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Molecular Assessment of Established Clonal Propagated Mulberry (*Morus nigra* L.)

Ismail A. Ismail (Corresponding Author)
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia
Email: i.aismail73@gmail.com

El Dessoky S. Dessoky
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

Attia O. Attia
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

Osama M. Saleh
Natural Products Research Department, National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Nasr City, Cairo, Egypt

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Abstract

Mulberry (*Morus* Sp.) is one of the economically important trees cultivated for the tasteful fruits, its several pharmaceutically important chemicals potential, timber, cosmetic and in silk industry for its foliage, also in various molecular breeding applications.. The origins of most cultivated mulberry varieties are believed to be in the area of China-Japan and in the Himalaya foothills. and it now has a very wide distribution range in Asia-Europe (from Korea to Spain, including China, India, Central Asia and Near East); in Africa (North and East Africa) and in America (from the U.S.A. to Argentina, including Mexico, Central America, Colombia and Brazil). In Saudi Arabia, Mulberry grown well and spread in different places such as Taif Province (El Shafa region), Eastern region; Al-Ehsaa Province and some southern areas. Nodal explants of *Morus nigra* were clonally propagated *in vitro* for plant regeneration. Auxiliary shoot buds have been promoted in Murashige and Scoog (MS) media in a variety of cultural contexts. The largest number of shoots (13.00 ± 0.47) with an average length of 2.00 ± 0.47 cm were initially obtained from a medium containing 2.0 mg / L N6-benzyladin (BA) and 3% sucrose. Recurrent subcultures provided the highest number of seedlings (approximately 29.30) for excavation after the fourth passage. Seedlings were rooted in 1/2 MS medium supplemented with 1.0 mg / 1 indole-3-butyric acid (IBA). Successfully, about 90% of the plantlets acclimatized. Along with determination of the genetic variations between three mulberry genotypes including two cultivated accessions (*Morus alba*) and one wild genotype (*M. nigra*) utilizing inter-simple sequence repeat (ISSR) markers. Genetic variation and phylogenetic relationship of mulberry germplasm collection have been studied. All ISSR markers used in this study revealed higher genetic diversity was in the wild species comparing with cultivated species. ISSR matrices reported that the mean genetic similarity coefficient was 0.7677 for all mulberry genotypes. Although some differences have been observed, much similarity has been obtained in dendrogram topology. Cluster analysis of the ISSR using UPGMA software revealed that wild species were genetically distinct. The correlation coefficients of similarity for the ISSR used are statistically important. The Principal Coordinate Analysis (PCA) for ISSR data also supports its UPGMA clustering. The average number of genetic variations recorded in mulberry genotypes was 0.287±0.096. Dendrogram (Un-weighted peer group method analysis) classifies mulberry accessions into two main groups; Admissions collected from western area of Taif, and the other comprised two sub-clusters including one isolate, i.e., *M nigra*, a collection from Al shafa. Contains access to another sub-cluster southwest regions of Taif, which belong to *Morus nigra* wild growing. These accessions of mulberry were found to be genetically similar from north and southwest Taif Province. These results have significant implications for improving the mulberry germ plasma characterization, conservation and investigates the genetic diversity among the mulberry species grown in Taif governorate and to establish a micro-propagation system as germplasm conservation to preserve the assets of local mulberry and thus develops an easy and effective method to identify native genotypes in a limited space and time frame.

Keywords: *Morus nigra; in vitro* propagation; Genetic distances; ISSR markers.

