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Professional paper

MOLECULAR STUDIES IN OLIVE: A REVIEW ON GENETICS AND GENOMICS DEVELOPMENTS IN *OLEA EUROPAEA L.*

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ABSTRACT

The olive tree (*Olea europaea* L.) is amongst the most widely cultivated crops in the Mediterranean Basin, presenting a major economic importance for the region. The identification process of olive trees used to be performed based on morphological markers, meanwhile during the last three decades molecular markers used in olive culture, have rapidly evolved and are already applied for various purposes. The usage of molecular markers is primarily related to genetic diversity and phylogenetic analysis, owing to their validity, stability, and simple usage. In addition, molecular studies in olive are also being applied for genome mapping, gene expression, inheritance patterns, and marker-assisted selection. Numerous techniques have been constantly developed and employed in olive studies, nevertheless, a handful of them have received positive outcomes. The recent advances in DNA sequencing, ESTs, and SNPs, as well as traditional DNA markers (e.g. RAPDs, AFLPs, ISSRs, and SSRs), have led to high throughput analyses of olive cultivars. Modern functional markers and transcriptomics will have a great impact and are expected to accelerate further progress in taxonomy, olive variety identification, genetic map construction, conservation strategies, and olive breeding. In this review we introduce the most relevant molecular markers, applied in the olive tree and discuss the recent techniques, focusing on utilization of each approach.

Keywords: Olive, *Olea europaea L.*, olive studies, molecular markers, genetic diversity, genome analysis.

INTRODUCTION

Olive is a native species originating from Asia Minor (Besnard *et al*., 2018) being a historical tree in the Mediterranean Basin. The olive tree is the most cultivated among temperate crops in the world and has been domesticated for almost 6000 years in Mediterranean countries, where the majority of the world's germplasm is found (Yadav *et al*., 2021) Olive genetic resources are extensively dispersed throughout the Mediterranean region and recently cultivated in other countries including China, Australia, South Africa, Argentina, USA (Chiappetta *et al.,* 2017). These resources consist of wild and domesticated olive cultivars and related subspecies. Different studies have indicated that the cultivation of olives is a process affected by time and regions because of climate variations. (Besnard *et al*., 2018). Over two thousand olive varieties are known worldwide, the majority of them cultivated in the Mediterranean basin where new ecotypes are identified. This germplasm resource is developed during a long process and has led to the conservation of many traits closely related to tolerance to various environmental stresses and diseases, that potentially could help in preventing biological diseases and environmental challenges in the future.

The cataloging process of accessions includes identification, characterization, authentication, and naming of the cultivars. This process should be mandatory before the distribution of any plant material from germplasm banks, thus avoiding confusion on the identity of varieties and providing the diffusion of true-to-type cultivars. In this context, the correct identification despite being challenging, at the same time is a first step in preserving genetic diversity (Sion *et al*., 2021). Another issue is the confusion in the nomenclature of varieties due to the process of adaption of cultivars in new climatic conditions and the use of local names for newly introduced plant material. This uncertainty in olive nomenclature has brought up the necessity to verify cultivars using reliable methods, such as molecular and morphological markers (Arranco *et al.*, 2000, Corrado *et al*., 2009). In addition, morphological and molecular data have shown that each marker has its unique aspects of variability thus the combination of all markers would be a useful approach for genotyping regional varieties and identifying novel accessions (Laaribi *et al*., 2017). The study of genetic diversity is of utmost importance and related to several factors including the selection of desired traits in breeding programs, and increasing disease resistance to different factors in crops. Furthermore, in conditions of climatic extreme occurrences is essential to conserve plant genetic resources as a valuable reserve of gene pools in the future. (Sion *et al*., 2021).

This review contains information regarding earlier and recently developed techniques based on molecular markers and application in olive culture.

