoulos et al., 2005; Martins-LOPES et al., 2007, 2009; Gomes et al., 2008, 2009; Khadari et al., 2010).

Previous studies have concluded that molecular markers and the ISSRs were useful in evaluating phylogenetic relationships in *O. europaea* (Hess et al., 2000; Gemas et al., 2004; Beiki et al., 2012; Hegazi et al., 2012; Linos et al., 2014; Golmohammadi et al., 2019) and for identification using olive leaves, fruits, and oils (Pasqualone et al., 2001; 2004; 2007; 2012).

The present study aimed to evaluate the genetic relationships of six olive genotypes grown in Chalkidiki, Greece, by the ISSRs marker system. The investigation is a preliminary step for the further characterization of micropropagated olive planting materials with cultivar authenticity, towards avoiding somaclonal variation and, consequently, optimizing the process of identifying and preserving the local olive genetic resources.

MATERIAL AND METHODS

Plant samples and DNA isolation

Plant material of six genotypes (Table 1) was collected from mature olive trees grown in the region of village Ormylia, Chalkidiki, Greece, and used in the study.

Molecular characterization was carried out at the Department of Genetics and Plant Breeding of AU-Plovdiv.

DNA was isolated from young, fully developed leaves. Each sample (200 mg) was ground after freezing in liquid nitrogen to a fine light green powder. The Amersham-Pharmacia commercial kit (PhytoPure DNA extraction kit) was used for obtaining genomic DNA of appropriate quality (lack of degraded fragments) and in approximately equal amounts of each extraction. DNA quality was tested in a 1% Agarose gel electrophoresis.

Table 1. Olive genotypes (1-6) used in the study

№/Genotype	Description
1 Chondrolia Chalkidikis	The main olive variety of Chalkidiki, Central Macedonia, and Northern Greece, large-fruited, cultivated mainly for consumption. It is harvested and processed green; therefore, it is known as "*aguraki*," which means little cucumber. It is also used for the production of stuffed fruit with almonds, pepper, carrot. Vigorous variety, the only one behaving as self-incompatible. The main pollinator is 'Dafnolia.' The small fruit rejected after calibration is used to produce excellent olive oil.
2 Kalamon (Kalamata)	The very ancient olive variety originated in Kalamai or Kalamata, where remains the leading one. The fruit is big, having a very characteristic shape similar to the nail of the eagle; therefore, its other name "*aetonycholia*." It obtains a typical black color, and it is processed as ripe - black. The olive fruit has the best organoleptic properties, the most prolific standard in Greece for olives' consumption.
3 Koroneiki	The main ancient variety of Peloponnese, now cultivated in many countries for the production of olive oil. The name comes from the city of Koroni in South Peloponnese. Despite its smallest size of all olive varieties, the fruit has a very high yield of polyphenol-rich olive oil with excellent quality. The cultivar is the primary source of Greek olive oil.
4 Prodromos	Variety originates from Aliakmon's valley in the county of Imathia, cold-resistant, very productive. Semi large fruit with a high yield in olive oil, obtaining typical black color suitable for processing. In the last years, it is planted in large numbers in Northern and Central Greece, suspected as a seedling/variety of 'Chondrolia Chalkidikis' found in mountain place at altitude 800 m.
5 Karidolia	A clonal form of 'Chondrolia Chalkidikis,' with a more rounded, nut-shaped fruit, found up to 10% all over the main variety plantations. The critical difference is the shape of the fruit. The fruit of 'Chondrolia Chalkidikis' is having an asymmetrical shape ending in a breast-like nipple. The fruit of 'Karidolia' is roundish, having more or less a walnut (*karidi*) form. It does not obtain the typical black color, ripens early, and harvested by the end of November - beginning of December for the procession as edible. It is also used as stuffed.
6 Leucocarpa (White olive)	Leucocarpa is a rare olive characterized by small fruits which assume a peculiar ivory-white color when ripe, with tiny silver leaves. It has an ornamental and ritual significance as a gift for weddings and christenings. It is a source for the production of special colorless extra virgin olive oil with ideal organoleptic characteristics.

PCR Amplification

PCR analysis was performed using ten ISSR primers (Table 2) in a reaction volume of 25 µl for each sample: PCR buffer -2.5 µl; dNTPs -1.5 µl; ISSR primer -1.5 µl; Taq-0.12 µl; H_20 -18.38 µl; 1µl genomic DNA in the following amplification mode: 94˚C initial denaturation for 3 min, subsequent 40 cycles: denaturation 94 ˚C - 1 min, annealing - 45 sec, extension 72 ˚C - 45 sec and a final extension 72 ˚C - 4 min. The annealing temperature was determined based on the melting temperature of each primer, according to Kochieva et al. (2002).

