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Chapter

SSR Markers in the Genus *Pistacia*

Salih Kafkas

Abstract

Simple sequence repeats (SSRs) are one of the most powerful molecular marker systems due to abundance in the genomes, its codominant nature, and high repeatability. *P. vera* L. is cultivated species in the genus *Pistacia* due to commercial value of its edible nuts. Other species in the genus are in the wild and are important especially for rootstock sources as well as for ornamental and forest trees. There were a very limited number of SSR markers for *Pistacia* species until several years ago; however, next-generation sequencing (NGS) technology has allowed to develop plenty of SSRs since 2016 in the genus. There are currently about 1500 published SSR markers developed from cultivated *P. vera*. There are also several studies generating SSR loci from wild *Pistacia* species. In a conclusion, there are currently an adequate number of SSR markers for cultivated pistachio and that can be used in wild *Pistacia* species due to their high level of transferability rate between *Pistacia* species. These SSRs can be used for assaying diversity in natural populations, marker discovery, germplasm characterization, parental identification, genetic linkage mapping, and evolutionary studies in the genus *Pistacia*.

Keywords: *Pistacia*, SSR, microsatellite, pistachio, repeats

1. Introduction

Pistacia is a genus in the Anacardiaceae family which also contains cashew, mango, poison ivy, poison oak, pepper tree, and sumac plants [1]. The genus is estimated to be about 80 million years old [2]. It contains at least 11 species, and *P. vera* L. (pistachio) is the only cultivated one for its edible nuts [3]. In addition, its nuts are considerably larger than all the other species in the genus. The other species grow in the wild, and several of them have been used for many years as rootstock seed sources for *P. vera*. Furthermore, most of them have also been used as forest trees [4]. According to the Food and Agriculture Organization of the United Nations, the world production of pistachios in 2017 was 1,115,066 MT, ranking fifth in world tree nut production behind cashews (*Anacardium occidentale* L.), walnuts (*Juglans regia* L.), chestnuts (*Castanea* spp.), and almonds [*Prunus dulcis* (Mill.) D.A. Webb], and currently, Iran, the United States, and Turkey are the main pistachio producers in the world [5].

Pistacia species are dioecious and wind pollinated; however monoecious individuals within *P. atlantica* were also reported [6]. *P. vera* is believed to be the most ancestral species in the genus, and the other species are probably its derivatives [7]. There are two centers of diversity of cultivated pistachio: one comprises the Mediterranean region of Europe, Northern Africa, and the Middle East countries. The second comprises the Eastern part of Zagros Mountains from Crimea to the Caspian Sea. Pistachio cultivation extended westward from its center of origin to

Italy, Spain, and other Mediterranean regions of Southern Europe, North Africa, and the Middle East, as well as to China and more recently to the United States and Australia [8–10].

The *Pistacia* genus is distributed mainly across subtropical regions of the northern hemisphere and consists of both evergreen and deciduous species with shrub and/or tree-like growth habits [7]. Although *P. vera* is a commercially grown species in a number of semi-arid regions worldwide, the species remains quite underexploited when its wide native range and inherent genetic diversity are considered [8, 11, 12]. For instance, commercial pistachio cultivation is done in only a few countries in the world. Besides, pistachio production is done with a very limited number of cultivars in those countries, and most of them are seedling selections from the nature [1, 13]. This narrow genetic base in the production presents a threat in pistachio against new diseases and pests as well as changing ecological conditions. Therefore, the germplasm collections have great potentials to increase the genetic diversity and to develop pistachio cultivars for current production areas and/or to expand the regions where reliable commercial production is possible.

Dioecy and a long juvenile period are the primary difficulties encountered in breeding and genetic studies of *Pistacia*. The long juvenile period in combination with dioecious character causes large investments of time and land for characterization and evaluation of progenies in a breeding program. Furthermore, the genetic control of the most economically significant traits is not clearly understood, including disease and pest resistance, yield, nut quality characteristics, and alternate bearing in pistachio. Therefore, current technologies such as molecular markers are good facilities to overcome such difficulties in the breeding programs. Pistachio is a diploid plant which has a haploid chromosome number of n = 15. It is also highly heterozygous species due to dioecy [1, 14].

DNA markers have played a major role in breeding programs for several decades in plants. Several molecular markers such as randomly amplified polymorphic DNA (RAPD) [15], simple sequence repeat (SSR) [16, 17], sequence-related amplified polymorphism (SRAP) [18], amplified fragment length polymorphism (AFLP) [4], inter-simple sequence repeat (ISSR) [19], selectively amplified microsatellite polymorphic loci (SAMPL) [20], and single-nucleotide polymorphisms (SNPs) [21] have been used to assess the genetic diversity, fingerprinting, phylogenetic relationships, germplasm characterization, sex determination, genetic linkage mapping, and QTL analysis in cultivated and wild *Pistacia* species.

In pistachio, most of the cultivars in the production are earlier selections either by growers or breeders. Pistachio cultivars from controlled crosses are in a limited number, and selected genotypes in the current breeding programs are under evaluations in different countries. Using molecular tools in conventional breeding programs can be a good advance in pistachio. Initial molecular studies in pistachio were mainly on germplasm characterization by using different molecular marker techniques. Identification of molecular markers linked to sex determination was also studied that allows early selections of females in a cultivar breeding program [21–23]. Recently, the markers linked to sex have been used in a cultivar breeding program in Turkey [24].