1. Introduction

Mulberry belongs to the genus Moras, which belongs to the genus Morse. Mulberry (*Morus* sp.) is commonly used to obtain silk for leaf feeding for silkworms. But the fruits of this species have an amazing ability to provide a variety of valuable industrial products. It is used in the food and pharmaceutical industry and opens new avenues for the industrial exploitation of mulberry fruits around the world. More than 150 species of mulberry have been reported, although their identity is still the subject of great debate. Conservation of all genetic resources, including cultivated and wild relatives, is essential. Three species are best known: Morus alba, rubra, and nigra, as widely distributed in India, China, Japan, North Africa, Arabia, and southern Europe, among other regions [1, 2]. In Saudi

Arabia (Morus nigra L.) is commonly found in the wild and grows in uncultivated places. Traditionally, mulberry is propagated by cuttings and seeds. Mulberry growers often find it difficult to root the cuttings. Propagation by seeds is undesirable due to the huge diversity of plants due to cross-pollination. Tissue culture techniques such as micro-amplification provide a fast and reliable method to produce many identical plants in a short period of time. In vitro production of plants from auxiliary buds by different workers has been reported in different species of morass [3-7]. Biotechnological applications have great promise in further improving the mulberry crop, conventional research has not been as successful as hoped. Biotechnological research in genome characterization with isozymes and DNA markers, micro-propagation, in vitro preservation technologies such as reproduction from callus, somatic hybridization, slow storage and cryopreservation, and genetic mutations [8]. Native genotypes found under similar agro-climatic conditions provide an advantage that can be used much easier for breeding purposes than in remote areas. Therefore, valuable genetic resources of existing indigenous genotypes must be preserved for their proper use. To obtain this, it is essential to assess the genetic variation between native genotypes. [9]. Studies on the mulberry gene were first started in Japan [10], where chloroplast DNA successfully isolated from mulberry. Various molecular symbols, such as direct amplification of mini-satellite DNA (DAMD) [11]. Randomly Amplified Polymorphic DNA (RAPD) [12], Inter Simple Sequence Repeats (ISSRs) [9], and Amplified Fragment Length Polymorphism (AFLP) [13] used to study the genetic variation of mulberry varieties. Improving yield and fruit quality through breeding relationships between genetic variants and selected mulberry genotypes (Moras sp.) have been evaluated. The main objectives of our study are developing an improved protocol for large scale production in vitro multiplication system of black mulberry (Morus nigra L.) as a first step for mulberry germplasm conservation, and genetically characterization of some mulberry (Morus sp.) genotypes which growing in Taif province, KSA using DNA based markers.

2. Materials and Methods

2.1. Plant Materials

Nodal explants (including black mulberry (*Moras nigra* L.) auxiliary buds and shoot tips collected from juvenile branches) are varieties of trees grown in El Shafa regions (21°05'04.2"N 40°21'45.6"E) in Taif Governorate, Saudi Arabia.

2.2. Micro-propagation

1-2 cm long pieces of explants have been surface-sterilized and placed vertically in MS medium is associated with different concentrations and combinations of auxin and cytokinin. MS media without growth regulators is used as a control treatment for all experiments.

2.2.1.Shoot Initiation Stage

Axillary buds and shoot tips were cultured in MS medium supplemented with vitamins for four weeks.

2.2.2. Shoot Multiplication and Elongation Stages

For shoot multiplication and elongation, induced subcultures were displayed in full strength MS medium for another 4 weeks with combinations of different cytokinins concentrations.

2.2.3. Rooting and Acclimatization Stages

The elongated branches (3 to 4 cm long) were transferred to half-MS medium to grow to different densities and roots and replaced with auxin compounds for 4 weeks. Then, the rooted plants are planted in clean peat moss plastic pots for adaptation.

2.3. DNA Isolation

The whole genetic DNA was isolated from the leaf tissue using the CTAB method with some modifications [14].

2.3.1. Inter - Simple Sequence Repeats (ISSRs) Analysis

For ISSR-PCR analysis, a total of ten primers were used to select the best amplification results with DNA samples.

The amplification products were separated by electrophoresis on 1.5% agarose gel mixed with ethidium bromide and the bands were visualized and documented in a UV gel doc. System.

