

Genetic diversity assessment of ancient mulberry (*Morus* spp.) in Lebanon using morphological, chemical and molecular markers (SSR and ISSR)

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: Lebanon has ancient mulberry trees which are the remnants of the abundant orchards that dominated its lands during the nineteenth century. Lebanese mulberry germplasm has not been assessed yet. This study aims to collect local old rainfed mulberry accessions from different geographical regions and assess their diversity by using morphological and molecular markers (SSR and ISSR). Genetic diversity of 70 accessions of mulberry were evaluated by using 27 morphological traits. The dendrogram based on the morphological attributes showed a relative separation of the different accessions based on fruits color and taste. Molecular analysis was performed for the accessions by using selected SSR and ISSR primers. The primers marked a high discriminating power (0.7 to 0.89). The dendrogram constructed on the base of UPGMA method showed 13 different groups. The clustering patterns indicated no location nor local name specificity among mulberry accessions. The combination of SSR and ISSR primers was informative for estimating the extent of mulberry genetic diversity. It can be concluded that there is a high level of genetic diversity within mulberry trees in Lebanon. These results will be useful for mulberry germplasm management in terms of biodiversity protection and as a valuable source of gene pool for crop improvement.

1. Introduction

Mulberry belongs to the genus *Morus* of the family Moraceae. It is a

multipurpose tree with a significant ecological, nutritional and economical high value. Mulberries are highly adaptable species in different soil and climatic conditions. They are generally quite tolerant to drought, pollution and poor soil. Therefore, they can be found in a wide area of tropical, subtropical, and temperate zones in Asia, Europe, North America, South America, and Africa (Kafkas *et al.*, 2008). The genus *Morus* regroups 24 species (Thabti *et al.*, 2014). The most widespread species in the Mediterranean climate areas are: *Morus alba* with fruit colors ranging from white to dark red, *Morus rubra* with mainly red/purple fruits and *Morus nigra* with dark purple to black fruits (Gerasopoulos and Stravroulakis, 1997).

Mulberry fruits have remarkable potential for providing various valuable industrial products of high economic value for human beings. They are used for direct fruit consumption (*Morus alba*, *Morus indica*, *Morus nigra*, and *Morus laevigata*). Most of mulberry species have distinct flavor with juicy and acidic characteristics making them attractive for use in the processing industry for products such as fruit juice, ice cream, jelly, and jam (Ercisli and Orhan, 2007). Interest in mulberry has increased considerably over the last 20 years as a healthy fruit. *Morus* species have great antioxidant potential due to their high content in phenolic compounds including flavonoids, anthocyanins, and carotenoids (Zhang and Ma, 2018). Mulberries present anticancer and anti-inflammatory properties and show as well significant effect on many chronic diseases like diabetes (Nakamura *et al.*, 2009; Kwon *et al.*, 2015; Qian *et al.*, 2015).

Mulberry is an economically important plant used for sericulture. It is the sole food plant for the domesticated silkworm, *Bombyx mori* (Zhao *et al.*, 2009). The genus *Morus*, is cultivated extensively in East, Central and South Asia for silk production (Awasthi *et al.*, 2004). Hence, mulberry is one of the most important components that decide the sustainability of this industry (Liu *et al.*, 2009).

At the turn of the century, Lebanon was known for its high-quality silk industry. Bestowed with an ideal climate and a fertile soil, mulberries were planted everywhere in Lebanon and mulberry production flourished (Firro, 2009). The silk tradition in Lebanon is more than two thousand years old. It goes back to the period of the famous purple dye (Ourjouan) extracted from the Murex shell by the Phoenicians of Sidon and Tyre and used to produce imperial purple

silk (Khater, 2009). In the 19th century, silk industry constituted almost 80% of Lebanon's economy. By the early 20th century, 70% to 80% of the cultivable land of the country mountainous regions (Mount Lebanon) became devoted to mulberry orchards. Due to the high demands in silk production, mulberry tree has an unsurpassed economic impact on rural communities. After 1940's, when silk began to be imported from the Far East, the sericulture industry declined sharply. Mulberry cultivation became marginalized. However, Lebanon still has very old rainfed mulberry trees which are the remnants of the abundant orchards that were once shaping the landscape of many villages. Mulberry trees are found in different Lebanese villages, mostly located at orchards periphery or in small gardens.

In Lebanon, mulberry genotypes are very diverse, as they were sometimes obtained in the past from seeds or from cuttings. This process has led to a great number of landraces adapted to different conditions and different uses throughout the country. In Lebanon, there are many local traditional accessions but no named cultivars. Mulberries are distinguished and denominated according to the fruit color: "Abyad" (white mulberry), "Mwachah" (purple mulberry), "Shami" and "Aswad" (black mulberries).