SSRs are useful as molecular markers and very polymorphic due to the high mutation rate affecting the number of repeat units [25]. They are very useful for assaying diversity in natural populations or germplasm collections and for fingerprinting and parental identification. They are also very valuable markers especially for genetic linkage mapping and evolutionary studies [26] and have a high level of transferability between closely related species. The development of SSR markers from *P. vera* [14, 16–17, 28–30] and from wild *Pistacia* species was performed in several studies [31–34].

The first complete genetic linkage map was constructed in pistachio by [35] who used SRAP, ISSR, and AFLP markers in an inter-specific F1 population derived from a cross between *P. vera* and *P. atlantica*. Recently, Khodaeiaminjan et al. [29] constructed the first complete SSR-based linkage map of pistachio using an intra-specific F1 population. More recently, Motalebipour et al. [36] constructed a genetic linkage map and performed the first QTL analysis in pistachio by using an inter-specific F1 population.

Next-generation sequencing (NGS) has provided a new perspective for research, owing to its high throughout and speed of data generation. It made easy to perform whole genome and transcriptome sequencing in a short time and low cost. Motalebipour et al. [14] performed genome survey study in pistachio and estimated genome size of pistachio as 600 Mb.

In this chapter, SSR abundance, distribution of their use as useful markers in the characterization of germplasm resources, taxonomy and phylogenetic analysis in the genus, as well as their transferability among *Pistacia* species are discussed.

2. SSR distribution in *P. vera*

Motalebipour et al. [14] performed a genome survey study in pistachio and estimated genome size of pistachio as 600 Mb. The authors used 26.77 Gb Illumina PE (pair-end) reads of Siirt cultivar to estimate genome size and to reveal SSR distribution in pistachio genome. Motalebipour et al. [14] detected a total of 59,280 di-, tri-, tetra-, penta-, and hexa-nucleotide SSR motifs, and the dinucleotide motifs were the most abundant type of repeats (63.2%) in pistachio (**Figure 1**), followed by tri- (18.0%), tetra- (12.8%), penta- (3.8%), and hexanucleotide motifs (2.2%).

Motalebipour et al. [14] searched repeat motifs in their genome survey study in pistachio, and AT/AT (23.0%) and TA/TA (21.6%) were the most abundant repeats, followed by AG/CT (7.6%) and GA/TC (6.2%), AAT/ATT (4.1%), CA/TG (2.6%), and TAA/ATT (2.6%) (**Figure 2**). The most abundant tetra- and penta-nucleotide

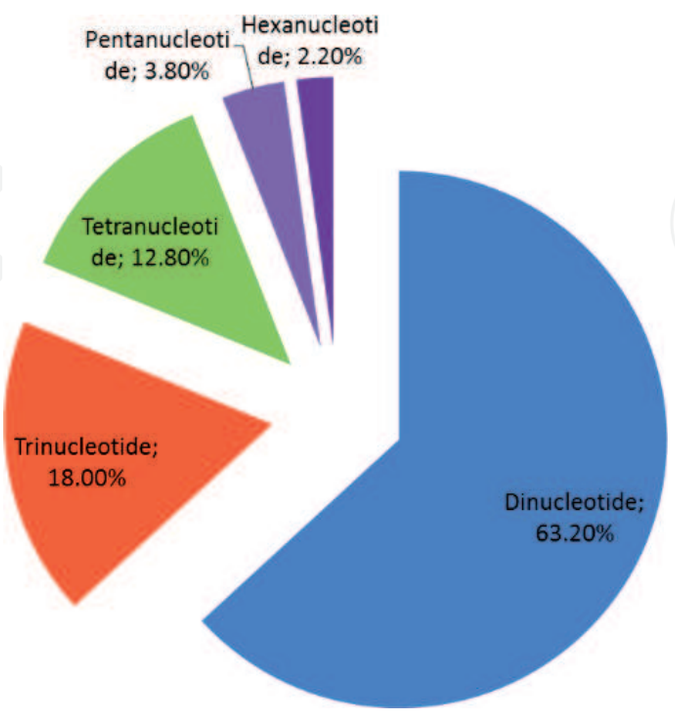

Figure 1.
Distribution of 59,280 SSRs in the pistachio genome based on repeat type. Obtained from Motalebipour et al. [14].

