

Article

Morphological and Molecular Characterization of Five Cultivars of *Ziziphus* spp. Cultivated in Southern Iraq

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ABSTRACT

Ziziphus spp. is one of the economic fruit trees whose cultivation spreads in southern Iraq. This study characterized the morphological, molecular, and genetic diversity of five cultivars (Malasy, Normal, Zaytony, Bambawi, Tofahy), using 12 morphological indicators for leaf and fruit and six primers for RAPD technology. The results of the morphological characterization showed that the two cultivars (Normal and Malasy) recorded the highest genetic kinship between them, amounting to 10.04. In comparison, the farthest genetic distance between the two cultivars (Zaytony and Normal) was recorded, which amounted to 29.3. As for the molecular characterization results, the primers produced 113 20 bands, with an average of 18.8 bands for each primer, and the percentage of polymorphism was 100% and unique bands totaling 51 bands appeared, including 17 bands with different molecular weights characteristic of the cultivars. The genetic tree diagram showed the distribution of cultivars in two groups. The combination of morphological and molecular characterization is one of the most important tools that can be relied upon in finding genetic differences and determining fingerprints, especially between types of genders or cultivars of the same species.

Keywords: fingerprints, genetic distance, Morphological, RAPD, *Ziziphus*.

INTRODUCTION

Sidr (*Ziziphus mauritiana* Lam. and *Ziziphus jujube*.) is a perennial evergreen tree and shrub. Its cultivation is spread in tropical, semi-tropical and temperate regions. It belongs to the family Rhamnaceae, and the genus is one of the most prominent genera of the family, as it includes about 170 species¹. Sidr trees are considered multi-purpose, as their fruits are eaten fresh due to their high content of vitamins, especially ascorbic acid, carbohydrates, fiber and minerals. It is rich in protein compared to apples and ripe fruits. Sidr trees are used as windbreaks or fuel, in addition to the medicinal benefits of their leaves and bark^{2,3}.

Morphological markers are the basis that researchers have followed since ancient times in the study of plant classification, and it is one of the methods used to study the genetic relationships between cultivars. These markers to characterize

the new cultivars of each plant depend on a set of traits that can distinguish the cultivars from each other ⁴. With the progress of molecular biology, many molecular markers have emerged that have been used in many fields, especially the study of genetic diversity, finding genetic fingerprints for plants, genetic mapping, finding genetic affinity, and in plant breeding programs ⁵. The flowering period in *Ziziphus* (up to 7 years) and during this period, the plant needs a lot of agricultural and economic service operations. DNA tags are a handy tool for cultivar identification during breeding and growth ⁶. Genetic diversity in the genus *Ziziphus* was assessed using different systems of DNA molecular markers, such as RAPD SRAP ⁷ and ISSR ⁸.

Molecular analysis was used to study the genetic diversity of three cultivars of *Ziziphus spp.* in Egypt. Three primers were used for RAPD, and ten primers for Sequence-related amplified polymorphism (SRAP). The results showed the emergence of 75 bands by RAPD primers, of which 32 (42.67%) were polymorphic and 43 (57.33%) were monomorphic. Also, 21 unique bands specific to the cultivar appeared, and 11 standard and unique bands were obtained between each of the two cultivars studied ⁹.

A study recorded a new species in Nepal based on finding differences in leaf shape, dimensions, tree shape and size and comparing it with other species ¹⁰. A study to assess the genetic diversity of five different ecotypes of *Ziziphus jujuba* Mill based on morphological characteristics in Isfahan Province, Iran, showed that the quantitative features were more important in the ecological patterns, such as the dimensions of the leaves and the weight, length and diameter of the fruit, the results also showed a significant negative and positive correlation between some traits ¹¹.

Morphological and molecular characterization was carried out for ten cultivars of Sidr trees (*Ziziphus nummularia* Burm. F.) grown in different regions of India. A large discrepancy was observed between the cultivars by analyzing five quantitative traits and 25 qualitative morphological traits for leaves, fruits and seeds. ISSR markers also showed polymorphism (86.58%), the genetic similarity value was in the range of 0.45-0.77, indicating a moderate level of genetic diversity among the cultivars, and two of the eleven ISSR primers were also able to generate unique cultivar-specific bands, which can be used to determine the genetic fingerprint of the cultivars Zadber-5 and Zadber-8. This revealed that morphological and molecular markers could be used successfully to determine the genetic diversity and relationship of Sidr cultivars and use them in breeding programs ¹².

