Analysis of Genetic Diversity of Myrtle (Myrtus Communis L.) by Using SSR Technique

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Abstract

This research carried out to compare some of the individuals of Myrtle from bushes in different environmental sites (Lattakia, Safita, Qusul Maaaf, northern Aleppo and at different altitudes from the sea surface). The genetic diversity of 19 genotypes was tested using simple sequence repeats (SSRs) technique with 10 primers. The results of DNA extraction showed a high molecular size fragment as a band at the top of each lane, additionally to a partial degradation. At the end DNA concentration, integrity and purity were enough for SSR marker. Genetic variations were detected by SSR marker with similarity coefficient ranged between 0.08 – 0.89 based on Dice coefficient. Total of 27 alleles were scored from 19 genotypes, and the number of alleles was ranged between 2 (myrcom8 and 9) and 4 (myrcom2 and 6). The calculated value of polymorphism information content (PIC) was ≤ 0.5. Nineteen genotypes were distributed on three main clusters, two of them II and III included minimum number of genotypes from humid climate sites, while the majority of genotypes was distributed on cluster I in mixed manner.

Keywords: Genetic diversity, Myrtus communis L., SSR, Similarity coefficient, Polymorphism information content.

1. Introduction

Myrtle belongs to the Myrtaceae exponent, its plants are either shrubs or trees and contains about 150 genera and more than approximately 5,500 species [1, 2]. The Myrtle family is represented by ornamental and aromatic Myrtle or the common (M. communis L.), in large areas of the Mediterranean basin [3,4] including Syria, where it spreads widely in nature in the hills and hills of Lattakia, as well as in Safita and Qusul Maaaf and northern Aleppo [5, 6]. The myrtle is among the medicinal and aromatic plants of very important ornamental and economic and environmental value [7]. Myrtle has been used for industrial, nutritional, medical, ornamental and environmental purposes since ancient times [8,9]. Currently, myrtle bushes are a naturally occurring source of raw material only for oil extraction and industrial processes such as making fruit juice of myrtle. The constant use of the natural resources of the Myrtle due to the great demand for it for its many uses, which led to its diminishing species Myrtle s in nature [10]. Here, steps must be taken to avoid species extinction from their natural environment and reduce genetic diversity and, at worst, species disappearance [7].

The Methods of characterizing and classifying plant species are based on the more advanced morphological and productive characteristics, but they are often affected by prevailing environmental conditions and give close and similar results that are difficult to rely on in distinguishing differences [11], and they require significant time and effort. Therefore, these methods must be supported by biotechnology methods. The modern [12] in characterizing genetic sources using Molecular markers as they are characterized by a large number and stability of their results and not affected by environmental conditions and can be used in analyzes of genetic diversity and estimating genetic similarities [13]. Iterations of simple chains (SSRs) are the most used molecular indices in the field of plant genotyping during the past twenty years, due to their ability to detect the morphological diversity of co-dominant or multi-allele genetic indicators that exist in the plant species or transmitted to it through the flow of genes among the types of relatives [14]. In particular, SSRs are useful for wild species to study the genetic diversity on the basis of measuring genetic distances, as well as to assessment gene flow and high rates of the hybridization. The possibility of SSR primers moving between species of the same species [15, 16], or between species of the same species have already been confirmed by Legume [17], and Myrtaceae [18] and Fagaceae [19].

Several genetic indicators were used in studying the genetic diversity of the myrtle, where the distribution of the studied Myrtle models according to the geographical spread region was obtained by using the AFLP technique [20], and the same technique was used earlier to study the wild Myrtle communities where it was possible to separate these communities are grouped according to fruit color [21]. Albaladejo [22], also revealed genetic variations, significantly, among 14 Myrtle communities when using this technique. [8], also using AFLP technology on Myrtle models collected from Italy and the Mediterranean region, reached a subunit classification level called M. communis sub sp. tarentina. [23], was able to separate

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The study of myrtle species was conducted in Italy using ISSR technology. The RAPD and isozyme technology was also used to include a climatic field that extends between tropical and subtropical regions. As a result, there has been more genetic diversity among societies than among individuals and prevailed the dominant species in tropical and subtropical regions. [24]. How possible, through the RAPD technique, to separate the wild and cultivated myrtle species in Turkey to separate them according to the color of the fruits. Genetic diversity was greater for the wild species than for the cultivated models [25].