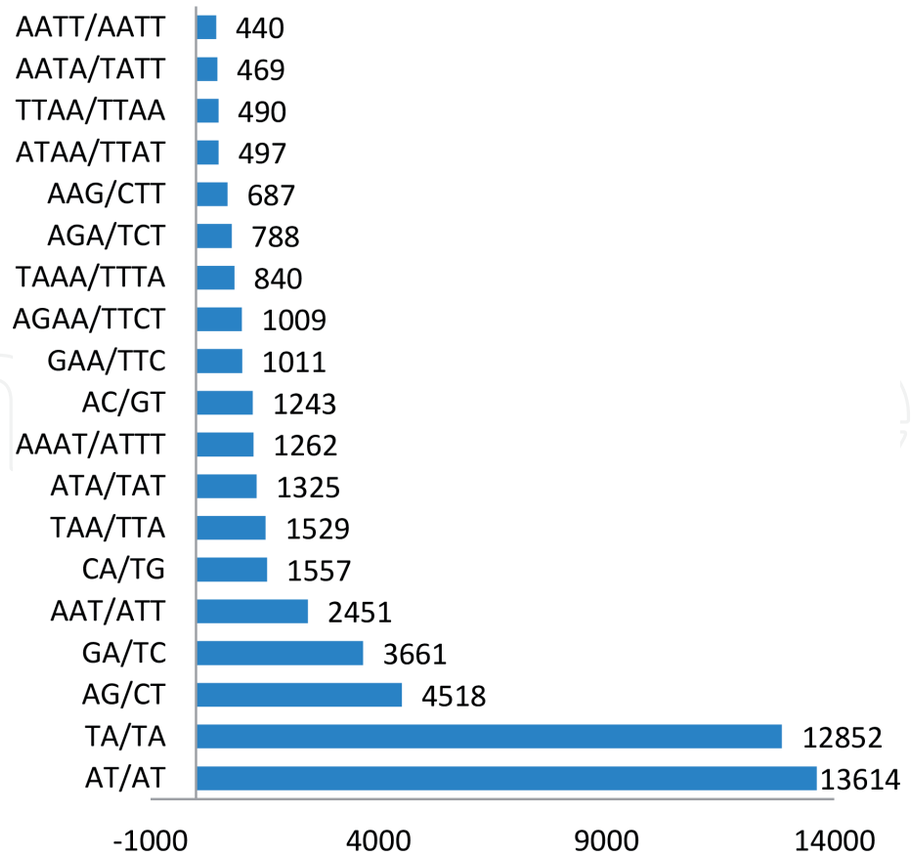

Figure 2.
Distribution of SSR motifs in pistachio at 40× coverage sequencing data. The X-axis represents motif types, and the Y-axis represents the count of motifs in the genome of pistachio. Obtained from Motalebipour et al. [14].

repeat motif types were AAAT/ATTT (2.1%) and AAAAT/ATTTT (0.44%), respectively (**Figure 2**). Among hexanucleotide motifs, AAAAAT/ATTTTT and GCCCAA/TTGGGC (0.07%) were the most abundant motifs. The authors calculated one SSR per 8.67 kb in the pistachio genome.

Khodaeiaminjan et al. [29] had about 10x Illumina data of three *P. vera* (Siirt, Ohadi, and Bağyolu) cultivars and one *P. atlantica* genotype (Pa-18) to find pairwise polymorphic SSR loci in silico. The authors detected a total of 3821, 3833, 2024, and 4597 SSRs in Siirt, Ohadi, and Bağyolu *P. vera* cultivars and in *P. atlantica* genotype, respectively. The dinucleotide motifs were the most abundant type of repeats (48.6%) in four genotypes followed by hexa- (21.3%) and trinucleotide motifs (19.4%) (**Figure 3**).

Khodaeiaminjan et al. [29] calculated the number of repeats in 4 genotypes, and 4 and 11 repeated motifs were the most abundant followed by 12, 8, 5, and 13 times repeated motifs (**Figure 4**).

Jazi et al. [30] performed a transcriptome study by RNA sequencing of a pooled sample representing 24 different tissues of 2 pistachio cultivars with contrasting salinity tolerance under control and salt treatment in pistachio. The authors searched SSR motifs in transcriptome sequences of pistachio, and 11,337 SSRs were defined as di- to hexanucleotide motifs in 11,130 contigs. Di- and trinucleotide repeats were the most abundant SSRs, accounting for 40–44% of total SSRs, followed by tetra- (9.5%), penta- (3.1%), and hexanucleotide repeats (2.2%). The pistachio transcriptome was rich in GA/TC (12.13%), AG/CT (11.02%), AT/AT (8.32%), TA/TA (8.04%), GAA/TTC (5%), and AGA/TCT (4.02%). To make these SSRs useful, a total of 7605 primer pairs were generated from the microsatellites with sufficient flanking sequences. However, none of them were tested in *Pistacia* species.

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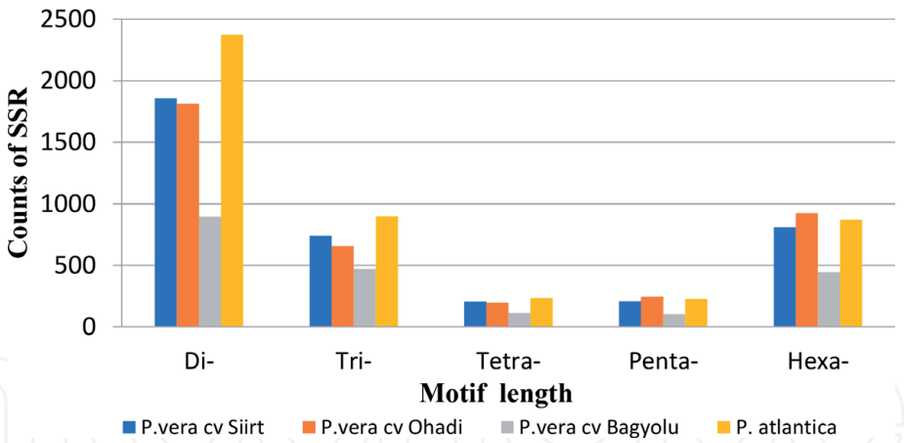

Figure 3.
The number of di-, tri-, tetra-, penta-, and hexanucleotide SSRs in Siirt, Ohadi, and Bağyolu P. vera *cultivars and in* P. atlantica*. Obtained from Khodaeiaminjan [37].*