A comparison of morphological and molecular traits was made to characterize three species of *Ziziphus* in Iran using a combination of morphological and molecular characteristics. Both morphological and molecular data revealed high internal qualitative differences between the accessions. The percentage of formal polymorphism was 90.49%, and the average content of the information polymorphism was 0.37. At the same time, a significant correlation was recorded between morphological and molecular markers, suggesting this could serve as a valuable tool in optimization programs ¹³.

Given the economic importance of the studied cultivars, the study was conducted to define the fingerprint, genetic variance, and a statement of genetic relationships and morphology between them. To benefit from them in preparing education and improvement programs for cultivars spreading in the south of the country.

MATERIALS AND METHODS

The study was carried out on five cultivars of Sidr (*Ziziphus mauritiana* Lam. and *Ziziphus jujube*.) trees (Malasy, Normal, Zaytony, Bambawi Tofahy) planted in different regions of southern Iraq. The study included morphological characterization using 12 leaf and fruit characteristics, which included (leaf length, leaf width, leaf holder length, leaf area, fruit weight, fruit length, fruit width, fruit size, fruit pulp weight, seed weight, seed length, and diameter seed). The molecular study included molecular characterization using six primers of the Random Amplified Polymorphic DNA (RAPD) technique. The molecular study was conducted in the Biotechnology Laboratory at the Department of Horticulture and Landscaping - College of Agriculture - University of Basra. Samples were collected from young leaves, sterilized and preserved until DNA extraction.

DNA was extracted according to the method of ¹⁴, and DNA concentration and purity were estimated using a Nano Drop ND-2000 Spectrophotometer (THERMO SCIENTIFIC, USA) at a wavelength of 260 nm, according to the ratio A260/A280. The polymerase chain reaction (RAPD-PCR) was carried out as follows: the initial denaturation phase of the DNA strand was one cycle at 94°C for a period of 7 minutes, followed by the replication phase was 40 cycles, each cycle included: 30 seconds for the template denatured at 94°C, then 60 seconds at 94°C. Heat (32) to bind the DNA template to the primers, then 2 minutes at a temperature of 72 to elongate the primers. Then the final elongation stage, one cycle at 72 °C, for 5 min.

Data analysis

The morphological characterization was carried out based on the studied traits, as the genetic distances were measured after the Eucliden disstandes matrix was created between the cultivars under study using the Eucliden coefficient. The genetic kinship tree was drawn by applying the unweighted pair group method with arithmetic average (UPGMA) to show the distribution of cultivars into clusters or clusters in the form of a dendrogram. A Principal coordinate analysis (PCA) was also carried out using Past software ver 3, ¹⁵.

As for the molecular characterization, they were first included in determining the molecular weights of the bands produced by the polymerase chain reaction (PCR) based on the DNA Ladder 100 bp volume guide produced by Promega company. Using Gel Analyzer 2010a Image Documentation Software. Then, the results of the bands obtained using the above techniques were recorded and converted into descriptive tables in Microsoft Excel by placing (1) when the band is present and setting (0) for its absence. The results were analyzed according to the pre-prepared tables using the biostatistical program Past software ver 3. The genetic dimension was analyzed using the Jaccard coefficient of genetic similarity between the cultivars under study.

RESULTS

Morphological characterization

The analysis results of the main components showed the presence of four factors that contributed to the total cumulative variance between the variables, and the first component had the highest contribution rate of 76.62% of the total variance Figure 1. At the same time, the second component contributed 17.65%, while, notably, other members had low contribution rates.