Mulberry genetic diversity is progressively being lost in farmers' fields and in nature. The threat results from the interaction of several factors and is processing at an alarming rate. The most crucial factors are urbanization, climatic changes, out breaks of new diseases and pests, and the frequent occurrence of natural calamities. Little information is available about the genetic diversity of Lebanese mulberries. To protect mulberry in Lebanon, a marginalized species, conservation programs should be initiated. In this study, we have collected local mulberry accessions from different geographical regions of Lebanon and assessed their genetic diversity by using agromorphological traits as well as molecular markers (SSR and ISSR).

2. Materials and Methods

Field survey

Samples of fruits for morphological and chemical analyses were collected from local trees of mulberry *Morus* from 21 sites covering different Lebanese regions (the North plain, Bekaa plain, Mount

Lebanon, the South). These sites are subjected to different climatic conditions (precipitation, temperature) and agricultural practices. They are situated at an altitude between 30 and 1620 m, a latitude ranging from N33° 16' 166" to N34° 21 '51.5" and a longitude between E36°10' 849" and E35° 01' 38.7" (Fig. 1). The number of individual trees sampled per site (population) ranged from two to sixteen cultivars. In total, 70 accessions of mulberry were studied. Collected samples consisted of mature fruits (approximately 500 g) and vegetative materials (young leaves, mature leaves and branches). The studied accessions included 'Abyad' (white mulberry), 'Mwachah' (purple mulberry), 'Shami' and 'Aswad' (black mulberries).



Fig. 1 - Geographic distribution of the studied mulberry accessions as visualized with DIVA-GIS program (Hijmans *et al.*, 2001).

Morphological and chemical characterizations

The characterization of the vegetative materials and the fruits was based on descriptors for investigation of mulberry germplasm's morphology produced by Agriculture and Consumer Protection FAO (Sohn, 2003). Thus 27 morphological characters were studied for the mulberry accessions. These studied traits included 13 qualitative characters (for the leaf: shape, margin, base, apex, surface, color, glossiness, phytotaxis, bud shape and color; for the fruit parts: shape, color, taste, seed color) and 14 quantitative characters (for the vegetative parts: leaf length,

width and thickness, petiole length, bud length, internode length; for the fruit parts: fruit length, diameter and weight, peduncle length, juice percentage (volume of the juice*100/weight of the fruits), sugar quantity (using refractometer), pH and acidity (by titration reaction).

Molecular characterization

DNA extraction. Genomic DNA was extracted from mulberry young fresh leaves using cetyl trimethyl ammonium bromide (CTAB) procedure described by FAO/IAEA (2007). The DNA quantity and quality was visually quantified using the agarose gel electrophoresis method as described by Maniatis *et al.* (1982). DNA samples were stored at -20°C.

PCR amplification of the DNA with ISSR primers. Six primers (UBC807, UBC810, UBC826, UBC827, UBC864 and BI3) were tested for DNA amplification (Emir, 2013). The ISSR (Inter Simple Sequence Repeat) amplification was carried out as per Vijayan and Chatterjee (2003) using 20 µl reaction mixture containing 2 µl of 10 X PCR buffer (750 mM Tris-HCl pH 8.8; 0.1% Tween-20), 0.2 mM dNTP, 2 Mm MgCl₂, 200 nM Primer, 50 ng genomic DNA and 1 U Taq DNA polymerase (MBI Fermentas Inc, Hanover, MD-21076, USA). The PCR schedule included an initial cycle at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and a final extension of 10 min at 72°C. The PCR products were resolved by electrophoresis on a 1.5% agarose gel in 1 X Tris Boric Acid buffer (TBE), stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

PCR amplification of the DNA with SSR primers. Microsatellite polymorphisms were identified using three SSR primers (primers: MulSTR1, MulSTR2 and MulSTR3) (Tikader *et al.*, 2009). Microsatellite amplification reactions were performed in a final volume of 25 µl in the presence of 2.5 µl of buffer, 200 µM of each dNTP, 0.4 µM of each primer pair, 1 unit (U) of Taq DNA polymerase, 50 ng template DNA, and 2 mM MgCl₂. The amplification reaction consisted of an initial denaturation step at 94°C for 4 min, followed by 45 cycles of 1 min denaturation at 94°C, 65 sec annealing at 50°C, 90 sec extension at 72°C with a final extension of 72°C for 10 min using thermal cycles. The PCR amplification products were separated on a 6% denatured polyacrylamide gel and visualized by silver staining.

Data analysis

For qualitative traits, scores were attributed

according to FAO mulberry descriptors. A phenotypic diversity index, h_{sj} (Shanon index) (Magurran, 1988) was calculated for each site to describe the phenotypic diversity of mulberry. The following formula was used for calculating h_{sj} for each trait with n categories $h_{sj} = -\sum P_i \ln P_i$, where p_i is the relative frequency in the i th category for the j th trait. The average diversity (H) over k traits of each site was estimated as: $H = \sum h_{sj}/k$. Traits evaluation was performed by using the Principal Component Analysis (PCA). The relationships between mulberry leaves and fruits based on their quantitative and qualitative traits were studied using Hierarchical Cluster Analyses executed using Euclidean Distance following the Ward's method implemented in PAST software (Hammer *et al.*, 2001).