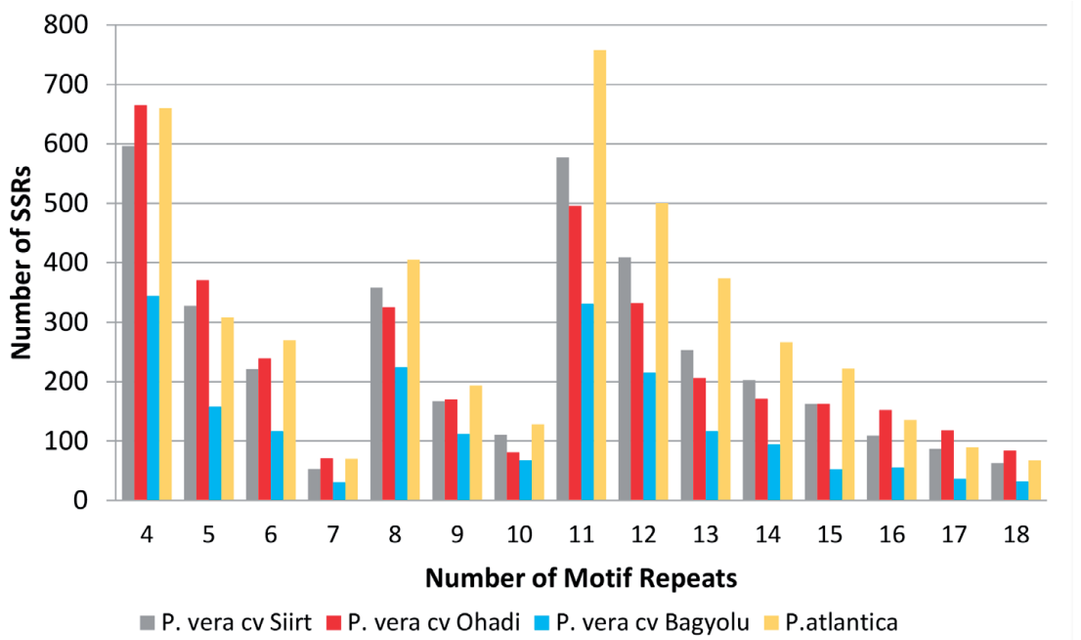

Figure 4.
The number of motif repeats in P. vera *Siirt, Ohadi, and Bağyolu cultivars and in* P. atlantica. *Obtained from Khodaeiaminjan [37].*

3. Development of SSRs from *P. vera*

In *P. vera*, the first SSR markers were developed by Ahmad et al. [27] using enrichment method from Kerman pistachio cultivar. The authors constructed dinucleotide (CT and CA) and trinucleotide (CTT)-enriched genomic DNA libraries. Eighty-nine clones randomly selected from 295 clones for "CT/GA"-enriched library and 57 clones (64%) contained the repeats. Sixty-two clones out of 250 clones, randomly selected from "CA/GT"-enriched library, and 37 (59%) clones contained the repeats. Of the total 151 clones, 67 (44%) clones had sufficient flanking sequence for primer design. Thirty-three clones were selected randomly for "CTT/GAA"-enriched library. Eight (24%) clones had sufficient flanking sequence for primer design. The authors tested these primer pairs in commercially important American, Iranian, Turkish, and Syrian pistachio cultivars, and 14 (56%) SSR loci successfully produced PCR products.

Kolahi-Zonoozi et al. [28] developed SSRs using the FIASCO protocol (Fast Isolation by AFLP of Sequences Containing Repeats) from genomic DNA of *P. vera* cv Akbari. A total of 234 clones were sequenced, and 125 (53.4%) contained SSR motifs. A total of 90 clones having repeats close to the edge of the insert was eliminated, and 35 clones remained and were used to design 42 primer pairs. The authors tested 42 SSR primer pairs in 45 Iranian pistachio cultivars for amplification and polymorphism. Sixteen primer pairs (38.1%) successfully produced scorable bands, and 12 pairs (28.6%) showed polymorphism in 45 pistachio cultivars. The most common repeat motifs in our study were dinucleotides. A total of 32 alleles was obtained with an average of 2.75. The *PIC* values ranged from 0.19 to 0.56 with an average of 0.33. The expected heterozygosity (*He*) varied from 0.081 to 0.518 with an average of 0.345, while the observed heterozygosity (*Ho*) ranged from 0.023 to 0.930 with a mean of 0.490.

Zaloğlu et al. [16] used enrichment method and developed SSR markers from *P. vera* cv Siirt. Genomic libraries enriched with the repeats of "CA," "GA," and "AAC" and "AAG" were constructed. A total of 94 clones (58.8%) contained repeats, and the CA-enriched library contained the highest number (32 clones; 86.5%), followed by the GA-enriched library (25 clones; 61%), whereas the AAC (16 clones; 45.7%)- and AAG (22 clones; 46.8%)-enriched libraries had the lowest number of clones containing repeats. From each library 57 clones were selected and a total of 228 clones was sequenced. A total of 84 primer pairs was designed, 59 generated (70.2%) bands, and 47 polymorphic in the characterization of 7 diverse pistachio cultivars. The number of alleles ranged from two to nine with an average of 3.6. A higher number and frequency of SSRs was obtained from dinucleotide-enriched libraries than trinucleotide libraries. The SSR loci from the CA enriched library (*Ho* = 0.49) were more homozygous than those from the other libraries (*Ho* = 0.59–0.61). The AAC-enriched library (*He* = 0.55) was the least informative, whereas the GA library (*He* = 0.71) was the most informative; the CA and AAG libraries had intermediate values. As a result, the GA-enriched library was the best, whereas the AAC library was the worst among the four libraries in terms of perfect repeats, number of alleles, polymorphism, and informativeness. The AAG-enriched library was also good because of its perfect repeats, observed heterozygosity, and numbers of amplified and polymorphic loci. The authors suggested to use the GA- and AAG-enriched libraries in further SSR marker development studies in pistachio. A higher frequency of SSRs was obtained from the dinucleotide-enriched (73.1%) libraries than the trinucleotide-enriched (46.3%) ones. The dinucleotide-enriched libraries had a higher number of alleles (*Na* = 3.8) and effective number of alleles (*Ne* = 3.0) than the trinucleotide-enriched libraries (3.3 and 2.5, respectively). The dinucleotide-enriched libraries (*He* = 0.67) were more informative than the trinucleotide ones (*He* = 0.59). The 59 SSR primer pairs were tested in 8 different *Pistacia* species, and 54 were transferable to at least 1 *Pistacia* species. The SSR loci in trinucleotide (72.7%)-enriched libraries had higher transferability than dinucleotide (54.7%) ones.