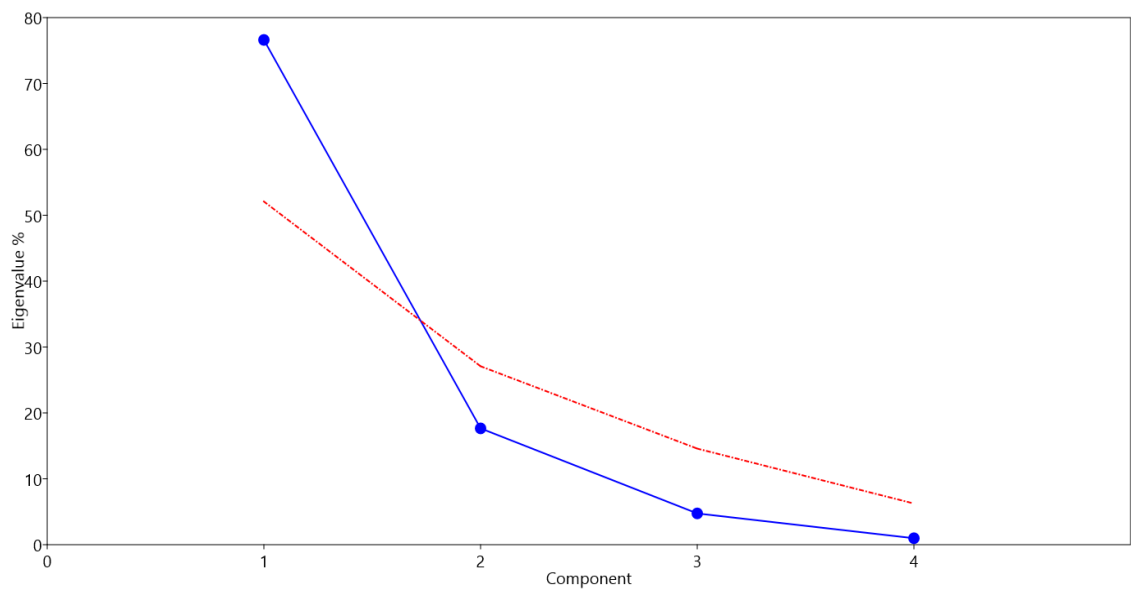


Figure 1. Diagram of imaginary roots for Sidr cultivars based on morphological characteristics.

Figure 2 shows the percentages of the contributions of the variables that represent the differences in the first component (which recorded the highest rate of contributions to the total variance) resulting from the phenotypic traits. It is noticed that the length of the fruit indicated the highest percentage of contributions in finding differences, followed by the characteristic of leaf area, while the rest of the attributes recorded varying rates of changes in the first component.

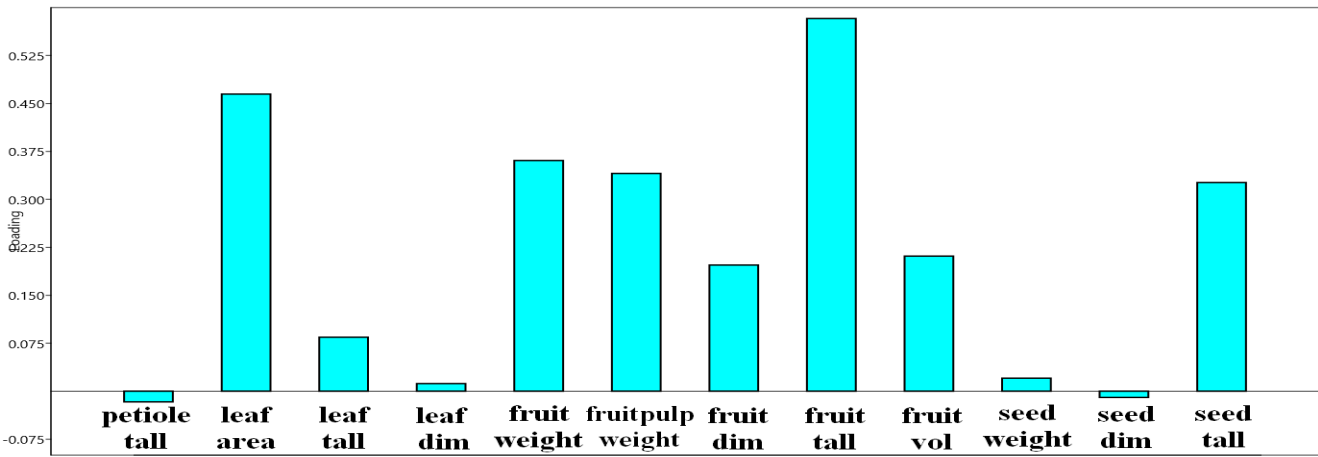


Figure 2. Variable Contribution Ratios in the variance of the essential components based on morphological characteristics.