MORPHOLOGICAL MARKERS

Morphological characterization is based on a systematic description of the morphological characteristics of plants, where the traits must be highly heritable, discriminative, easily distinguishable, and uniformly expressed both at taxonomic and agronomic levels (Bartolini *et al*., 2006). Morphological and phenological characterization is earlier performed. Olive cultivar identification has traditionally relied on fruit features however, this approach may encounter several challenges due to their stage of growth and environmental conditions influencing fruit quality (Ipek *et al.,* 2012). Trujillo *et al*., 2013 evaluated 11 traits of endocarp, which are the most discriminative and solid, and can be conserved for a long period, meanwhile fruit characteristics fluctuate based on the state of the environmental factors, considering endocarp important in olive catalog descriptions. A recent study of Greek olive cultivars has developed a semi-automatic algorithm to evaluate qualitative and quantitative traits related to fruit, leaves, and endocarp (Blazakis *et al.,* 2017). Another recent study performed by (Castro *et al*., 2019) developed a high-throughput system consisting of UAV imagery and an algorithm allowing automatic, rapid, and accurate identification of olive tree height, age, and architectural traits in large-scale cultivation. Galvez *et al.,* 2021 used a high-resolution imaging system, a reliable UAV (unmanned aerial vehicle) methodology to examine tree canopy traits in olive germplasm banks, aiming to help in the selection of potential cultivars for different growing systems. Although a useful tool in modern mapping population studies, morphological markers suffer some limitations including environmental ones, and for this reason is essential to progressively use and combine this technique with other DNA assay techniques (Sion *et al*., 2021).

Importance of using molecular markers

Molecular markers are fragments of DNA associated with a genome and are utilized to determine a specific DNA sequence. Since the first discovery in the 1980s, they have provided a necessary, cost-effective, rapid, and valuable method for genetic diversity and polymorphism estimation (Sion *et al*., 2021).

Once molecular markers are discovered they have helped in DNA fingerprinting, creating genetic linkage maps for protein-coding genes, population studies, recognition of linked traits, and QTL (quantitative trait locus) mapping (Nickle and Barrete-Ng, Sion *et al*., 2021). Several features such as neutrality, high density, and polymorphism make molecular markers appropriate to use in genetics due to their frequent distribution throughout the genome. They represent an excellent tool for genetics research and can be used for several purposes including DNA polymorphism detection and fingerprinting, paternity analysis, genetic mapping, cultivar and olive oil traceability, phylogenetics studies, and marker-assisted selection (Bracci *et al*., 2011, Sion *et al*., 2021). Molecular characterization and genotyping are essential tools contributing to the quick and precise identification of olive varieties and wild forms, through molecular fingerprinting platforms. Since being developed, microsatellite markers have been widely applied in olive characterization. Several traits, particularly co-dominant inheritance, specificity per locus, multiallelic traits, distribution across the genome, and transferability make microsatellites a powerful tool in the evaluation of genetic diversity. The assessment of intra- and inter-cultivar polymorphisms can be useful in the determination of the standard olive cultivars and may offer valuable insight for upcoming selection programs (Ipek *et al*., 2012).

1. DNA-based molecular markers in olive

Molecular markers are classified based on; their inheritance pattern (dominance or co-dominance), methods of detection (PCR-based or hybridization-based), and the inheritance's transference (paternal, maternal). As well molecular markers are classified based on the polymorphism in the length of a DNA sequence or the variation in the identity of nucleotides at a specific site, in chromosomes (Nickle and Barrette-Ng).

1.1. Hybridization based markers

1.1.1. Restriction Fragment Length Polymorphism

RFLPs are the first markers being developed and use the hybridization method to detect polymorphisms, employing restriction enzymes to cut DNA fragments in specific sites and generate different fragment lengths. This technique is used in taxonomic studies, phylogeny, and the study of plant genetic variability (see Table 1). (Sion *et al.,* 2021)

1.1.2. Diversity Arrays Technology

DArT is a hybridization-based method. It was first developed in rice and later in oats, sorghum, and woody plants, allowing whole genome profiling without performing sequencing, and annotating genetic variability. In olives, DArT markers have shown a remarkable rate of reproducibility. Referring to Domínguez-García, *et al*., 2012, these markers have discriminated among 62 olive cultivars, constructing a genetic map of crossed populations. Genetic diversity

annotated using DArT markers in olive contributes to the proper management of the olive collections. This technique is considered to be appropriate in genome fingerprinting, has a low cost per sample, and is practical, robust, and highly reliable (Grover *et al*., 2014). Belaj *et al.,* 2012 applied DArT markers in combination with agronomical traits and other molecular markers to construct a core collection in olives.