To assess the information given by SSR and ISSR markers, the following parameters were calculated: number of alleles per locus, percentage of observed heterozygosity (H_o), expected heterozygosity ($H_e = 1 - \sum p_i^2$, where p_i is the frequency of the i th allele) and the power of discrimination ($PD = 1 - \sum g_i^2$, where g_i is the frequency of the i th genotype). Genetic distances were calculated according to Jaccard (1908). Trees were produced by clustering the data with the

unweighted pair-group method (UPGMA) with SAHN-clustering and tree programs of PAST software (Saporta, 1990).

3. Results

Mulberry trees were distributed over various agro-climatic areas of Lebanon (Fig. 1, Table 1). A total of 70 mulberry accessions were studied belonging to 'Abyad' (22 accessions), 'Mwachah' (25 accessions), 'Shami' (20 accessions) and 'Aswad' (3 accessions). Among mulberry species found in Lebanon, *Morus alba* was the dominant species in cultivation (95%). Around 85% of the surveyed mulberry trees were rainfed, old and inherited from family.

Morphological analysis

Leaves morphological characterization. Mulberry trees tend to have short trunks with large, low, spreading limbs. Leaves were alternately arranged and simple. The majority of cultivars had a cordate leaf shape, except the leaves of two accessions were reniform ('Abyad' and 'Shami') and two other were cordate to oval ('Abyad' and 'Mwashah'). All leaves

Table 1 - Climatic and geographic characteristics of the 21 locations surveyed to characterize the Lebanese mulberry trees

Site	Latitude (N)	Longitude (E)	Altitude (m)	Annual average temperature (°C)	Annual Rainfalls (mm)	Varieties	Number of accessions
Douris	33°59'588"	36°10'849"	1131	14.9	441	Abyad Mwachah	4
Rayak	33°51'751"	35°59'591"	927	15.1	544	Aswad	2
Nabishet	33°52'10.4"	36°06'34.7"	1233	13.6	570	Abyad Mwachah Sami	6
Jenta	33°51'23.5"	36°06'26.2"	1114	13.2	580	Abyad Mwachah	2
Britel	33°56'02"	36°08'54.3"	1154	14.7	471	Abyad Mwachah Shami	4
El borjein	33°39'27.2"	35°29'11.3"	1620	12.7	630	Shami	1
Baassir	33°39'30.1"	35°26'54.7"	1094	12.9	630	Abyad Mwachah Shami	2
Hawsh Nabi	33°55'28.6"	36°04'23.8"	990	15.2	544	Abyad Mwachah	2
Hawsh Refaa	33°55'23.7"	36°02'34.2"	971	15.1	530	Abyad Mwachah	5
Kfar Dabash	33°56'43.2"	36°02'13.7"	1079	15.1	540	Abyad Mwachah	1
Chmistar	33°57'49.6"	36°01'07"	1145	14.9	550	Shami	2
Beit Chama	33°55'07"	36°01'25"	1011	15.2	541	Abyad Mwachah	1
Tammen taata	33°52'43.3"	35°59'45.9"	937	15.1	542	Abyad Mwachah	1
Chlifa	34°05'109"	36°06'098"	1012	14.7	461	Abyad Mwachah Shami	5
Flaoue	34°04'934"	36°03'761"	1139	14.6	461	Abyad Mwachah Shami	3
Dayr Lahmar	34°07'077"	36°07'940"	1012	14.5	461	Baladi	1
Zahle	33°48'59.9"	35°57'32.6"	882	15.2	646	Abyad Mwachah Shami	5
Ali ennahry	33°51'21.04"	35°01'38.7"	958	15.1	544	Shami	1
Sour	33°16'166"	35°13'133"	30	20.2	697	Abyad Mwachah Shami	4
Kfar Chakhna	34°21'51.5"	35°51'50.7"	198	13.9	754	Shami	2
Hasbaiya	33°32'74"	35°64'373"	467	15.1	590	Abyad Mwachah Shami	16

had dentate margins. They presented mainly a cordate base and an acute apex. Leaf surface of 70% of the accessions was slightly rough. Only nine accessions of 'Shami' presented rough surfaces.

Leaves generally presented an average length between 6.83 and 18.9 cm and width between 3.94 and 17.02 cm. Petiole length average was between 0.72 and 5.07 cm. Leaves of black mulberry accessions 'Shami' (0.02 and 0.03 cm) were thicker than those of white mulberries (0.01 cm).

Fruits morphological characterization. For the 70 accessions, the pomological characteristics investigated showed a great diversity. Concerning the fruit shape, 35.7% of the mulberry fruits had oblong shape, 27.1% were round, 24.2% were reniform, and 13% were oval. For the local variety 'Mwashah', nearly half of the accessions had oblong shape while the majority of 'Shami' had round one. White mulberry 'Abyad' presented mainly oblong and reniform shape.