As for the relationship between the varieties and the morphological features that participate in studying diversity among the cultivars, Figure 3 shows this relationship on the two orthogonal axes. It is noted from the figure that the length of the fruit and leaf area were among the most significant characteristics and greatly affected the association and distribution of varieties, Table 1. The determination of the genetic relationship indicated that these two characteristics result from the expression of genes of common origin. In addition, it had a major role in the spacing and convergence of genetic distances between cultivars.

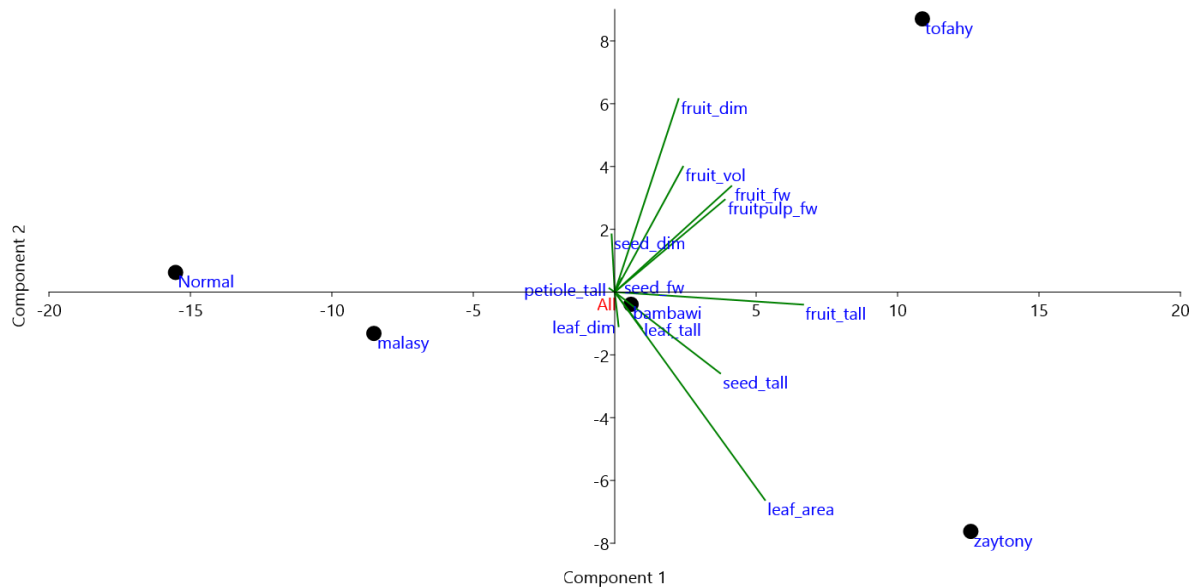


Figure 3. Principal Coordinates Analysis of the contribution of morphological characteristics to the association and distribution of cultivars

	<i>petiole tall</i>	<i>leaf area</i>	<i>leaf tall</i>	<i>leaf dim</i>	<i>fruit fw</i>	<i>fruit pulp fw</i>	<i>fruit dim</i>	<i>fruit tall</i>	<i>fruit vol</i>	<i>seed fw</i>	<i>seed dim</i>	<i>seed tall</i>
Malasy	1.4	18.09	4.42	3.7	5.03	4.81	22.08	19.62	5	0.22	6.67	10.29
Normal	1.71	10.11	4.34	3.8	3.42	3.17	18.2	17.12	4.55	0.25	9.81	10.89
Zaytony	1.1	29.12	7.3	4.75	11.09	10.62	20.35	33.19	7.5	0.47	7.39	20.88
Bambawi	1.5	17.15	6.5	5.21	7.51	6.78	22.13	28.41	7.55	0.73	8.71	16.74
Tofahy	1.28	19.22	5.45	3.19	15.18	14.13	29.26	31.53	12.8	1.05	9.68	16.27

Table 1. Morphological characteristics of the leaf, fruits and seed of the five-sidr cultivars.

The results of the cluster analysis Figure 4 showed that the cultivars were distributed in two groups (A, B). Group A included three cultivars, as the two cultivars (Normal and Malasy) combined into one cluster and recorded the highest genetic kinship between them, amounting to 10.04. In contrast, the Bambawi cultivar was isolated alone. Group B included the two cultivars (Tofahy and Zaytony). The farthest genetic distance measured between the two cultivars (Zaytony and Normal) was 29.38. As for the rest of the cultivars, different genetic dimensions were recorded in Table 2.