Topçu et al. [17] sequenced more clones from GA- and AAG-enriched libraries based on suggestion done by Zaloglu et al. [16]. A total of 192 clones was sequenced from each library, 135 primer pairs were designed, and 110 generated PCR products. Topçu et al. [17] tested 110 SSR loci in 12 diverse pistachio cultivars for amplification and polymorphism. A total of 46 loci from the GA library and 18 loci from the AAG library was polymorphic in *P. vera*. A total of 64 polymorphic loci produced 264 alleles with an average of 4.13 alleles per locus. The observed (*Ho*) and expected heterozygosity (*He*) values were 0.52 and 0.56, respectively, while average polymorphism information content (*PIC*) was 0.51. One hundred out of 110 SSR loci were transferable to at least one of the tested 10 *Pistacia* species. *P. eurycarpa* that is the closest species to *P. vera* had the highest number of transferable loci, whereas *Pistacia texana* and *P. lentiscus* that are the farthest species to *P. vera* had the lowest number of transferable loci.

Motalebipour et al. [14] obtained 40× sequencing data from *P. vera* cv. Siirt to develop SSR markers. A total of 59,280 SSR motifs was detected with a frequency of 8.67 kb in pistachio. The authors randomly selected 950 SSR loci and screened them in three *P. vera* cultivars (Siirt, Ohadi, and Bağyolu) in one *P. atlantica* genotype (Pa-18). A total of 610 loci (64.2%) generated amplification products, 197 (20.7%) loci were monomorphic, and the remaining 143 (15.1%) SSR loci failed to generate amplification products. Of the 610 that amplified, 204 produced polymorphic and easily scorable bands. Of these, 193 were perfect (94.6%), 8 (3.9%) were compound, and 3 (1.5%) were interrupted repeats. Dinucleotide motifs were the most abundant (63.2%), followed by tri- (18.0%), hexa- (12.8%), tetra- (3.8%), and pentanucleotide motifs (2.2%). Two SSR primer pairs amplified at two loci, and 206 SSR loci were obtained and used to study genetic diversity in *Pistacia*. The authors tested these 206 loci in 24 pistachio cultivars along with 20 wild accessions belonging to five *Pistacia* species (four accessions from each one). A total of 2036 alleles was obtained from 206 SSR loci ranging from 2 to 19 alleles with an average of 9.88 in testing 44 *Pistacia* accessions. Of the analyzed 206 SSR loci, 41 were polymorphic, and 136 had amplifications in all tested 6 *Pistacia* species. The observed heterozygosity (*Ho*) ranged from 0.0 to 0.82 with an average of *Ho* = 0.41. The average *He* value was 0.74, which ranged between 0.08 and 0.91. The *PIC* values ranged from 0.08 to 0.90, with an average of 0.71. All 206 SSR loci generated amplification products in 24 *P. vera* culticars, and a total of 897 alleles was produced with an average of 4.5 alleles per locus. Two hundred (97.1%) SSR loci were polymorphic in 24 pistachio cultivars. The observed heterozygosity (*Ho*) value ranged from 0.00 to 1.00 with an average of 0.46. The expected heterozygosity (*He*) values varied from 0.04 to 0.87 with an average of 0.55. The polymorphism information content values ranged between 0.04 and 0.85 with an average of 0.50.

Khodaeiaminjan et al. [29] used in silico approach to develop polymorphic SSR markers in pistachio. The authors compared 10× Illumina sequencing data of three *P. vera* (Siirt, Ohadi, and Bağyolu) cultivars and one *P. atlantica* genotype (Pa-18) to find pairwise polymorphic SSR loci in silico, and 750 loci were detected. The authors tested all 750 loci in 18 *P. vera* cultivars and 6 *P. atlantica* genotypes, and they obtained 625 polymorphic loci from 618 SSR primer pairs. A total of 3947 alleles was obtained from 625 loci with an average of 6.2 allele per locus. A total of 613 (98.1%) SSR loci in 18 *P. vera* cultivars and 544 SSR loci (87.0%) in six *P. atlantica* genotypes was polymorphic. The numbers of alleles were 2631 within *P. vera* and 2183 in *P. atlantica*. The lowest genetic diversity values were obtained from the Bagyolu-Pa-18 pairwise combination, while in silico SSR loci from Ohadi-Pa-18 pair produced high genetic diversity values. In the analysis of 24 *Pistacia* genotypes, a total of 613 (98.1%) loci was polymorphic, and the average number of alleles (*Na*) was 6.3 ranging from 2 to 20. The average expected heterozygosity (*He*) and observed heterozygosity (*Ho*) values were 0.67 and 0.53, respectively. The average polymorphism information content value was 0.63. The highest genetic diversity values were produced by SSR loci from the Ohadi-Pa18 and Ohadi-Siirt pairwise combinations, whereas the lowest values were in SSR loci from the Bagyolu-Pa18 pair.

4. SSRs in wild *Pistacia* species

4.1 P. atlantica

There is only one study using *P. atlantica* DNAs to develop SSRs by Khodaeiaminjan et al. [29]. The authors used 10× coverage sequencing data of monoecious *P. atlantica* genotype, namely, Pa-18, and generated 4597 SSR loci.