The accessions showed significant differences in the fruit weight ranging from 1.1 g ('Abyad' from Doris) to 7.9 g ('Mwashah' from Hawshrefaa). Fruit length varied from 1.7 ('Shami' from Flewa) to 4.9 cm ('Mwashah' from Hawshrefaa-Bekaa) and fruit width from 1.1 ('Abyad' from Doris) to 2 cm ('Shami' from Baaser). Minimum length of fruit peduncle was 0.11 ('Shami' from Flewa) and maximum length 1.28 cm ('Aswad' from Tyr).

Fruit color of mulberry accessions was diverse: 'Abyad' accessions were white and 'Mwachah' accessions were violet. The fruit color of 'Shami' accessions were darker and varied between red-purple to black or black. Seed color varied between light yellow and yellow-brown; 'Mwashah' fruits had mainly light yellow seeds and 'Shami' presented yellow-brown seeds.

The percentage of juice yields differed within the accessions of the same local variety. The lowest and greatest juice yields varied from 30.1% ('Abyad' from Nabishit) to 72.3% ('Shami' from Baaser) and 73.1% ('Abyad' from Shlifa). As for the chemical characteristics of mulberry accessions, sugar content ranged from 7 ('Mwashah' from Janta) to 19.5 Brix ('Aswad' from Tal Amara). pH varied widely from 2.29 ('Abyad' from Hasbaya) to 6.42 ('Abyad' from Douris). Titrable acidity was very diverse in the different mulberry accessions. Titrable acidity values were from 0.01 ('Mwashah' from Zahle) to 0.14 g/l ('Shami' from Flewa).

Morphological characterization PCA

The characterization of the collected mulberry accessions using different morphological characters showed high level of variation among the accessions. The Principal Component Analysis (PCA) revealed that the first 3 components explained 37% of the total variation, based on the 27 morphological characters (Table 2). The first component represented 18 % of the total variation and included fruit and leaves characteristics. It comprised fruit length, color, taste, pH, acidity and peduncle length, besides to the petiole length, glossiness and thickness of leaves. The second component represented 10% of the total variation and is mainly influenced by leaf width. The third component was characterized by a percentage of variation of 9% and is dominated by the bud length character.

Table 2 - Principal component analysis (PCA) of the 27 morphological characters evaluated for the 70 different mulberry accessions. The characters in bold are discriminant

Variables	Factor 1	Factor 2	Factor 3
Fruit length	-0.622011	-0.428543	0.385704
Fruit weight	-0.422828	-0.548276	0.534493
Fruit diameter	-0.279504	-0.469198	0.362977
Peduncle Length	-0.639179	-0.10646	-0.393485
Percentage of sugar quantity	0.126663	0.055644	0.121337
pH	-0.720232	0.290375	0.027219
Acidity	0.605034	-0.131547	0.165099
Leaf length	-0.014178	-0.543281	-0.48354
Leaf width	-0.038798	-0.719527	-0.23667
Petiole length	-0.700726	-0.161604	-0.190875
Leaf thickness	-0.739758	-0.294618	0.241539
Date of maturity	-0.085013	-0.253214	0.241209
Bud length	0.069769	-0.177441	-0.629778
Bud width	0.136856	-0.265182	-0.59383
Bud shape	-0.192576	0.061054	-0.048574
Internodal distance	-0.141876	-0.525259	-0.327898
Leaf shape	-0.109165	-0.008143	-0.223988
Leaf base	-0.074223	0.208101	0.302694
Leaf apex	0.007149	-0.251893	-0.077977
Leaf surface	-0.50104	0.446809	-0.164365
Leaf color	-0.260375	-0.135751	0.031335
Leaf glossiness	0.670155	0.023403	-0.053846
Fruit shape	-0.498979	-0.040292	0.105085
Fruit color	-0.6271	0.204057	-0.072799
Fruit taste	0.663459	0.058647	0.089481
Seed color	-0.210256	-0.238095	0.409438
Exp.Var	4.797.858	2.719.786	247.986
Prp.Tot	0.177698	0.100733	0.091847

Classification of accessions based on morphological attributes

The accessions could be separated into groups based on the 11 most discriminant traits. The hierarchical cluster analysis classified mulberry accessions in 6 groups at -6 similarity of Euclidean distance (Fig. 2, Table 3).