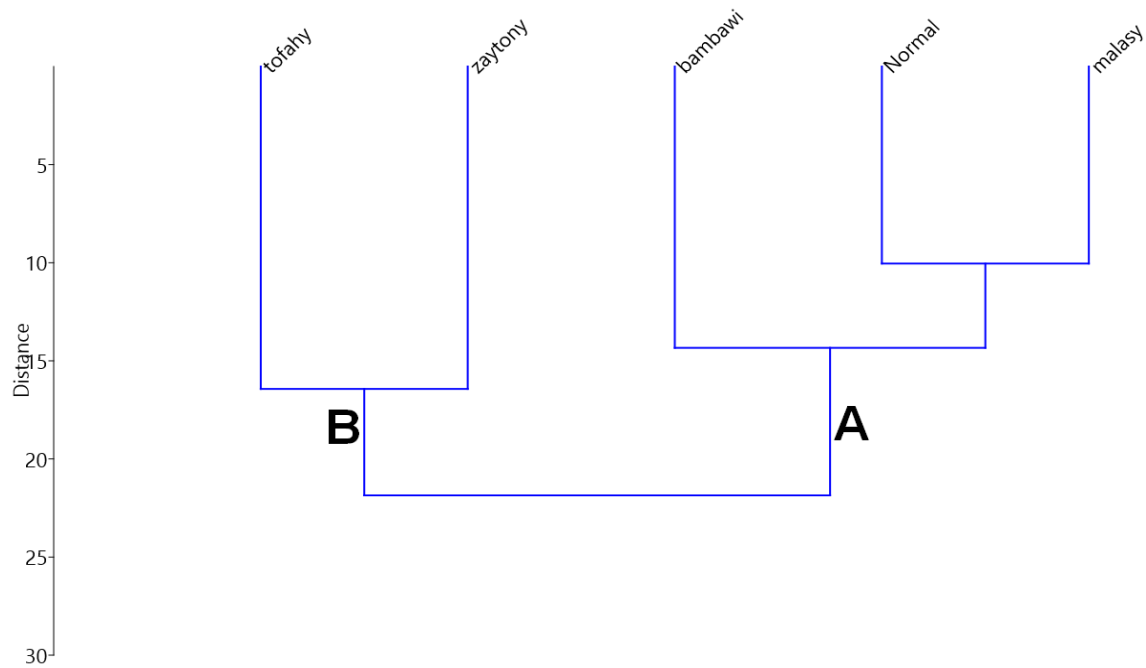


Figure 4. Dendrogram of Sidr varieties depending on morphological characteristics

	Malasy	Normal	Zaytony	Bambawi	Tofahy
<i>malasy</i>	0	10.04	22.53	12.14	22.19
<i>Normal</i>	10.04	0	29.38	16.54	27.76
<i>zaytony</i>	22.53	29.38	0	14.72	16.43
<i>bambawi</i>	12.14	16.54	14.72	0	14.55
<i>tofahy</i>	22.19	27.76	16.43	14.55	0

Table 2. Matrix of genetic dimensions of Sidr cultivars based on morphological characteristics.

Molecular characterization

The results of cultivar amplification by RAPD primers showed that they varied in the number of amplified bundles, as they produced 113 bands, with an average of 18.8 bands for each primer Table 3. The OPB-09 produced the most significant number of bands and amounted to 24 bands, while the OPB-11 gave less than the number of inflated bands, which amounted to 14 bands Figure 5. As for the number of polymorphic bands amounted to 76 bands, with an average of 12.6 bands for each primer. The OPA-09 produced the highest number of polymorphic bands, amounting to 16, while the OPB-07 gave 9 bands of polymorphism, the lowest number of bands generated by the primers.