The authors tested 625 loci in 6 *P. atlantica* genotypes, and all had amplification, while 544 (87.0%) were polymorphic. The number of alleles (*Na*) ranged from 1.0 to 8.0 with a mean of 3.5. The mean of observed heterozygosity (*Ho*) was 0.51, while the average *PIC* and expected heterozygosity (*He*) values were 0.53 and 0.52, respectively.

Motalebipour et al. [14] developed 206 SSR loci from *P. vera* and tested them in 4 diverse *P. atlantica* genotypes. A total of 200 SSR loci generated amplification products with a high rate of transferability (97.1%). Thirty-nine (19.5%) of the amplified SSR loci were monomorphic, and the rest were polymorphic (80.5%). A total of 527 alleles was produced by 161 polymorphic SSR loci, ranging from 2 to 7 per locus with an average of 3.3 alleles per locus. The observed heterozygosity (*Ho*) ranged from 0.0 to 1.00 with an average of *Ho* = 0.48. The average *He* value was 0.56, which ranged from 0.22 to 0.84. The *PIC* values ranged from 0.19 to 0.82, with an average of 0.49.

Zaloğlu et al. [16] and Topçu et al. [17] developed 59 and 110 SSRs from *P. vera* and tested them for amplification in *P. atlantica*, and a total of 47 and 96 SSR loci was amplified, respectively. So, the authors published a total of 143 SSR loci for further studies in *P. atlantica* from both studies. The authors did not test their polymorphism levels in *P. atlantica*.

4.2 P. khinjuk

Arabnezhad et al. [32] were the first in developing SSRs from *P. khinjuk*, who constructed two enriched DNA libraries with dinucleotide (AG) and trinucleotide (ATG) microsatellite motifs. Thirty-six contained microsatellite motifs from 44 sequenced clones. Among them, a higher proportion of microsatellites (71%) were simple perfect, and the remaining SSRs identified as interrupted perfect (17%) and complex imperfect (11%) repeats. Nine of the sequences contained too short flanking DNA sequence to design primer pairs; thus only 27 primer pairs were designed and tested in six *Pistacia* species. Of 27 primer pairs, 25 pairs successfully amplified SSR loci in *P. khinjuk* with expected size. Five primer pairs were subsequently discarded due to low rate of amplification across six *Pistacia* species. The authors tested the remaining SSRs in a total of 18 *Pistacia* genotypes (13 *P. vera* cultivars and 1 genotype from each of *P. khinjuk, P. atlantica, P. mutica, P. integerrima*, and *P. palaestina*). The primer pairs produced 114 alleles in 18 *Pistacia* genotypes. In 13 *P. vera* cultivars, the average number of alleles per locus was 2.8, ranging from one to six. In all *Pistacia* accessions, the average values of *He* and *PIC* were 0.61 and 0.56, respectively, while values of these diversity parameters calculated 0.45 and 0.38 when only *P. vera* genotypes were considered.

Zaloğlu et al. [16] and Topçu et al. [17] developed 59 and 110 SSRs from *P. vera* using enrichment method, and a total of 39 and 96 SSR loci had amplification, respectively. It is still necessary to test 135 SSR loci for polymorphism in *P. khinjuk* for further studies.

4.3 P. lentiscus

The first SSR development study in *P. lentiscus* was published by Albaladejo et al. [31] who used di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC), and tetranucleotide (GATA, CATA, ATAG) genomic-enriched libraries. The authors randomly selected 163 clones and 75 (46%) had microsatellite motifs. A total of 21 primer pairs was designed and tested in 16 individuals. Eight of 21 primer pairs displayed consistent and polymorphic patterns, whereas the others were discarded due to producing monomorphic and multibanding patterns and failing in amplification. Forty-two

individuals from two populations sampled in Southern Spain were used for characterization of eight loci. A total of 59 alleles was detected, ranging from 3 to 13 per locus. The expected heterozygosity ranged from 0.139 to 0.895.

Motalebipour et al. [14] developed 206 SSR loci from *P. vera* and tested them in four *P. lentiscus* genotypes. A total of 151 SSR loci was amplified with a 73.3% transferability rate. Of the amplified SSR loci, 83 (55.0%) were polymorphic. A total of 217 alleles was obtained from 83 polymorphic SSR loci in *P. lentiscus,* ranging from 1 to 6, with an average of 2.6 alleles per locus. The observed heterozygosity (*Ho*) values ranged from 0.00 to 1.00 with an average of 0.50. The expected heterozygosity (*He*) values varied from 0.22 to 0.78 with an average of 0.49. The polymorphic information content (*PIC*) values ranged between 0.19 and 0.75 with an average of 0.41.

Zaloğlu et al. [16] and Topçu et al. [17] developed 59 and 110 SSRs from *P. vera* and tested them for amplification in *P. lentiscus*, and a total of 31 (52.5%) and 76 (69.1%) SSR loci was amplified, respectively. The authors did not test their polymorphism levels in *P. lentiscus*. Therefore, there are 107 SSR loci for *P. lentiscus* from both studies to use them in the future.

4.4 P. chinensis

Ding and Lu [34] developed 12 polymorphic microsatellite loci from *P. chinensis* using a microsatellite-enriched genomic library based on magnetic beads. These loci were characterized in 24 individuals from 3 populations located on Thousand Island Lake, Zhejiang Province, China. The number of alleles per locus varied from 3 to 16. The mean number of alleles per locus was between 3.3 and 4.0 at the population level. The observed and expected heterozygosity ranged from 0.12 to 0.87 and 0.23 to 0.89, respectively.