‘Shami’ accessions were classified separately into 3 main groups (G1, G4 and G5). The first group G1 included ‘Shami’ and 2 accessions of ‘Aswad’. G1, G4 and G5 accessions were characterized by a sour fruit taste and a dark black-purple or black-red fruit color. Fruits of G4 and G5 presented significantly the lowest pH mean values (3.78 and 3.85 respectively). G5 fruits presented the lowest sugar content (9.62 °Brix). Accessions of G4 and G5 had the shortest fruit length (2.32 cm and 2.45 cm respectively) and the shortest peduncle (0.24 cm and 0.46 cm respectively). Regarding leaf characteristics, G4 and G5 accessions were characterized by low mean value of leaf length, while G1 were characterized by a significantly high leaf length (17 cm). G1, G4 and G5 accessions had also the thickest limb (0.02 - 0.03cm) and the shortest peduncle (1.4 - 1.48 cm).

The group G2 consisted of 12 accessions of ‘Abyad’ and one of ‘Mwashah’. These accessions were characterized by a white fruit color and a sweet taste. The group G3 consisted of 19 accessions of ‘Mwashah’ and 2 of ‘Shami’. They were characterized by purple fruits. They presented the highest value of fruit length (3 cm) and medium values for pH and

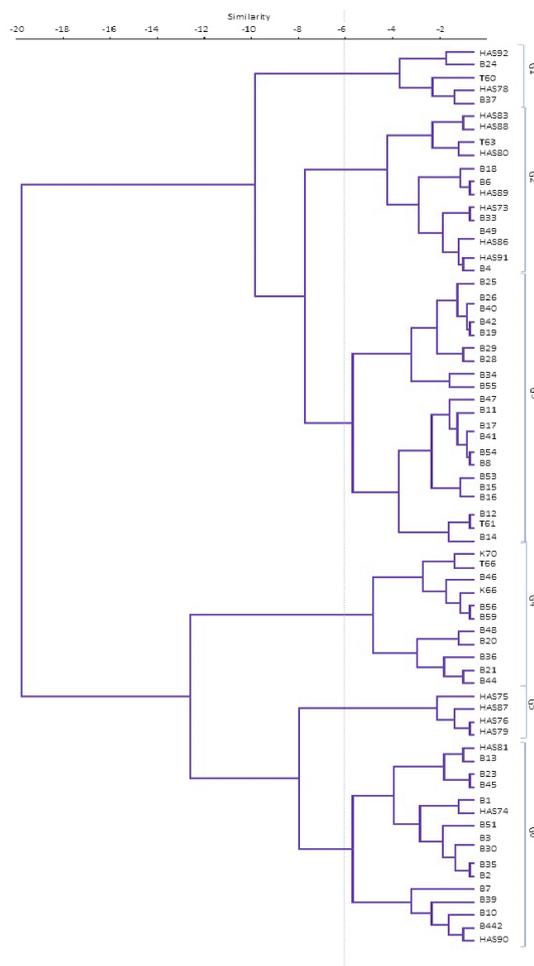


Fig. 2 - Dendrogram of Jaccard distance based on the characters that presented high variability in the principle component analysis, depicting the genetic relationship among the 70 different mulberry accessions.

Table 3 - Variability of the quantitative morphological fruit characteristics for the accessions clustered within the same group (G1, G2, G3, G4, G5 and G6) minimum, maximum and mean values (with standard deviation)

Group (number of accessions)	Fruit length (cm)	Fruit width (cm)	Fruit weight (cm)	Peduncle (cm)	Percentage of juice (%)	Sugar quantity (Brix)	pH	Acidity (g/l)
G1 (3 Shami, 2Aswad)	2.11< L<4.05 2.91±0.81 a	0.96< w <2.05 1.59±0.42 a	0.99< W <7.81 3.91±2.60 a	0.93< P <1.28 1.13±0.14 a	51.4< J <72.3 59±7.77 a	7< S<15 11.6±3.84 bc	3.4<pH <5.97 4.79±1.03 b	0.028< N < 0.13 0.05±0.04 b
G2 (12 Abyad, 1 Mwashah)	2.14< L< 3.45 2.77±0.39 ab	1.49< w <1.88 1.61±0.20 a	2.05< W <5.72 4.00±1.17 a	0.43< P <1.4 0.81±0.26 b	34.2< J <58.2 52.68±8.50 a	11< S<16.5 13.15±1.87 ab	2.29<pH <6.47 5.70±1.28 ab	0.018< N < 0.076 0.05±0.02 b
G3 (19Mwashah, 2 Shami)	2.22< L <4.95 3.00±0.63 a	1.4< w <2 1.75±0.15 a	2.6< W <7.98 4.90±1.42 a	0.51< P <0.99 0.76±0.18 b	35.4< J <64.1 51.67±8.18 a	7< S<15 11.33±2.21 bc	3.5<pH <6.35 5.47±1.04 ab	0.014< N < 0.116 0.03±0.02 b
G4 (11 Shami)	1.71< L <3.1 2.32±0.39 b	1.3< w <1.86 1.57±0.19 a	1.3< W <5.38 3.74±1.30 a	0.1< P <0.90 0.24±0.23 d	36.2< J <76.7 53.69±12.20 a	7< S<18.5 13.73±3.08 ab	3.14<pH <5.97 3.78±0.39 c	0.04< N < 0.2 0.10±0.04 a
G5 (4 Shami)	2.13< L <2.68 2.45±0.255 ab	1.5< w < 1.93 1.72±0.19 a	3.4< W <5.77 4.68±1.09 a	0.38< P <0.56 0.46±0.09 c	49.4< J <58.5 52.95±4.09 a	8.5< S<11 9.62±1.11 c	3.1<pH <6.12 3.85±1.51 c	0.038< N < 0.05 0.045±0.006 b
G6 (5 Mwashah 10Abyad, 1Aswad)	1.88< L <3.33 2.68±0.44 ab	1.10< w <1.95 1.59±0.25 a	1.11< W <5.64 3.76±1.26 a	0.46< P <1.14 0.82±0.22 b	30.1< J <73.1 50.74±10.31 a	10.5< S<21 14.91±3.07 a	5.3<pH <6.51 6.05±0.45 a	0.014< N < 0.08 0.041±0.019 b