<i>primers</i>	<i>Sequence (5'-3')</i>	<i>No total bands</i>	<i>Mono</i>	<i>Poly</i>	<i>Unique bands</i>	<i>Poly</i>	<i>Primer Efficiency%</i>	<i>Primer Discrimination power %</i>
			<i>morphism bands</i>	<i>morphism bands</i>		<i>morphism %</i>		
OPA-09	GGGTAACGCC	18	0	16	14	100	15.93	21.05
OPA-11	CAATCGCCGT	14	0	12	10	100	12.34	15.79
OPB-07	GGTGACGCAG	20	0	9	4	100	17.70	11.84
OPB-09	TGGGGGACTC	24	0	14	8	100	21.24	18.42
OPC-08	TGGACCGGTG	16	0	14	12	100	14.16	18.42
OPC-09	CTCACCGTCC	21	0	11	3	100	18.58	14.47
total		113	0	76	51			
Mean		18.8	0.0	12.6	8.5	100	16.66	16.66

Table 3. RAPD primers from the bands, their efficiency ratios and their discriminatory ability in Sidr cultivars.

The primers also produced unique bands totaling 51 bands and an average of 8.5 bands for each primer. OPA-09 gave 14 unique bands, while the OPC-09 gave only three unique bands. Some unique bands distinguished for the cultivar appeared at different molecular weights. It did not appear in the rest of the cultivars, which could be a genetic fingerprint for these cultivars that appeared in it. Also Table 3 shows that the six cultivars gave a high percentage of formal polymorphism in the cultivars amounting to 100%. As for the efficiency of the primers, the OPB-09 recorded the highest efficiency rate of 21.24%, while the lowest efficiency rate was 12.34% recorded by the primer OPA-11. As for the diagnostic ability of the primers only, the OPA-09 gave the highest diagnostic ability of 21.05%, while the OPB-07 gave the lowest discriminatory ability of 11.84%.