The obtained products were analyzed by electrophoresis on a 1% agarose gel. At each start, 5 µl of PCR product was loaded. The fragments were stained with ethidium bromide and visualized on a UV transilluminator. A molecular marker (M) with a length of 100 to 10 000 bp (Fermentas) was used to determine the fragments' size.

Table 2. Sequences of ISSR primers

Primer	DNA sequence	Length (bp)
E1	(CA)8AA+GG	20
E2	(CA)8AA+GC+T	21
E3	(GA)8C+TC	19
E4	(AG)8C+TC	19
E5	(AC)8C+TA	19
E6	(AC)8C+TG	19
E7	(AG)8C+TG	19
E8	(AC)8C+TT	19
E9	(AG)8C	17
E10	(GA)8T	17

Data and phylogenetic analysis

The data obtained by PCR analysis were used to construct a dendrogram. DNA fragments of ISSR-PCR reactions were scored by their presence (1) or absence (0), and the ones at low intensities were counted when they were reproducible in the PCR runs. Cluster analysis was completed using the statistical package "SPSS for Windows."

RESULTS AND DISCUSSION

The DNA extraction protocol used resulted in obtaining genomic DNA with similar quality (lack of degraded fragments) and approximately equal amounts.

ISSR marker system is easy for performance and requires purified genomic DNA, proper primer selection, and appropriate amplification mode. The results' analysis demonstrated the potential of five of the chosen ISSR primers (E4, E5, E7, E9, and E10) to differentiate the genotypes studied (Table 3). The other five primers (E1, E2, E3, E6, and E8) did not give satisfactory results.

A total of 34 ISSR amplified fragments were identified using the selected informative primers. The number of visualized amplified products for each primer ranged between 5 (E7) and 10 (E4), with an average of 6.8 bands. By the analysis of the amplification, 26 polymorphic sequences were differentiated. The polymorphic bands varied between 3 (E5) and 8 (E4), with a mean of 5.2 for the study. The primer E4 was selected as the most informative and able to differentiate the studied genotypes. Figure 1 shows the electrophoregrams of the informative for the study primers.

With the ISSR primers used, the counted percentage of the polymorphism was 76.5. It is higher than the established 53.3%, as Hagazi et al. (2012) reported, studying the genetic diversity of 22 native and Egyptian olive varieties. As compared, Kaya (2015) reported 94.9% polymorphism in 40 genotypes (8 cultivars, each having five clones) specific to Turkey.

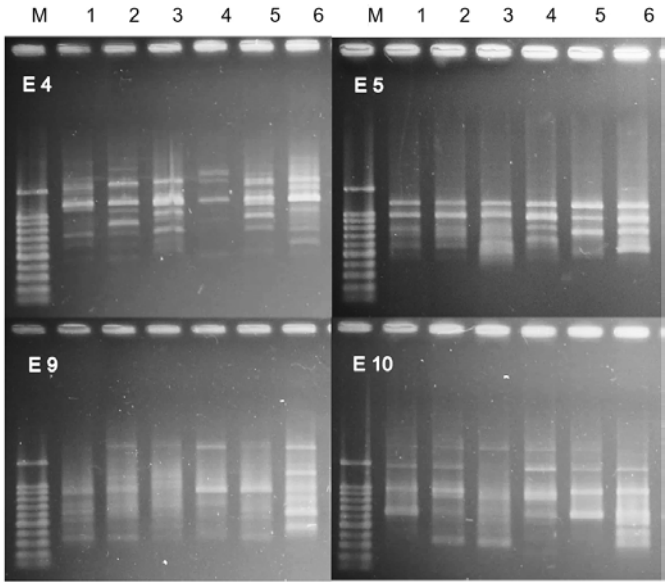

Figure 1. Agarose gel image of PCR products using informative ISSR primers. Lines: M - λ 100 bp marker, 1-6 – studied olive genotypes

Table 3. Amplified products using ISSR primers

Primer	DNA sequence	Total number of amplified products	Polymorphic bands	Monomorphic bands
E4	(AG)8C+TC	10	8	2
E5	(AC)8C+TA	6	3	3
E7	(AG)8C+TG	5	4	1
E9	(AG)8C	6	5	1
E10	(GA)8T	7	6	1
Total		34	26	8
Average		6.8	5.2	1.6
Polymorphism (%)			76.5	

Established here rate of polymorphism could be explained by the limited number of the studied genotypes and primers used. The percentage of polymorphism within the screened samples confirms the existing olive genetic diversity and, therefore, the ISSR system's effectiveness for cultivar and clonal identification of olive germplasm.