Motalebipour et al. [14] developed 206 SSR loci from *P. vera* and tested them in 4 diverse *P. chinensis* genotypes, and 177 SSR loci produced amplification products with 85.9% transferability rate. Of the amplified loci, 119 loci (67.2%) were polymorphic. A total of 365 alleles was amplified by 119 polymorphic SSR loci with an average of 3.1 alleles per locus. The average observed heterozygosity (*Ho*) was 0.48, and it ranged between 0.00 and 1.00. The expected heterozygosity (*He*) values varied from 0.22 to 0.84 with an average of 0.54. The polymorphism information content (*PIC*) values ranged between 0.19 and 0.82 with an average of 0.48 in *P. chinensis.*

Zaloğlu et al. [16] and Topçu et al. [17] developed SSRs from *P. vera* and tested them for amplification in *P. chinensis*. A total of 121 SSR loci had amplification PCR products in *P. chinensis,* with 71.6% transferability rate. However, the authors did not test polymorphism levels of the amplified SSR loci in *P. chinensis*. Chen et al. [33] developed 14 SSR loci from *P. weinmannifolia*, and 9 of them (64.3%) were amplified in *P. chinensis*.

4.5 P. weinmannifolia

Chen et al. [33] developed SSRs using the FIASCO protocol (Fast Isolation by AFLP of Sequences Containing Repeats) from genomic DNA of *P. weinmannifolia*. A total of 205 clones was sequenced, 147 contained SSR motifs, and 94 allowed primer design with sufficient flanking regions. The primer pairs were tested for polymorphism in 24 individuals from 2 populations, and 14 produced polymorphic microsatellite loci with an average of 4.1 alleles (ranging from 1 to 9) per locus in *P. weinmannifolia*. The expected (*H*e) and observed (*H*o) heterozygosities ranged from 0.000 to 0.906 and from 0.000 to 0.933, respectively. Ten of these loci

contained dinucleotide repeat motifs, and four loci had complex repeat motifs. The authors tested 14 primer pairs in *P. chinensis* and *P. mexicana* for their transferability, and 9 (64.2%) loci in *P. chinensis* and 4 (28.6%) loci in *P. mexicana* were successfully transferable.

Zaloğlu et al. [16] developed 59 SSRs from *P. vera* and tested them for amplification in *P. weinmannifolia*, and a total of 23 SSR loci was amplified with a 39.0% transferability rate. However, the authors did not test their polymorphism levels within *P. weinmannifolia*.

4.6 P. integerrima

There is no study in the literature developing SSRs from *P. integerrima* tissues. Arabnezhad et al. [32] developed 27 SSR loci in *P. khinjuk*, and 18 (66.7%) were successfully amplified in *P. integerrima*. Two previous studies [16, 17] developed a total of 169 SSRs from *P. vera* and tested them for amplification in *P. integerrima*. A total of 147 SSR loci had amplification products with 87.0% transferability rate. There is a necessity to test the amplified loci for polymorphism within *P. integerrima*.

Motalebipour et al. [14] developed 206 SSR loci from *P. vera* and tested them in four diverse *P. integerrima* genotypes. A total of 193 SSR loci generated amplification products with a high rate of transferability (93.7%). Of the amplified SSR loci, 157 (81.3%) were polymorphic in *P. integerrima*. A total of 416 alleles was produced by 157 SSR loci with an average of 2.70 alleles per locus. The average observed (*Ho*) and expected (*He*) heterozygosities were 0.50 and 0.52, respectively. The expected heterozygosity (*He*) values varied from 0.22 to 0.78 with an average of 0.55. The polymorphism information content (*PIC*) values ranged between 0.19 and 0.75 with an average of 0.44.

4.7 P. terebinthus

There is no SSR development study also from *P. terebinthus* tissues in the literature. Motalebipour et al. [14] generated 206 SSR loci from *P. vera* and tested them in *P. terebinthus* genotypes, and 183 SSR loci produced amplification products with 88.8% transferability rate. Of amplified SSRs, 142 were polymorphic. A total of 416 alleles was produced by 142 polymorphic SSR loci. The number of alleles (*Na*) ranged from 1 to 7 with an average of 3.4 alleles per locus. The observed heterozygosity (*Ho*) ranged from 0.0 to 1.0 with an average of 0.47. The expected heterozygosity (*He*) values varied from 0.22 to 0.84 with an average of 0.56. The polymorphic information content (*PIC*) values changed between 0.19 and 0.82 with an average of 0.50.

Zaloğlu et al. [16] and Topçu et al. [17] developed 169 SSRs from *P. vera* and analyzed them in *P. terebinthus* for amplification. A total of 128 SSR loci had PCR products in *P. terebinthus,* with 71.6% transferability rate. However, the authors did not test polymorphism levels of the amplified SSR loci in *P. terebinthus*.