The justifications and objective of the research: Given the importance of the ornamental and aromatic myrtle plant and its multiple uses, especially from the decorative and medicinal side, in addition to the deterioration of the plant as a result of unexplained aggressions in its natural environment. The aim of the research is to determine genetic variation and to determine the degree of genetic relationship between Myrtle members grown in different environmental conditions using SSR technology.

2. Materials and methods

2.1 Plant material

The plant material included leaves from the myrtle bushes (white and black fruits), including (50) shrubs, covering several areas of the natural grown of the myrtle with different environmental conditions, and at different altitudes from the sea surface. The young leaf samples were collected from the Myrtle shrubs in December 2015, and stored directly in frozen folders until transported to the laboratory, then the leaf samples were stored in the freezer at a temperature of -80 °C, for the purpose of DNA extraction.

2.2 Extraction of the DNA

The DNA genetic material was extracted by CTAB method (Cetyl Trimethyl Ammonium Bromide) from (50) paper samples in December 2015. In the biotechnology laboratory in the Faculty of Technical Engineering at the University of Aleppo.

2.3 Estimating the quantity and quality of DNA from electrophoresis in the agarose gel

The amount of extracted DNA was estimated, its quality determined, and its integrity ensured in terms of the size of the pieces produced, by electrophoresis using the (agarose gel). Molecular DNA molecules appear on the agarose gel as clear bands / bundles above the gel, while partially fractured shapes in Smear appear distorted at long and small sizes that extend along the lane path.

2.4 Reparation of the agarose gel

- The agarose mold casting plate was used to prepare the gel, where a suitable comb was putted according to the number of samples to form holes after the gel solidification, so these holes or wells are used to load the samples.
- Agarose gel prepared at a concentration of 1%, at a weight of 2.5 g agarose and added to the amount of 250 mL of 1x TAE solution.
- Preheat the previous mixture in a microwave oven for a short period (about three minutes), until the agarose has completely melted, then cooled to 55 °C.
- Pour the gel into the plate quietly, taking care not to create air bubbles in the gel.
- After the gel is completely solidified, the gel and the plate are putted in an electrophoresis device that has been filled with sufficient amount of the gel solution so that the gel is well immersed.

2.5 Loading DNA samples into the gel

The samples were prepared for loading by taking 2 microliter of DNA extract and 2 microliter of loading solution (6x loading dye) to a small 0.2 ml tube, then complete the volume to 12 microliter by adding 8 microliter distilled and sterile water, and mixing the ingredients for each sample by drawing in the pipette Several times when adding water. The samples were carried on the gel, and at the beginning of each comb a known standard index sample was loaded to compare the concentration and quality (100 nanogram of the standard indicator Lambda DNA was used).
2.6 Coloring and development

The gel was washed with distilled water to get rid of the salts in the solution of translation then it's immersed in a solution of Ethidium Bromide of 0.5 g / ml for a period of 20-30 minutes, after that washed with water to get rid of the coloring solution, then it was photographed by a digital camera attached to the gel documentation under ultraviolet radiation and the image was stored in the computer connected to the device to be analyzed later.

2.7 SSR-Simple Sequence Repeats

2.7.1 Primers used

This test is based on the polymerase Chain Reaction (PCR) sequence reaction, where 10 pairs of primers [26] were used in separate reactions to reveal 10 genetic sites in the myrtle genome of table(1).

| Interaction program icon | Repetition motif | The primer sequence (5'--------3') | Genetic location |
|--------------------------|-----------------|-----------------------------------|-----------------|
| B                        | (AC)6           | F: CGTGATGCACACTGAAGCTGA          | Myrcom 1        |
|                          |                 | R: AACCCCTTTGCAACACATTT           |                 |
| B                        | (TC)18          | F: ATAGCTTTTACCCGCCATTG          | Myrcom 2        |
|                          |                 | R: GTGCATGGTCCTCAGTAGGT           |                 |
| B                        | (CT)13          | F: GGCTCGTTACAGTCATACC           | Myrcom 3        |
|                          |                 | R: TTTGCAACCTTTAAGCTGG           |                 |
| C                        | (TC)20          | F: CAAACATCTACCCATAGA           | Myrcom 4        |
|                          |                 | R: CCACAGTCAGAGGGAGAGC           |                 |
| A                        | (CT)8           | F: TGAGAGATCGAAACAAAAAG         | Myrcom 5        |
|                          |                 | R: CATGAATGGGCAACGTAAA           |                 |
| B                        | (A)12(CA)10     | F: AAATGAAAAAGCTAAAAGTTAAAACA    | Myrcom 6        |
|                          |                 | R: AACAGGAGGAGCAAGCCAAAA         |                 |
| B                        | (GA)19          | F: AGACATGCTCAACCTGTATGC         | Myrcom 7        |
|                          |                 | R: AATGTATCCACACATGCAGA          |                 |
| B                        | (TA)6           | F: TGCTCGGTCAATAGGTTG           | Myrcom 8        |
|                          |                 | R: TCAAAAACGTCTTCACATGAA         |                 |
| B                        | (A)15GG(CA)9    | F: GAAAGTTGCACTGTTATTTCCAA      | Myrcom 9        |
|                          |                 | R: TCTTCTTTCAATCCTATCAA          |                 |
| B                        | (CT)17          | F: TTAAGTGCTTTGCGCATTTG         | Myrcom 10       |