Different letters were significantly different at the 0.05 level (Duncan's Multiple Range Test).

sugar content. The group 6 regrouped 5 accessions of 'Mwashah', 10 of 'Abyad' and one of 'Aswad'. These accessions were characterized by their sweet taste (high sugar content and high pH). Groups 2, 3 and 6 presented a medium leaf thickness.

Molecular characterization

ISSR analysis. The molecular analysis of mulberry accessions presented a high variability. The ISSR markers showed distinct polymorphism between the different mulberry accessions, only primers UBC-826 and UBC-864 showed no amplification. A total of 18 polymorphic bands were detected across the 70 accessions of mulberry through the use of four ISSR primers UBC807, UBC810, UBC827 and BI3. The size of amplified products ranged from 700 bp to 1600 bp. The number of scorable markers produced per primer ranged between 4 (UBC810, UBC827) and 5 (UBC807, BI3). This study showed that (AC), (GA) repeat primers generated excellent band profiles. Primers synthesized from (ATG) repeats failed to amplify.

The power of discrimination calculated for each primer (Table 4) enabled us to evaluate the genetic diversity of our locus. The calculated power of discrimination PD values were between 0.75 and 0.89, showing that the studied loci are of high diversity.

These primers could be effectively used to study polymorphism between mulberry accessions.

SSR analysis. Primer MulSTR1 showed no amplification within our accessions. A total of 10 polymorphic bands were detected across the 70 accessions of mulberry through the use of two SSR primers (MulSTR2, MulSTR3). Upon using MulSTR2 SSR primer, the acrylamide gel showed three bands across the different accessions (192bp, 200bp, and 208bp), while MulSTR3 SSR primer generated 7 different bands ranging from 192bp to 275bp (Table 5). The results presented high polymorphism in all the amplified loci. In their assessment of mulberry genotypes by SSR marker profile, Wani *et al.* (2013) used MulSTR2 and MulSTR3 primers which generated each 2 alleles among the 17 mulberry genotypes tested. Our results showed a higher number of polymorphic bands for a higher number of accessions. The power of discrimination was relatively high for each primer, PD>0.5. MulSTR3 presented higher polymorphism than MulSTR2, with a PD of 0.8 and an expected heterozygosity of 0.713. These two primers and especially MulSTR3 could be effectively used in genetic diversity studies of mulberry.

Classification of accessions based on molecular markers

The allelic diversity data was used to produce a

Table 4 - Primer sequences, number and sizes (bp) of the produced bands and discriminating power (Dp) of the six ISSR markers used in the study

Primers	Sequence	Number of bands	Band sizes (bp)	Dp
UBC-810	3'GAGAGAGAGAGAGAGAT 5'	4	800-1500	0.80
UBC-807	3'AGAGAGAGAGAGAGAGT 5'	5	900-1600	0.89
UBC-827	3'ACACACACACACACAG 5'	4	700-1700	0.85
BI3	3'ACACACACACT 5'	5	500-1500	0.75
UBC-826	3'ACACACACACACACAC5'	-	-	-
UBC-864	3' ATGATGATGATGATGATG5'	-	-	-

Table 5 - Primer sequences, number and sizes (bp) of the produced bands and discrimination power (Dp) of the three microsatellite markers used in the study

Primers	Sequence	Number of bands	Band sizes (bp)	Dp
MulSTR1	F: 5'GCCGTGTACCACTGGAGTTTGCA 3' R: 5'TGACCGTTTCTTCCACTTTACC-	-	-	-
MulSTR2	F: 5' CGTGGGGCTTAGGCTGAGTAGAGG R: 5' CACCACCACTACTTCTTCTTCCAG	3	192-208	0.52
MulSTR3	F: 5' GGGTTGGGTAGATGGGCTTATGT- R: 5' CCCTATTAACCTTTTGGTCACTCTA	7	192-275	0.83

dendrogram *via* the distance matrix-UPGMA (Fig. 3), thus revealing the genetic relationship among mulberry accessions. The dendrogram constructed on the base of the SSR and ISSR amplification product of