1.2. PCR-based molecular markers

These markers are based on DNA amplification using oligonucleotide sequences and do not include probe hybridization. These markers include; Random Amplified Polymorphic DNA, microsatellites or Simple Sequence Repeats, Inter Simple Sequence Repeats, Single Nucleotide Polymorphism, Random Amplified Microsatellite Polymorphism, Sequence Characterized Amplified Regions, and Expressed Sequence Tags. The analysis is PCR- based and includes DNA isolation, quality and quantity assessment, and gel electrophoresis. Following the amplification step, a gel examination is performed to analyze the DNA pattern fragments. If sequencing is performed, further purification of amplified products is essential, prior to sequencing (Hasan *et al*., 2021).

1.2.1. Random Amplified Polymorphic DNA

RAPD procedure first developed in 1990 by Williams *et al.,* (Table 1) is based upon the usage of random short primer sequences (10 nucleotides) to detect DNA polymorphism. (Bracci *et al*., 2011). Simplicity, cost-effectiveness, and ease of usage are some of the main factors of RAPD markers being widely used.

1.2.2. Amplified Fragment Length Polymorphism

The AFLP technique merges the restriction enzyme reactions step, with PCR amplification. This method has been applied to evaluate inter and intra-cultivar variety and genetic relatedness between wild and cultivated forms (Table 1). This method integrated with other molecular markers similar to RAPD, ISSR, and SSR has contributed to establishing an olive linkage map (Sion *et al*., 2021)

1.2.3. Inter Simple Sequence Repeats

ISSRs are DNA fragments varying from 100 bp to 3000 bp in length. Being highly polymorphic, and reproducible, the straightforward and rapid method enables studying genetic diversity, evolutionary biology, and genome mapping (Table 1) (Marwal and Gaur, 2020). Without previous sequence information, an ISSR primer detects polymorphisms in inter-microsatellite DNA regions (Uddin *et al*., 2015)

1.2.4. Sequence characterized amplified regions

This type of molecular marker requires locus-specific oligonucleotides to bind DNA, these fragments are developed from sequencing of amplified products of RAPDs and AFLP techniques (Sion *et al*., 2021). Despite being not as polymorphic as SSRs, the SCARs markers have shown to be a useful tool in genotype identification. Furthermore, they can be used in plant breeding programs, and forensic analysis (Busconi *et al.,* 2006). These markers are developed to analyze olive oil traceability and are frequently used in olive fingerprinting (Table 1).

1.2.5. Simple sequence repeats

Microsatellites were first introduced in the early 1990s, known as SSR are short tandem repeats oligonucleotides. Polymorphism is expressed from the variability in the number of repetitions. SSRs are used to analyze the relationship between wild and cultivated olives and among different

olive subspecies. These markers are codominant, reproducible, robust, ubiquitous, and reliable (Table 1). Several SSR markers are developed and considered the most reliable and powerful tools in the genetic characterization of olives (Ipek *et al*., 2012). Germplasm conservation is a matter of the uttermost importance, preserving genetic resources from erosion. World Olive Germplasm Banks in Cordoba and Marrakech and others are established to serve this purpose (Kaya *et al.*, 2013, Diaz-rueda *et al*., 2020, Khadari *et al*.,2003), and SSRs are considered a valuable tool for studying olive accessions.

The creation of Next Generation Sequencing has greatly streamlined the isolation of microsatellites, enabling the rapid and effective development of markers (Grover *et al*., 2014). Positive null allele frequencies for several loci similar to ssrOeUA-DCA4, UDO99-39, ssrOeUA-DCA17 are reported by several authors including Muzzalupo *et al*., 2014 and Belaj *et al.,* 2010 and ssrOeUA-DCA17 locus by Baldoni *et al*. 2009. Baldoni *et al*. 2009 have examined and proposed a set of 11 SSRs primers (UDO-043, DCA9, GAPU103A, DCA18, DCA16, GAPU101, DCA3, GAPU71B, DCA5, DCA14, and EMO90) for olive genetic studies. Another study performed by Trujillo *et al.,* 2013 has reported that ten SSR primers (GAPU89, GAPU101, sseOeIGP07, UDO99-043, UDO99- 005, ssrOeUA-DCA9, ssrOeUA-DCA16, ssrOeUA-DCA3, ssrOeUA-DCA11, and ssrOeUA-DCA4) could discriminate between 93 % of the accessions, whereas only five of them can identify 79 % of the olive accessions (UDO99-043, GAPU101, ssrOeUA-DCA9, ssrOeUA-DCA16, and ssrOeUA-DCA3). 466 alleles were identified across the olive collection, where 67 of these alleles were detected in one genotype, and an average of 14.12 alleles per locus were detected with an average PIC value of 0.77. A larger set of 17 SSR primers was found to be necessary to identify genetically all accessions of the World Olive Germplasm Bank.