4.8 P. texana, P. mexicana, and P. eurycarpa

Chen et al. [33] developed 14 SSR loci from *P. weinmannifolia*, and 4 of them (28.6%) were amplified in *P. mexicana*. Zaloğlu et al. [16] and Topçu et al. [17] developed 59 and 110 SSRs from *P. vera* and tested them for amplification in *P. mexicana*, and a total of 33 and 77 SSR loci was amplified in *P. mexicana*, respectively. Topçu et al. [17] generated 110 SSRs from *P. vera* and tested them for amplification in *P. eurycarpa* and *P. texana*. A total of 100 and 76 SSR loci was amplified with a 90.9 and 69.1% transferability rates. However, Zaloğlu et al. [16]

and Topçu et al. [17] did not test polymorphism levels of the SSR loci in *P. mexicana, P. texana*, and *P. eurycarpa.*

5. Use of SSR markers in the genus *Pistacia*

There are only several studies characterizing *Pistacia* species and *P. vera* accessions using SSR markers. Pazouki et al. [38] used SSR markers and characterized 304 *Pistacia* accessions belonging to *P. khinjuk*, *P. vera,* and *P. atlantica* subsp. *kurdica*. The authors indicated lower level of polymorphism and variation within *P. atlantica* subsp. *kurdica* than *P. vera* and *P. khinjuk*. Motalebipour et al. [14] used 1505 alleles amplified by 136 SSR primer pairs for phylogenetic analysis of 6 *Pistacia* species. The closest species to *P. vera* was *P. atlantica*, and *P. integerrima*, *P. chinensis*, *P. terebinthus* followed it, while *P. lentiscus* was the most diverse species to the cultivated pistachio. The structure analysis confirmed the cluster analysis as well. Albaladejo et al. [31] used 8 SSR loci to characterize 42 *P. lentiscus* accessions belonging to 2 *P. lentiscus* populations. The number of alleles changed between 3 and 13 by obtaining a total of 59 alleles. The expected heterozygosity ranged from 0.139 to 0.895. Two *P. lentiscus* populations were separated clearly in cluster analysis. Chen et al. [33] characterized 24 *P. weinmannifolia* accessions using 14 SSR loci. The number of allele changed between one and nine with an average of 4.1. *Ho* values were between 0.000 and 0.933, while *He* values ranged from 0.000 to 0.906.

Ahmad et al. [27] used 14 SSR loci to characterize Iranian, Turkish, and Syrian pistachio cultivars. A total of 46 alleles was produced by 14 SSR loci ranging from 2 to 5 allele per loci. Cluster analysis placed most of the Iranian samples in one group, while the Syria samples were the most diverse and did not group in a single cluster. Kolahi-Zonoozi et al. [28] described 45 Iranian pistachio accessions by 12 SSR loci. The *PIC* values changed between 0.19 and 0.56 with an average of 0.33. The average *Ho* and *He* values were 0.490 and 0.345, respectively. Khodaeiaminjan et al. [29] characterized 18 pistachio cultivars from different origins by 2631 SSR alleles using 625 SSR loci. The constructed dendrogram separated pistachio cultivars mainly in two groups according to their geographical origin: one group contained the cultivars originated from Iran, and the second group included cultivars originated from Mediterranean countries such as Turkey, Syria, Greece, and Italy. Siirt cultivar (origin is Southeast part of Turkey) placed between two main groups. The results were in agreement with a hypothesis on diffusion of pistachio cultivars suggested by Kafkas et al. [19] who hypothesized that the Siirt cultivar is in a transition subcluster between Iranian and Mediterranean cultivars and pistachio cultivation diffused from its center of origin, the Iranian-Caspian region, via southeastern Turkey to Syria, the Mediterranean region of Europe, and North Africa. Motalebipour et al. [14] used 1505 alleles from 136 SSR primer pairs for genetic diversity analysis of 24 pistachio cultivars, and similar results were obtained with the study performed by Khodaeiaminjan et al. [29].

The SSR markers in the genus *Pistacia* were also used for genetic linkage map construction and QTL analysis. Khodaeiaminjan et al. [29] constructed the first SSR-based genetic linkage map of pistachio using an F1 segregating population derived from a cross between "Siirt" and "Bağyolu" cultivars. A total of 385 SSR markers was mapped along with 15 chromosomes, and the consensus map had 1511.3 cM length with an average of 25.6 SSR markers per LG, and the average distance between the markers was 3.9 cM with a 0.25 marker density. The first QTL study in pistachio was performed by Motalebipour et al. [36] who constructed a genetic linkage map of pistachio using an inter-specific F1 population and SSR markers. The authors mapped a total of 388 SSR markers along with 15 linkage

groups. The length of consensus map was 1492 cM with an average marker distance of 3.7 cM. The QTL analysis was performed for 5 morphological traits such as leaf length, leaf width, number of leaflets, young shoot color, and leaf color, and 17 stable QTLs during 2 consecutive years were identified. The released SSR-based genetic linkage maps and reported QTLs can be useful genetic resources for future genetic studies in pistachio.

6. Conclusions

SSR is a very useful molecular marker system due to abundance in the genomes and its codominant inheritance as well as high repeatability. They have also a high level of transferability between closely related species as in the genus *Pistacia*. They have been used for assaying diversity in natural populations, marker discovery, germplasm characterization, parental identification, genetic linkage mapping, and evolutionary studies. There were a very limited number of SSR markers for *Pistacia* species until several years ago; however, next-generation sequencing (NGS) technology has allowed to develop plenty of SSRs since 2016 in *Pistacia*.

P. vera is the most important species in the genus *Pistacia* due to commercial value of its nuts. There are about 1500 published SSR markers, and 2/3 of them are polymorphic that were developed from *P. vera* tissues. There are also published polymorphic SSR markers for wild *Pistacia* species. They were developed mostly from cultivated pistachio due to their high transferability rate. The published SSRs were also used to construct SSR-based genetic linkage maps in pistachio.

In a conclusion, there are currently an adequate number of SSR markers for cultivated *P. vera* and for several wild *Pistacia* species such as *P. atlantica*. It is still necessary to develop polymorphic SSR loci for some other *Pistacia* species such as *P. integerrima* and *P. eurycarpa* which have been used as rootstock for cultivated pistachio.

Conflict of interest

The authors declare no conflict of interest.

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