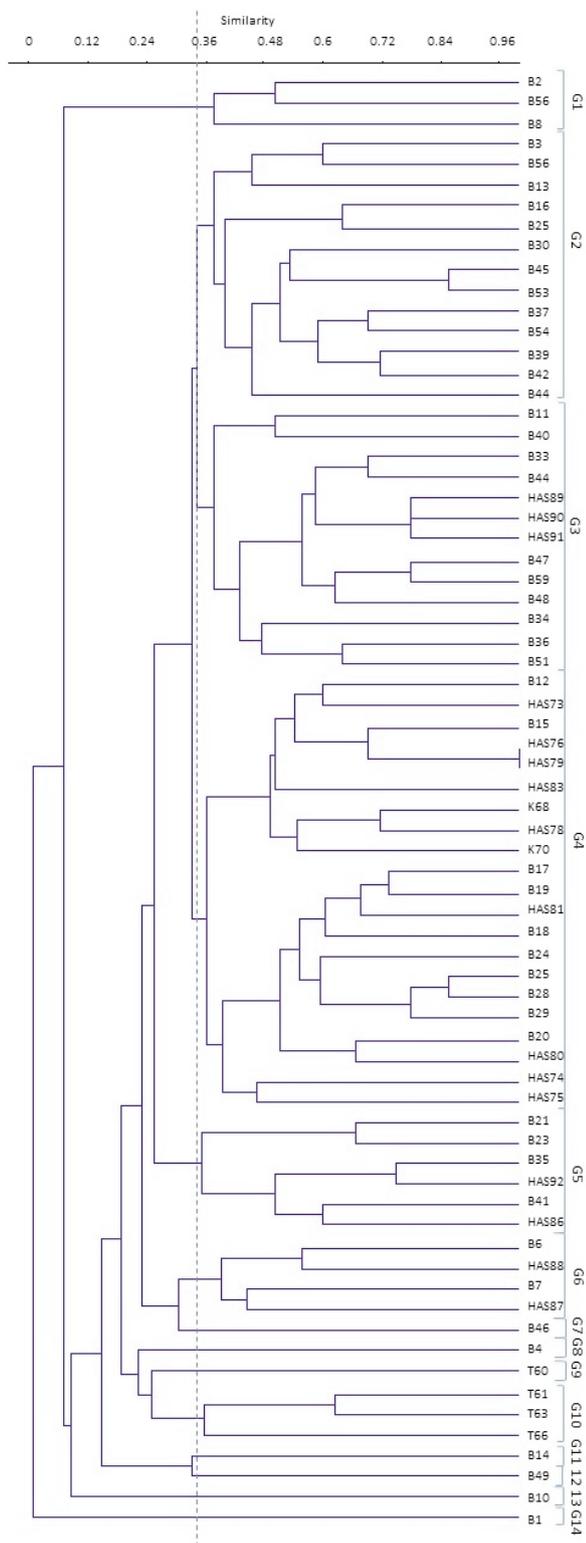


Fig. 3 - Dendrogram constructed from SSR and ISSR markers, using PAST program, Jaccard distance and UPGMA clustering of 70 mulberry accessions.

the different mulberry accessions showed 14 different groups at the Jaccard distance of similarity 0.34. Clusters G1, G2, G3, G5, G6 and G10 regrouped accessions of the three varieties ‘Mwashah’, ‘Shami’ and ‘Abyad’. G4 contained the three varieties as the previous groups in addition to an accession of ‘Aswad’. One single accession constructed individually the groups G7 (‘Shami’), G8 (‘Abyad’), G9 (‘Aswad’), G11 (‘Shami’) G12 (‘Abyad’), G13 (‘Mwashah’) and G14 (‘Abyad’).

4. Discussion and Conclusions

The conservation of the genetic variability of the ancient Lebanese mulberry trees is of utmost importance for germplasm preservation and for future breeding programs. Lebanese mulberries germplasm has not been assessed yet. This study is the first genetic diversity assessment of the Lebanese mulberry germplasm using a set of morphological traits and genetic markers. Our inventories recenssed accessions of four vernacular names ‘Abyad’, ‘Mwachah’, ‘Shami’ and ‘Aswad’ across different Lebanese regions. This shows that a limited number of traditional varieties was cultivated since decades, however morphological and molecular characterization of these 70 accessions revealed high diversity of this germplasm collection.