2.7.2 Preparation of the reaction and PCR programs

Attended the reaction mixture in a final volume of 10 microliter, the mixture for each sample contained 3 microliter from DNA (25 nanogram / microliter), 1 microliter of the buffer solution for 10 PCR buffer reaction x 0.4 (50 mM MgCl₂), 1 microliter of dNTP's four nucleotides (concentration of 2 mmol for each), 0.2 microliter of both the anterior and reverse primers (concentration of each primer 10 micro molar), 0.2 micro liter of Taq (5U/µl) polymerase, 5 sterilized dehydrated water to complete the size. The process of thermal recycling was done in a device of the type (Eppendorf Mastercycler, Germany) according to three models (A, B, C) of the program: Table (2)

| Phase       | Step                   | Temperature (° x) | Time (sec) | Number of cycles |
|-------------|------------------------|-------------------|------------|-----------------|
| First step  | secondary class        | 94                | 30         | 1               |
|             | Docking prefixes       | 60                | (30/-1/cycle) | 10             |
|             | Extension               | 72                | 60         |                 |
|             | Secondary class        | 94                | 30         |                 |
| Second step | Docking prefixes       | 50                | 30         | 30              |
|             | Extension               | 72                | 60         |                 |
|             | Final extension         | 72                | 420        |                 |
|             | Save the reaction       |                   |            | ∞               |

Table 2. Model (A, B, C) of PCR device programming (decline¹⁰ c).
2.7.3 The PCR polymerase chain reaction

After the reaction was completed, 2 microliter of loading solution (6x loading dye buffer) was added to the propagation product, then the entire reaction mixture (12 microliter) was carried on the agarose gel 2.5% concentration, and then the gel was colored with ethidium bromide and appeared under ultraviolet, in a device Gel documentation. The volumes of resulting segments (the genetic sites) of the electrophoresis process were compared with a measurement index of 50 molecular weight marker 50 bp.

2.8 Analyzing the results

briny system, where the number 1 was given if the genetic site exists and 0 in the absence thereof. The results were analyzed Paleontological Statistics (PAST) analysis program, ver. 1.90. The kinship tree was drawn and the similarity ratios between samples were calculated using the Jaccard coefficient. Whole sample, so it was ignored from estimating the concentration (Figure 1)
The strength of the Polymorphic information content (PIC) was calculated from the relationship

\[
PIC = 1 - \sum PnP^2 / n - 1
\]

where: n: number of individuals, P2: square of the frequency of existing packets present and absent.

3. Results and discussion

3.1 The concentration and quality of DNA

The results of DNA extraction from the studied Myrtle samples showed that a portion of the DNA extracted from the samples was intact except for the two samples (20 and 48), where the DNA appeared as a bundle above the agarose gel. The samples appeared in clear bundles with high density at the top, indicating a good amount of DNA, with some segmentation that appeared in the form of a Smear along the lane path for each sample. Therefore the results were ignored from the focus estimate.