Genotyping methods using SSR markers have evolved since the primary usage of agarose gel electrophoresis, to polyacrylamide gels and replacing them progressively with more advanced, advantageous, robust, reproducible, highly automated techniques, including capillary electrophoresis and automated sequencers (Yadav *et al*., 2021).

Novel techniques like High-Resolution Melting, have established another pathway of microsatellite annotation to exploit further potential beyond fragment polymorphism. The latest applications of SNP markers pose authentic validity and importance, nevertheless, SSRs are a preferred choice in genetic studies in olive collections and when laboratory resources are limited (Yadav *et al.,* 2021). Gomes et al., 2018 applied a High-resolution melting (SSR-HRM) DNA assay to accurately identification of the olive varieties in olive oil samples, based on the development of nuclear microsatellites (gSSR) and plastid DNA (cpDNA). With the application of SSRs and cpDNA discriminative loci (molecular markers), curve analysis generated 14 HRM profiles sufficient to genotype all varieties, thus proving SSR-HRM to be a reliable and suitable method for varietal tracing of olive oils.

1.3. Sequence-targeted techniques

1.3.1. Expressed Sequence Tags

EST-SSR markers are derived from transcribed regions of the genome, located in genes, developed and used along with SSR or not. These markers are used in map-based cloning, genome analysis, and comparative genomics as well as in plant breeding and marker-assisted selection programs (Table 1) (Arbeiter *et al.,* 2017, Yadav *et al*., 2021). Mariotti *et al*., 2016 have developed a set of 26 EST-SSR markers contributing to olive genotyping, specifically ten OLEST

SSR markers have resulted to be discriminative and highly polymorphic. However, due to the fact of derived from more conserved transcribed regions of the genome, these markers show a lower genetic variability and higher frequency of null alleles compared to SSRs. (Parthiban *et al.,* 2018, Mariotti *et al*., 2016). De la Rosa *et al*., 2013 have shown the relevant application of EST-derived SSR sets of primers in paternity evaluation in olive breeding programs. In recent years, these markers, being highly automated bioinformatics tools, easily developed, and cross-transferable are becoming important and suitable to examine problems regarding variety identification (Mariotti *et al.,* 2016, Yadav *et al*., 2021). A set of SNP markers derived from expressed sequence tags has analyzed the genetic diversity of olives helping in the detection of polymorphism in evolutionary biology and breeding programs (Mariotti *et al.,* 2020).

1.3.2. Single Nucleotide Polymorphism

A Single Nucleotide Polymorphism is a variation in a single nucleotide substitution in DNA fragments. These markers are codominant, highly reproducible, and distributed across the genome (Table 1). Recent developments using Next Generation Sequencing in *O. europaea* genome sequencing have enabled a rapid and wide application of SNPs in phylogenetic studies (Rao *et al.*, 2021, Cruz *et al.*, 2016, Bracci *et al.*, 2011, Biton *et al.*, 2015), genetics map, varietal identification (Belaj *et al*.,2012, Kaya *et al.*, 2013, Ayed *et al.*, 2019) have demonstrated the application of SNP primers olive identification quality and authenticity analysis.