A cluster analysis was performed based on the molecular characterization results, and a dendrogram was constructed using average linkage (Figure 2).

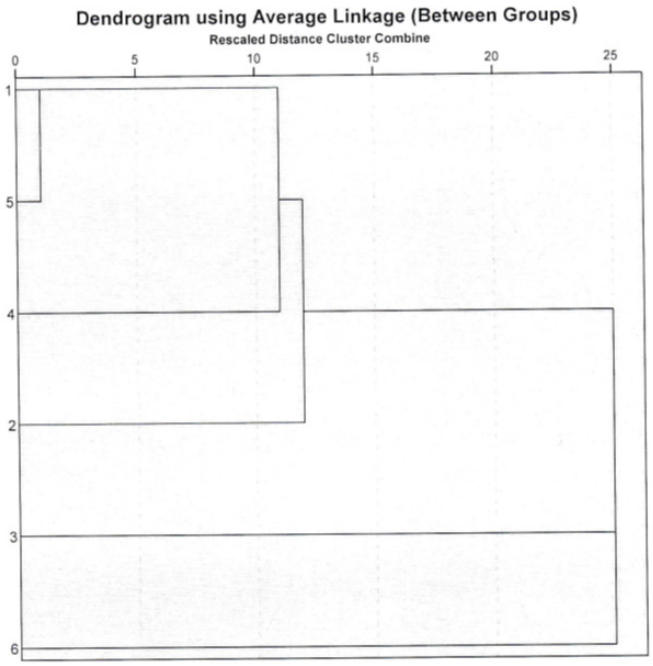

Figure 2. Phylogenetic relationship of the studied olive genotypes (1-6)

'Chondrolia Chalkidikis,' 'Karidolia,' and 'Prodromos' were included in one group, while 'Kalamon,' Koroneiki,' and 'Leucocarpa' were distinctly separated. The clustering demonstrated the similarity between the cultivar 'Chondrolia Chalkidikis' (1) and the clonal form 'Karidolia' (5), as a proposed clonal variation/mutation distinguished phenotypically only by the more oval fruit shape. The genotype 'Prodromos' (4) is suggested as a possible accidental seedling of 'Chondrolia Chalkidikis,' which has been discovered as a single tree, adapted to high altitude mountain conditions, with a putative cold resistance. The cultivar 'Kalamon' (2) is clustered separately but shows a genetic relationship to the genotypes described above, while Koroneiki (3) is separated into another cluster. White olive (6) seems to be genetically distant from all studied genotypes. According to botanical subclassification, the name of the cultivar Leucocarpa originates from the Greek "*leukos*" (white) and "*karpos*" (flesh or pulp). In Leucocarpa, the anthocyanin synthesis is blocked (Lavee, 1986), affecting the examined oils and olives' pigments and chromatic parameters (Pasqualone et al., 2012).

Investigating 103 olive genotypes, Linos et al. (2014) revealed the Greek olive germplasm's genetic structure by RAPD, ISSR, and SSR markers. Their study suggested that both sexual and vegetative propagation have contributed to the evolution of the Greek olive germplasm, providing a useful clarification on synonyms and homonyms, facilitating the identification of duplicates.

According to Pafundo et al. (2007) and Marieschi et al. (2011), when certifying olive orchards and regions, the availability of rapid and effective methodology for identifying olive varieties is crucial for obtaining a protected designation of origin. A thorough and accurate genotype profiling represents a crucial prerequisite to assisting breeding programs, performing comparative studies, and assessing innovative research (Mousavi et al., 2017). The validation of varietal-specific DNA markers is particularly important for the industry to avoid falsification, affecting olive oil quality and price.

CONCLUSION

The elucidation of the genetic links between olive varieties allows the competent management and usage of germplasm resources. The performed ISSR analysis demonstrated that each one of the genotypes was distinguishable from one another. The marker system applied, and the primers proved to be suitable for olive germplasm evaluation. Although the present study was conducted with a limited number of samples, it confirmed the ISSR marker system's reliability and efficiency for olive cultivar and clonal identification. Developed and adapted protocol will be used for further characterization of micropropagated olive planting materials, cultivar authenticity management, and, consequently, optimizing the process of identifying and preserving the local olive genetic resources.

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