![Figure 1. Shows the electrophoresis of the DNA extracted from the Myrtle samples in the 1% agarose gel. 1-50: studied Myrtle samples, M: 100 ng from the lambda benchmark (50 ng). A dilution of extracted DNA was carried out to 25 ng / based on bands reading compared to the concentration of 100 ng of the lambda standard index, to standardize DNA concentrations in all of the studied samples. 19 samples representing the studied sites and their environmental distribution were chosen to study their genetic diversity using the simple sequence repetition index (SSR).](image-url)

3.2 Number and size of alleles

The polymorphism was assessed among the studied MYRTLE members by calculating the number of alleles and values of the PICs for each of the ten genotypes of the studied SSR. The primers differed significantly in their ability to determine the differences between the individuals studied (Table 3). Some primers produced several molecular indices, while others gave fewer indices. Among the indicators that gave a polymorphism, two indicators gave 2 allele, five indicators gave 3 alleles, and two indicators gave 4 alleles, while the myrcom10 index did not give clear results on the individuals studied.
The SSR indicators gave a total of 27 alleles in 19 genetic individuals. The number of alleles in the genetic site ranged from 2 (myrcom 9, myrcom 8) to 4 (myrcom 6, myrcom 2), and they were on average 3 alleles for each genetic site. The size of the reproductive segments in all indicators used ranged between a minimum of 171 nucleotide pairs (myrcom 6) as a minimum and 288 nucleotide pairs (myrcom 5) as a maximum.

The difference in size varied between the smallest and largest allele in a specific SSR site from 4 nucleotide pairs (myrcom 1) to 38 nucleotide pairs (myrcom 8). The allele was also considered unique when viewed in one of the 19 studied individuals. The presence of 2 single allele (7.4%) was detected at 2 genetic sites (myrcom 5, myrcom 1) out of 9 SSR sites, with an average of 0.22 single alleles in the single genetic site (Figure 2). This single site was revealed to the individual in Drekwish (Dre14w), who may be to his different origin compared to the individuals. The absence of a large number of single alleles within this group of genetic individuals indicates a lack of genetic diversity among them.

![Bands from multiples of the use of primers Myrcom (1-2-3-5) on the agarose gel.](image)

**Figure 2.** Bands from multiples of the use of primers Myrcom (1-2-3-5) on the agarose gel.

| PIC  | Genetic awareness | Variations (nucleotide pair) bp | Allele size | Rare Alleles | number of alleles | Motif replication | Sequence of starters | 5’-3’ Site name |
|------|------------------|---------------------------------|-------------|--------------|------------------|-------------------|---------------------|-----------------|
| 0.206| 0.217            | 4                               | 227-231     | 1            | 3                | (AC)₆             | F:CGTGATGCACACTGAAGTGA | Myrcom 1       |
| 0.265| 0.299            | 26                              | 187-213     | -            | 4                | (TC)₁₈           | R:ACACCCCTTTTGCAACATT | Myrcom 2       |
| 0.467| 0.443            | 6                               | 180-186     | -            | 3                | (CT)₁₃           | F:ATAGCTCTTACCCGTTGAGT | Myrcom 3       |
| 0.369| 0.350            | 22                              | 197-219     | -            | 3                | (TC)₂₀           | R:GTGCATGTGCCTCGTATAGT | Myrcom 4       |
| 0.206| 0.196            | 14                              | 274-288     | -            | 3                | (CT)₈            | F:GGCAGCTACAGTTCCATT | Myrcom 5       |
| 0.394| 0.374            | 16                              | 171-187     | -            | 4                | (A)₁₂(CA)₁₀      | R:TTTGACAGCATTTCAAGTGA | Myrcom 6       |
| 0.416| 0.395            | 22                              | 169-191     | 1            | 3                | (GA)₁₉           | F:CAACCATCCACCATAGA | Myrcom 7       |
| 0.402| 0.382            | 38                              | 232-270     | -            | 2                | (TA)₆            | R:CCACAGTCAGAAAGGAGGAGC | Myrcom 8       |
| 0.507| 0.482            | 5                               | 181-186     | -            | 2                | (A)₁₅GG(CA)₉     | F:TAAGAGATCAGCAACAAAG | Myrcom 9       |
| 0.359| 0.349            | -                               | -           | 0.22         | 3                |                   | R:CATGAATGGCAACAGATGAA | Middle         |
3.3 Polymorphic Information Content (PICs) values

The PIC value reflects the diversity and frequency of the allele among the individuals studied. The value of the PIC can be evaluated on the basis of its alleles and varies widely for all tested genetic SSR sites. The level of polymorphism was assessed among 19 genetic individuals by calculating the PIC value for all hereditary SSR sites. The PIC value ranged between 0.198 (myrcom 5) and 0.5 (myrcom 9), while the mean was 0.360 Table (3). The two highest genotypes of Drekish and Zayniyya (Dre14w, Zen5b) gave the highest number of alleles (15), while the smallest number of alleles (2) was obtained from the genotype Safita (Saf16w).