The results of morphological characterization revealed a high level of variation among mulberry characters. Among the 27 descriptors studied, 11 specific characters of fruits (Fruit taste, fruit length, fruit color, pH, titrable acidity and peduncle length) and leaves (petiole length, leaf thickness, leaf glossiness, leaf width and bud length) revealed to be the most discriminating characters. The first component of the PCA was dominated by the fruit characteristics. A broad morphological diversity of the fruit was reported for mulberry germplasm (Yilmaz *et al.*, 2012; Peris *et al.*, 2014; Aljane and Sdiri, 2016; Krishna *et al.*, 2020). In our study, fruits exhibited distinct variations. Fruits shapes were diverse. Fruit color, titrable acidity, sugar content, juice yield and pH content were the most discriminating characters to differentiate mulberry accessions. Similar results were reported and significant differences were observed between the fruit characteristics (Yilmaz *et al.*, 2012; Peris *et al.*, 2014; Aljane and Sdiri, 2016; Krishna *et al.*, 2020). Fruit color is a desirable character for commercial acceptance of a variety. Fruit

color of our accessions varied from white, red, purple to black. The percentage of juice yields were within the limits of Yilmaz *et al.* (2012) study (between 39% and 72%). All black mulberries had the highest fruit juice yield ratio, the highest acidity values and the lowest sugar content. This is consistent with previous researches (Ozdemir and Topuz, 1998; Gunes and Cekic, 2004; Aljane and Sdiri, 2016). Therefore, black mulberries are preferred for processing into juice. Black colored mulberry species received recently a great importance due to higher contents of phenolic compounds and to their delicious taste (Aljane and Sdiri, 2016).

The dendrogram constructed on the base of the most discriminant morphological characters divided the accessions into 6 distinct groups. The evaluation of the relationship among accessions reduced their differentiation to fruit color and taste. A non-negligible variability of other traits influenced the grouping involving the length of the fruit, leaf and peduncle. The accessions grouping was marginally correlated to the accessions vernacular names with many exceptions. The dendrogram revealed that accessions within each cluster belonged to different regions suggesting that there was no clear relationship between accessions and geographical diversity. This is the case of the group G4 that included 'Shami' accessions growing in North Lebanon (Kfarchakhna) and in South Lebanon (Sour). Such results have been reported in different crops by several studies, e.g. on chestnut (Marinoni *et al.*, 2013), almond (Chalak *et al.*, 2007; Halasz *et al.*, 2019) and olives (Chehade *et al.*, 2015). This variability could be attributed to the free exchange of planting material between different Lebanese villages and emphasizes the adaptability of mulberry to different ecological conditions.

In this study, we evaluated the genetic diversity and the relationships among the collected mulberry accessions using SSR and ISSR markers. The results showed high polymorphism in all the amplified loci. The power of discrimination values was high showing that the studied loci are of high diversity. The observed SSR markers heterozygosity were high. Earlier studies using amplified fragment length polymorphism (Sharma *et al.*, 2000), ISSR (Awasthi *et al.*, 2004), and RAPD (Xiang *et al.*, 1995; Feng *et al.*, 1996; Zhao and Pan, 2000; Esha and Shirish, 2001) also showed a large genetic variation among different mulberry genotypes. Such a high level of polymorphism reflects the outcrossing nature of the species. In this work, the ISSR profiles generated by

(AC) and (GA) repeat anchored primers showed that these repeats are abundant in our accessions. Vijayan and Chatterjee (2003) observed amplification of (AC) rich repeat based ISSR primers. Awasthi *et al.* (2004) concluded that (CA)/(TG) repeats are abundant in *Morus* genome.

Cluster analysis of SSR and ISSR data using UPGMA revealed high genetic distances between the studied accessions. Five groups were constructed by one single accession. The other clusters regrouped accessions of 'Mwashah', 'Shami' and 'Abyad' within each group. The distanced genetic relationships among mulberry accessions are in consistence with their high heterozygosity due to their outbreeding reproductive system (Dandin, 1998). Accessions from different varieties and from different sites were grouped together. The molecular results emphasized that genetic diversity among mulberry accessions is not influenced by their geographical origin nor by their local names. This finding is in agreement with other researchers who studied genetic diversity using SSR markers on different crops, almonds (Distefano *et al.*, 2013), mung bean (Wang *et al.*, 2018) and torch Ginger (Ismail *et al.*, 2019). The analysis of the genetic parameters showed the high diversity of mulberry in Lebanon.

The comparison between morphological and molecular diversity indicated that morphological descriptors provide different information than the molecular one. In comparison with other works in woody species there were also no correlation. For example, two 'Shami' accessions (code B24 and HAS92) were in the same group in the morphological dendrogram however they belong to different groups in the molecular one. One 'Aswad' (black mulberry, code B6) accession and another 'Abyad' (white mulberry, code B7) accession were in the same group in the molecular dendrogram but they were not in the morphological one. It is probably that our markers sampled mainly a non-adaptive diversity.

The results of this study revealed a large morphological diversity and a high genetic variation among the Lebanese mulberry accessions. The combination of SSR and ISSR primers was informative for estimating the extent of mulberry genetic diversity. Morphological and molecular clusters have distinguished different lines of mulberry which may help in the selection of the most diverse profile. This germplasm would enhance the local gene pool and expand genetic variation for mulberry breeding program in the future.

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