2.4. Data Analysis for ISSR

DNA polymorphisms of the ISSR were analyzed. Only stable and clear bands in replicas are considered potential polymorphic markers. Data obtained by scoring the presence (1) or absence (0) of amplified fragments from ISSR analysis. The similarity coefficient (F) and dendrogram formula were reported using the unpaired pair method and the arithmetic mean (UPGMA) [15].

2.5. Statistical Analysis

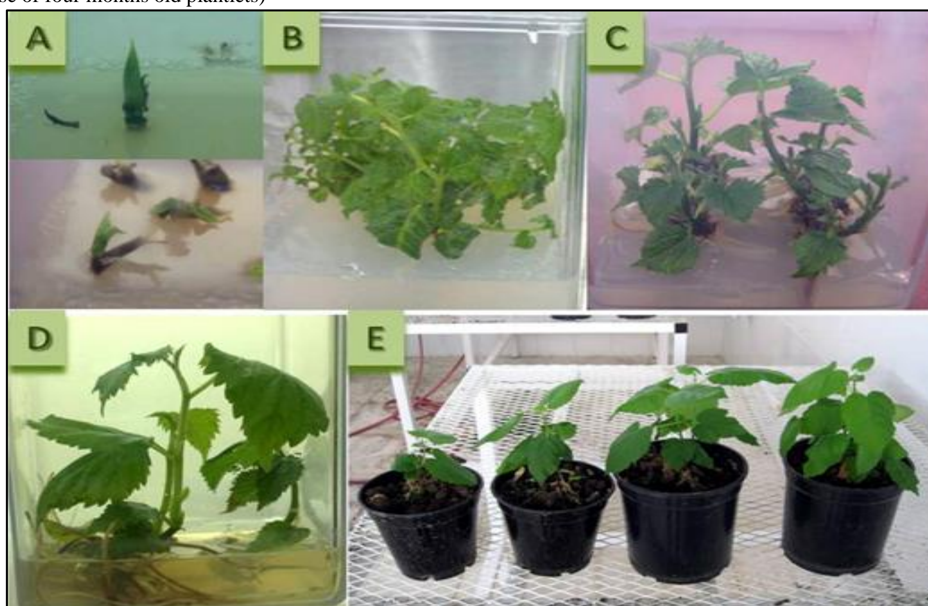
A completely random design was used for the *in vitro* campaign of all experiments. Analysis and comparison of differences between treatment mean values for the *in vitro* diffusion experiment was performed using the Duncan test at a 5% probability (0.01 = <p <0.05) level using the Statistical Support Software (ASISSAT) version 7.6 beta vas [16].

3. Results and Discussion

Two to three axillary buds' nodal segments and epical buds from juvenile branches of *Moras nigra* L. have been grown in MS medium containing the most effective concentrations of BA (0.5 and 2.0 mg / L) and 2 i.p. (0.5 and 1.0 mg / L) (Table 1).

Associated with 1 mg / L IP and 2 mg / L BA in 2 MS basal media. The shoot buds sprouted in a maximum rate in the cultures after two passages of inoculation, the current research of *M. nigra* demonstrates the possibility of large-scale mulberry promotion through nodal and shoot tip cultures. Auxiliary buds or shoot tip cultures are preferred according to ease of meristem development to shoots while maintenance of clonal fidelity. Light to moderate nodal transplant of medium thickness (0.5 - 0.6 cm) responds more favorably to bud germination and differentiation with emerging green axillary buds. 35-80% of nodules and 20-70% of shoot tip details on MS associated with various plant growth regulators have a seedling survival percentage and their subsequent development (Table 1).

Figure-1. Mulberry (M. nigra) micro-propagation stages (A: Shoot initiation, B: multiplication, C: elongation, D: rooting and E: acclimatization stage in greenhouse of four months old plantlets)



Two weeks after vaccination the shoot buds spread to several shoots in the same medium. These sprouts were transferred to a new medium of the same composition and maintained for four weeks. When vaccinated against MS basal medium fortified with BA (2.0 mg / l) in combination with (1.0 mg / l) 2 i.p., these descriptions showed the appearance of shoot buds in cultures six days after vaccination showed maximum response.