Mariotti *et al*., 2020 in their study have identified 124 possible genes having an impact on the flower development process, metabolic pathways, and environmental stress responses through the EST-SNP analysis, leading to a deeper comprehension of the genetic and genomic diversity of olives, polymorphism gene detection, emphasizing germplasm conservation. SNPs have high reliability due to their abundance and diallelic type. Advancements in sequencing have made SNPs, a marker of choice in the genetics study of olives, constructing genetic maps, and studying geographical relationships and are expected to be advantageous in future studies (Sion *et al*., 2021). Belaj *et al*., 2022 have developed 96 EST-SNP markers to characterize World Olive Germplasm Bank Collections. Authors suggest that SNP-developed markers would be essential in correct identification within collections and in to detect of identical genotypes among collections. This set of markers derived from NGS techniques would contribute to the discovery of unique genotypes and are of great help in marker-assisted selection studies. The recent development of this set of markers will facilitate establishing a public SNP database, having a great impact on cost-effective management and better conservation of the genetic resources of olives.

Table 1. List of the application, advantages and drawbacks of principal DNA markers used in olive studies.

Molecular Marker	Developers	Application in olive studies	Advantages	Limitations	References
RFLP	Williams *et al.,*1989	Genome mapping Phylogenetic studies Wild and cultivated olive variability	Co-dominant Reliable and informative Unique locus identification	Expensive High quality and quantity of DNA required High technical expertise Labor and time consuming	Williams 1989, Grover and Sharma 2014, Sion *et al.,* 2021, Bracci et al., 2011

RAPD	Williams *et al.,* 1990	Phylogenetic studies DNA fingerprinting of olive cultivars The relationship between varieties Intra-cultivar variability	Small amount of DNA required Less technical expertise Distributed all over genome	Dominant markers Poor reproducibility	Sebastiani and Busconi 2017, Bracci *et al* 2011., Sion *et al.,* 2021
SSR	Morgan and Olivieri, 1993	Phylogenetic studies DNA fingerprinting of olive cultivars Paternity analysis Subspecies analysis Construction of linkage maps	Widely distributed in genome Ubiquitous and easy to automate Codominant Robust Reproducible Highly polymorphic	Difficulties discriminating between alleles	Bracci *et al*., 2011 Grover *et* al.2014 Sion *et* al., 2021 Sebastiani and Busconi 2017.
SCAR and CAPS	Paran and Michelmore, 1993	Breeding program, DNA fingerprinting of olive cultivars Olive oil traceability	Definition of individual varieties	Dominant inheritance Detect a small number of different genotypes	Busconi *et al*., 2006
ISSR	Zietkiewicz *et al* 1994	Phylogenetic studies Detection of intra-cultivar variation Germplasm characterization, Ecological and evolutionary studies Construction of genetic linkage maps	Abundance in genome, Hypervariability among individuals No prior target sequence knowledge, Application to non-model species with ease	Mainly used in combination with other markers	Grover and Sharma, 2014
AFLP	Vos *et al* 1995	DNA fingerprinting of cultivars Construction of linkage maps QTL identification Cultivar traceability of olive oil Phylogenetic studies Germplasm characterization	Distributed throughout genome Rapid analysis High reproducibility, High polymorphism (lower compared to SSR),	Dominant inheritance Expensive High technical expertise Labor intensive	Bonin *et al*., 2007, Grover and Sharma 2014
EST-SSR	Wang *et al* 1998	Genetic diversity evaluation Genotyping Paternity analysis Self-compatibility assessment in olive cultivars Marker- assisted selection	High level of polymorphism, Accurate genotyping Easy development Cross-transferability across species	Less polymorphic and lower variability compared to SSR	Sebastiani and Busconi, 2017 Ellis and Burke 2007 Yadav *et al.,* 2021, De la rosa *et al*., 2013
SNP	Wang *et al* 1998	Analysis of genetic diversity DNA fingerprinting of cultivars Genetic maps Cultivar identification and traceability	High polymorphism High reliability High throughput analysis	High automation and technical expertise Prior sequence knowledge	Sebastiani and Busconi 2017 Sion *et al.,* 2021