3.4 Cluster analysis

The relationship tree was created by UPGMA using the genetic similarity values among the studied individuals to show the genetic relationship between them. The cluster analysis showed that there were genetic differences between the nineteen individuals from the common Myrtle whose results were analyzed (the Afrin sample was excluded) where the similarity coefficient according to the Dice coefficient ranged between 0.08 to 0.89.

![Figure 3. Cluster analysis diagram that shows the diversity and relationship between 19 individuals of the common Myrtle according to the Dice coefficient.](image)

The samples were distributed at the similarity level of 0.51 over three clusters of major clusters (I, II, (III). The cluster (I) included most of the studied samples (15 samples) were distributed on the wet and sub-humid bioclimatic floors, while the cluster (II) included three samples from the floors Humid biomechanics, while cluster (III) included a single sample of semi-humid biomass, cluster I was distributed at a similarity level of 0.63 to three under clusters (Ia, Ib, Ic), where the 15 samples that contained the main cluster were distributed into 9 samples Under the cluster (Ia), 5 samples under the cluster (IIb), and a single sample under the cluster (Ic).

3.5 Similarity Matrix

The similarity matrix was used to determine the level of relationship between the individuals studied. Pairwise similarity ranged from 0.08 to 0.89 with an average of 0.42. The similarity rate was 0.80 or more for each individual KAS10b on the one side with Dar6w, Ray7w, Ray8w and Man20w on the other side, and between Sam9w and between Dar6w and Ray7w and between Ray8w and Bda4b, between Ray7 and Dar6w. The similarity matrix of the studied individuals showed a genetic distance among the individual Drekish Dre15 and the basil diameter of Kat19b, and among Zarzur Zar3b on the side and the basil diameter Kat19b and the Mos17b and Safita SAF16w on the other, and between Zarzur Zar2w and Enab18b jujube, which recorded the lowest similarities of table (4). SSR indicators were used in this study to analyze the genetic diversity of
19 individuals from the Myrtle. These indicators were able to detect the presence of a genetic distance or non-similarity betwen the individuals studied according to the area of its

Table 4. Similarity matrix for studied Myrtle individuals.

|     | Zar | Za | Bn | Ze | Da | Ra | Ra | Sa | Kas | Sam | Mas | Zen | Dre | Dre | Saf | Mos | Ena | Kat | Man |
|-----|-----|----|----|----|----|----|----|----|-----|-----|-----|-----|----|----|----|----|----|----|----|
| 2w  | r3  | a4 | n5 | r6 | y7 | y8 | m9 | w  | 10b | h12 | 13b | 14w | 15w | 16w | 17b | 18b | 19b | 20w |
| Man | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 20w | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Kat1| 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 9b  | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Etn | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 8b  | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Mos | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 17b | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 14w | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 15w | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 16w | 0.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |

Also in this study, a smaller number of alleles were recorded in the studied individuals, not exceeding 4 alleles compared to [26], which was able to detect at a greater number of alleles reached 8 alleles when using the myrcm7 index, as well as a high percentage of genetic diversity and case of heterozygosis. This can be attributed mainly to the method used to separate the outputs of the PCR reaction, where the researcher resorted to the use of capillary electrophoresis, which gives the product of propagation in through which any changes in the length of the allele can be monitored. It should be noted here that the differences in the length of the allele with respect to the indicators used ranged between 4 and 38 nucleotides (Table 4), which it is not possible to obtain a separation strength for such type of alleles on the agarose gel. However [27] indicated the possibility of following certain procedures in the process of preparing the gel electrophoresis so that it was possible to separate the PCR reaction products of SSR indicators by a difference of 5 nucleotides between one allele and the other.
Conclusions

- The molecular marker SSR showed richness in the genetic base of myrtle, where the similarity ranged between 0.08 - 0.89.
- The nine indicators gave a total of 27 alleles in 19 genetic subjects, and the calculated value of the polymorphic content (PIC) reached a maximum of 0.5.
- The studied Myrtle members were divided into three main clusters, and a limited number of them were distributed associated with the bioclimatic floor.
- SSR technology can be used to distinguish between studied Myrtle individuals and thus show genetic diversity among them.
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