Table-1. Effect of different concentrations and combinations of two cytokinin's (2 ip & BA) on shoot differentiation, multiplication, and average shoot length of (*Morus nigra*)

Cytokinins conc. (mg/l)		**Number of Explants /treatments**	**Mean of No. Explants differentiation**	**Mean of No. of multiple shoot**	**Mean of Average shoot length (cm)**
2ip	**BA**				
0.0	**0.0**	60	$00^{i}\pm00$	00±000	$00^{e}\pm00$
0.5	**0.5**	60	$13^{f}\pm1.55$	$1.0^{g}\pm1.00$	$1.0^{d}\pm1.32$
	1.0	60	$17^{e}\pm1.62$	$1.3^{f}\pm1.11$	$1.3^{d}\pm1.38$
	1.5	60	$22^{d}\pm1.70$	$2.6^{e}\pm1.17$	$1.6^{cd}\pm1.42$
	2.0	60	$37^{c}\pm1.74$	$9.0^{b}\pm1.47$	$2.0^{cd}\pm1.42$
1.0	**0.5**	60	$23^{d}\pm1.78$	$3.0^{d}\pm1.29$	$2.0^{cd}\pm1.45$
	1.0	60	$36^{c}\pm1.80$	$5.0^{c}\pm1.41$	$3.0^{bc}\pm1.47$
	1.5	60	$41^{b}\pm1.82$	$8.0^{b}\pm1.53$	$4.0^{ab}\pm1.48$
	2.0	**60**	$\mathbf{55^{a}\pm1.83}$	$\mathbf{13^{a}\pm2.33}$	$\mathbf{5.0^{a}\pm1.49}$

Means with the same letter are not significantly different according to Duncan Test at a level of 5% of probability (.01=<p<.05).

Similar results were obtained by Zafar, *et al.* [17] From the nodal researchers of *Moras levigata*. The production of shoots from nodal sections was also established [18]. But they achieved shoe multiplication by growing plants in a fortified MS medium with glutamine (1 mg / l) as well as BA (2.5 mg / l). This medium facilitated the germination of nodal segments from shoots elongated and increased micro-cuttings *in vitro*.

These buds generated into several shoots after 2 weeks of inoculation. Lobed and un-lobed leaves like naturally mother plants were produced from micro shoots. After 15 days of inoculation, the shoots had been established grown with light green color. Explants were cultured on basal MS medium supplemented with BA (0.5-1.0 mg/l) + NAA (1.0- 2.0 mg/l) to develop complete plantlets. After 6-8 days of inoculation, explants appeared 70-80% of morphogenetic potential for shoot and root differentiation. After four weeks, enhanced growth along with the production of roots. In media containing BAP + 2IP in combination with 0.5 / 0.5 mg / l, the frequency of shoot buds was lower and a slight calling from the lower cut edge of the specification was also observed (Fig. 1). Consistent with these results, Kn is less effective than BAP for both shoot tip and nodal exfoliation shoot induction for three different types of mulberries, these results agreed with [6, 7, 19]**.**

Table-2. Effect of different concentrations and combinations of two auxins (IBA & NAA) on Rooting differentiation, number of roots/ explant and length of roots of Mulberry (Morus nigra L.)