1.4. Ribosomal and Organelle Based Markers

Ribosomal and other organelle-based markers have been used in olives for several purposes. Ribosomal markers are used in analyzing nucleotide variation in internal transcribed regions of ribosomal genes 18S, 5,8S, and 16S (Bracci *et al*., 2011). Ribosomal DNA sequences are used to describe population relationships within the genus *Olea Europaea* and the family of Oleaceae to analyze the evolution patterns involved. Chloroplastic and mitochondrial markers have haploid and maternal inheritance and the polymorphism detected is used to detect male sterility in several varieties of olives. The diversity in ribosomal and cytoplasmic non-coding regions of the DNA including the internal transcribed spacer and intergenic spacer make them a suitable tool in phylogenic studies. Several cultivated types of olive trees, throughout the Mediterranean basin, were found to contain a chlorotype-specific marker from the Eastern basin, indicating human traces effect on the geographical distribution of olives. Mitochondrial RFLP analysis has confirmed a distinctive genetic diversity in wild olives between those cultivated in the Eastern and Western parts of the Mediterranean basin (Sion *et al.,* 2021).

1.5. Transcriptomics

Transcriptome annotates gene expression, based on qualitative and quantitative variability of RNA transcripts that differs amongst individuals, exposed to the same conditions, highlighting the diversity in single individuals. Throughout the years transcriptome analysis has evolved from Northern Blotting to RNA sequencing data. Transcriptome studies, as well as comparative transcriptomics, provide essential data on how the genetic plant network responds under certain stress conditions (Imadi *et al.,* 2015). Researchers have applied transcriptome to analyze RNA response and gene regulation toward *Xylella* infection in two different olive varieties (Giampetruzzi *et al*., 2016). Balthazard *et al*., 2019 studied the evolutionary history of olive tree domestication across the Mediterranean basin using data from RNA sequencing for 68 wild and cultivated olive trees. Intending to study the genetic diversity along with the transcription and sequence levels, it was concluded that a major domestication event occurred in the Eastern part of the Mediterranean basin and was further distributed towards the Western part. A comparison between wild and cultivated olives domestication has shown alterations in gene expression, in moderate genomic consequences. Time-series expression analysis using RNA sequencing is applied in olives to analyze the variation of transcriptomic response in early development stages. AGL gene, involved in the repression of germination, and ARL1-like gene with implication in olive embryonic development, were identified through this method (Jimenez-Ruiz *et al*., 2018). Transcriptomics analysis is used to reveal candidate-associated genes in plant architecture. Data from (Gonzales-Plaza *et al*., 2016) recognized 2252 differentially expressed genes possibly involved in this process, assisting in olive plant breeding programs. Cruz *et al.,* 2016 have reported the first genome sequencing of the Mediterranean olive trees, providing valuable data for future studies related to developmental and physiological processes, analyzing the history of domestication, improving crucial phenotypic traits, and assisting in novel variety introduction. Using Oxford Nanopore third-generation sequencing and Hi-C technology, Rao *et al.,* 2021 updated the draft genome of olive, identifying a total of nine gene families, with 202 genes linked to biosynthesis pathways, a larger number, compared to previous data of Shotgun sequencing. This discovery would assist in genome studies on gene functions and molecular breeding.

Quantitative trait loci are regions in the DNA that affect the phenotypic alteration of complex traits because of genetic interactions and environmental influences (Powder 2020). Atienza *et al*., 2014 used the QTL mapping technique to detect crucial alleles for the development of a marker-assisted selection program for plant vigor and fruit-associated traits. They have detected five QTLs linked to fruit weight, and trunk diameter, that could help in further application in olive MAS breeding programs. Studies performed by Kaya *et al*., 2016 have demonstrated the usage of association mapping to identify relevant molecular markers linked to important traits in olives. Hernandez *et al*., 2017 have identified loci responsible for fatty acids, including oleic and linoleic acids. These findings are of great importance in olive breeding programs.

CONCLUSIONS

During the last decades, a large number of studies have been performed on olives, aiming to assist in molecular characterization, genetic relationships evaluation, diversity analysis, gene expression, inheritance patterns, marker-assisted selection, domestication process, evolutionary biology, and studying genetic resources, with numerous molecular markers used throughout this process. The innovation related to molecular markers-based techniques has an immense contribution to further advances in breeding programs, cultivar identification, investigating

genetic relationships, and olive oil traceability. Genomic sequencing data will accelerate further development in taxonomy, variety identification, and olive breeding programs. It is expected that functional genomics integrated with transcriptomics, proteomics, and metabolomics will provide helpful explanations for issues of great significance related to olive species.

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