Auxin conc. (mg/ l)		**No. shoot tips/treatment**	**Mean of No. of Rooting differentiation**	**Mean of Rooting %**	**Mean of No. of roots/ explant**	**Mean of length of roots (cm)**
IBA	**NAA**					
00	**00**	30	$00^{e}\pm000$	$00^{e}\pm000$	$00^{d}\pm000$	$00^{e}\pm000$
0.5	**0.5**	30	$2.1\pm^{1.22}$	$17^{d}\pm7.60$	$1.6^{c}\pm1.34$	$1.7^{d}\pm0.55$
1.0		30	$3.9^{d}\pm^{1.87}$	$24^{d}\pm8.08$	$1.8^{c}\pm1.51$	$3.0^{bc}\pm0.61$
1.5		30	$9.0\pm^{2.33}$	$33^{c}\pm8.27$	$3.0^{b}\pm1.69$	$3.9^{c}\pm0.68$
2.0		30	$15^{b}\pm^{2.78}$	$87^{b}\pm8.65$	$5.0^{b}\pm1.87$	$4.7^{ab}\pm0.70$
2.5		**30**	$\mathbf{19^{a}\pm^{3.11}}$	$\mathbf{98^{a}\pm8.9}$	$\mathbf{8.0^{a}\pm1.99}$	$\mathbf{5.8^{a}\pm0.77}$

Means with the same letter are not significantly different according to Duncan Test at a level of 5% of probability (.01=<p<.05).

The long multiple branches (2 - 3 cm) have been cut and transferred to rooting media (Table 2). MS medium supplemented with 0.5 mg/l NAA in combination with 2.5 mg/l IBA was optimal for root development for 3 weeks (Fig. 1). NAA was an effective rooting agent for *M. alba* as reported by Anuradha and Pullaiah [20]**.**

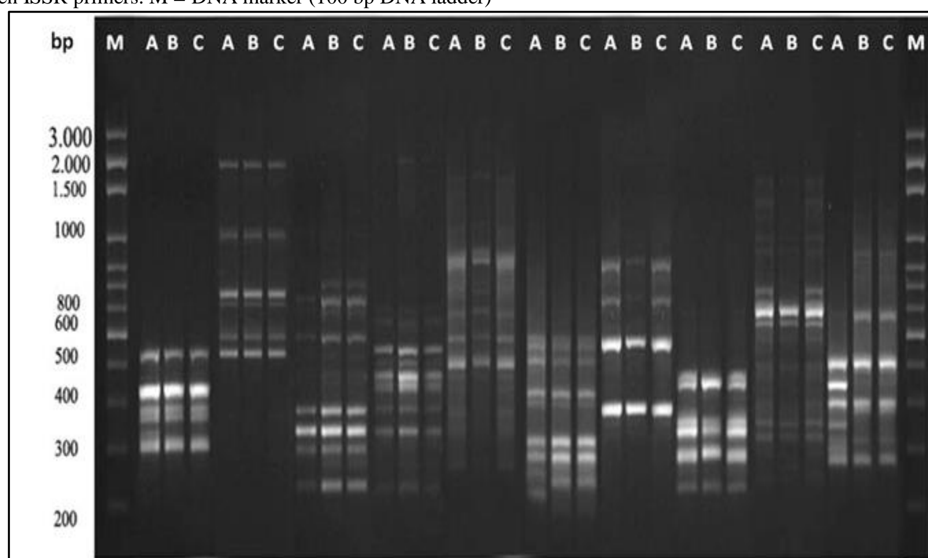
In small plastic pots, the well-developed shoot-roots plantlets with 90% survival rate (Fig. 1) transferred to a mixture of sand: soil: organic manure (2: 2: 1). The technique described here provides a good method for the rapid propagation of this crop on a commercial scale as well as for the inclusion of bouquets from economically important plant species and cultured auxiliary buds.

3.1. Mulberry Genetic Diversity

Various molecular markers have very important role in plant breeding programs and genetic conservation through detection of genetic distances of genotypes or plant germplasms [21]. From these molecular markers, RAPD [22, 23] and ISSR [22, 24-30] are popular tools for identification of differences between plant accessions. In our study, ISSR technique used to identify genetic variation in 30 plant specimens of three mulberry genotypes two (*M. alba* and *M. nigra*) from common cultivated in Taif region in KSA, and one wild growing (*M. nigra*). Fifteen primers produced bright amplification products and polymorphisms from 22 previously demonstrated ISSR primers and were selected for further analysis. A total of 138 reliable fragments were obtained (Figure 2). Of these, 126 bands are polymorphic (91.3%), giving a score of more than 9.2 per primer.

Average number of allele variants observed in one locus (1.93). Of the mulberry varieties, 15 ISSR primers produced 104 bands, of which 84 bands (80.7%) were polymorphic. Similarly, among wild species, 15 ISSR primers spread over 104 bands, of which 87 (83.7%) were polymorphic, indicating that wild species samples contained more polymorphisms than cultivated species. Here is the PIC value of each ISSR primer averaging (0.0966 to 0.3049) (0.2006). Among the 126 polymorphic bands, we found some specific bands that are used for mulberry mixing and differentiation. 29 fragments, 23.01% polymorphic fragments, putative accessibility-specific markers (data not shown). Considered exclusively for fourteen species (data not shown).

Figure-2. DNA polymorphisms amplified from the DNA extracts of three mulberry genotypes [M. alba (A), cultivated M. nigra (B) and wild M. nigra (C)] using ten ISSR primers. M = DNA marker (100 bp DNA ladder)



246 fragments ranging from 6–12 bands per primer for each cultivar have been produced from ten ISSR primers (Table 3). The size of the expanded products ranged from 314.33 to 2299.9 bp and the percentage of total polymorphic markers and polymorphisms was 18 and 6.7%, respectively. Primer CGC(GATA)4 amplified maximum number of polymorphic unique bands (3). The PIC values, the reflection image of the allelomorphic variation, and the frequency between types, are not the same for all ISSR locations tested. The minimum similarity between the two native genotypes *M. alba* and wild-growing *M. nigra* (70.2) and the maximum similarity to *M. nigra* (89) and the result of the wild-growing *M. nigra* cultivated in the genotype were shown. The average similarity index is 87.9.

Data obtained from ISSR analysis is subject to UPGMA analysis. The co-phenetic correlation coefficient (87.9) shows a slight distortion between the actual similarity values from the matrix and the values used to construct the dendrogram. Cluster analysis was performed based on a consistent matrix of jacquard similarity calculated from ISSR markers. With 91% similarity, the two genotypes were classified as One group, the rest of the cultivators were put into separate groups.

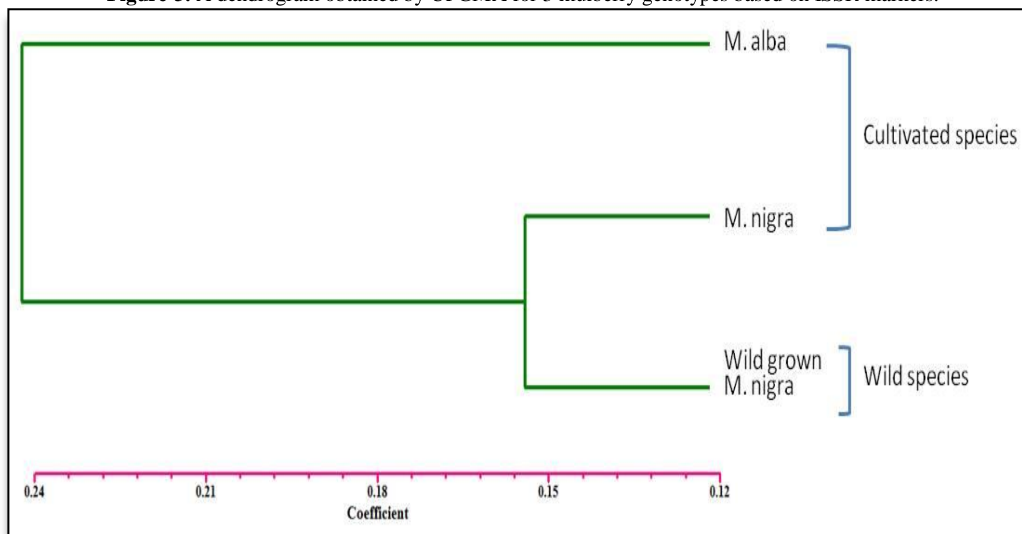
Table-3. Unique bands for each Mulberry cultivar with each ISSR primer.

No.	ISSR primer	Sequence	*M. alba*		*M. nigra* (cultivated)		*M. nigra* (wild grown)		*TUPMs for all cvs.
			+ m	-m	+ m	-m	+ m	-m	
1	ISSR- 03	5'-ACACACACACACACACYT-3'	1	0	0	0	0	0	**1**
2	ISSR- 05	5'- GTGTGTGTGTGTGTGTYG -3'	0	0	0	0	0	-1	**0**
3	ISSR- 06	5'- CGCGATAGATAGATAGAT -3'	1	1-	0	0	0	-2	**1**
4	ISSR- 09	5'- GATAGATAGATAGATAGC -3'	-1	0	0	0	0	0	**0**
5	ISSR- 10	5'- GACAGACAGACAGACAAT -3'	1	-1	0	0	0	0	**1**
6	ISSR- 11	5'- ACACACACACACACACYA -3'	0	0	2	0	2	0	**4**
7	ISSR- 12	5'- ACACACACACACACACYC -3'	0	0	0	0	1	0	**1**
8	ISSR- 15	5'- CTCTCTCTCTCTCTCTRG -3'	1	0	0	0	0	-1	**1**
9	ISSR- 16	5'- TCTCTCTCTCTCTCTCA -3'	0	0	0	0	1	0	**0**
10	ISSR- 19	5'- HVHTCCTCCTCCTCCTCC -3'	0	0	0	0	0	0	**0**

*(TUPMs) Total unique positive markers.

Furthermore, the four distinct bands produced by primers (GATA) 4GC at 530 bp, (GT) 8YG at 1910 bp, (GAG) 4GC 2500 bp and 990 bp were clearly distinguished between mulberry cultivars and wild species are separated (Table 3). The similarity coefficient ranged from 0.6014 to 0.9493 with an average of 0.7677, revealing a high level of genetic variation in the mulberry entries studied and it was possible to discriminate among all the mulberry entries analysed. The highest genetic similarity coefficient (0.9284) was found between cultivated *M. nigra* and wild *M. nigra*, indicating that they had almost identical genetic components. The lowest genetic similarity coefficient (0.5113) was found between *M. alba* and wild *M. nigra*, indicating that they are relatively far apart.

Figure-3. A dendrogram obtained by UPGMA for 3 mulberry genotypes based on ISSR markers.



Not much progress has been made in understanding the breeding behavior of mulberry. These interspecific hybridizations among, M. nigra, and M. sinensis revealed varied results as in some crosses fertility was very high, whereas in some others it was very poor, Tikader and Dandin [31], despite the agreement of those species in the conditions of the habitats in which they grow and compete, but due to the common presence of Morus nigra in the wild and without influential density, small populations, in addition to the weak ability to overlap between different species in those environments, at the same time, molecular tools for identifying the new types and their relationship have developed, so it is possible to identify any hybrid offspring that may be emerging in those different environments..

4. Conclusion

The ISSR profile was used to determine the genetic similarity standard, which was used to construct the dendrogram by the UPGMA method. The first and second major coordinates accounted for 52.5% and 10.0% of the total variation, respectively. Among the cultivars cultivated in the first group, *M. alba* belongs to the genus *Macromorse* in the morphological classification of the genus *Morus* and differs independently from other mulberry species, cultivated *M. nigra* and wild *M. nigra* are grouped together. Close correlation with 0.9348 genetic similarity coefficient. Dendrograms obtained with ISSR markers (Figure 3) show similar topology with ISSR markers from other authors, although there are some differences in the placement of some genotypes. From PCA analysis, as in other marker methods, cultured mulberry genotypes are not fully integrated, possibly since different molecular markers are formed due to differences.

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