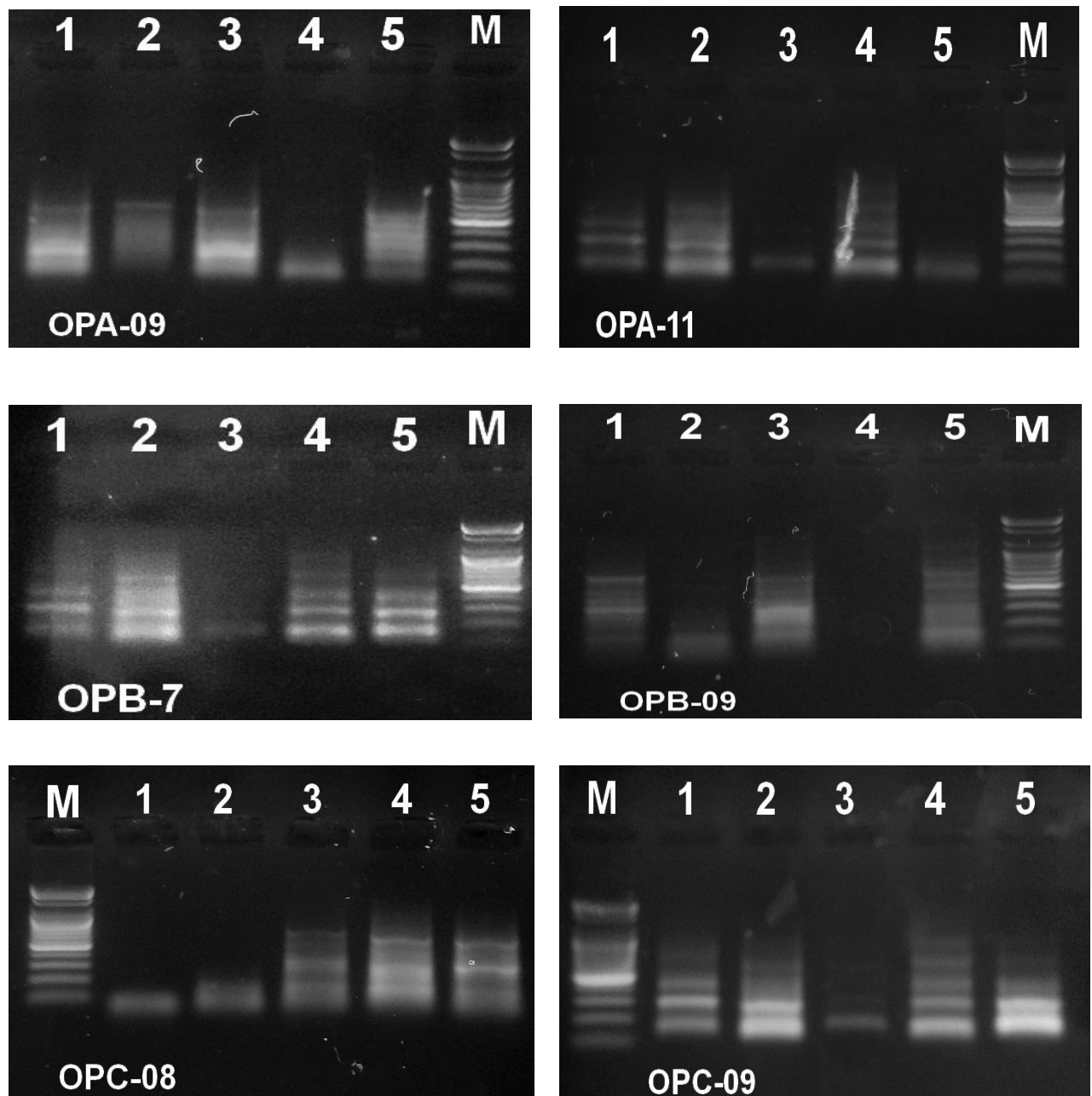


Figure 5. PCR amplification of RAPD six primers in five Sider cultivars on 2% agarose gel, M=100bp plus DNA ladder (1. Malasy, 2. Normal, 3. Zaytony, 4. Bambawi, 5. Tofahy)

The tree cluster analysis diagram for the cultivars, based on the results of the primers, showed that the cultivars were distributed in two main clusters Figure 6. The first cluster A included two cultivars (Tofahy and Bambawi). In contrast, the second cluster B, had the Normal cultivar, which was isolated and linked to a sub-cluster that included the two cultivars (Zaytony and Malasy).

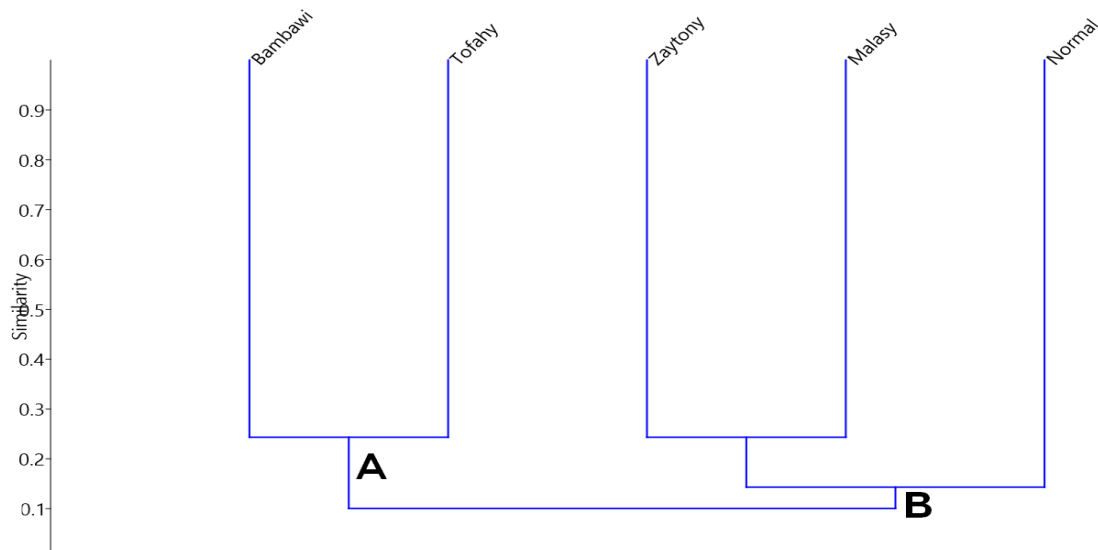


Figure 6. Tree cluster analysis diagram of Sidr cultivars depending on six RAPD primers.

Table 4 indicates the genetic similarity values between the cultivars, as the highest was between the two cultivars (Zaytony and Malasy) and amounted to 0.243. The lowest value of gene similarity was recorded between the two cultivars (Bambawi and Zaytony), and it was 0.049. The genetic similarity values for the rest of the cultivars ranged between the highest and the lowest, as the values were low, indicating the genetic diversity among the cultivars.

	<i>Malasy</i>	<i>Normal</i>	<i>Zaytony</i>	<i>Bambawi</i>	<i>Tofahy</i>
Malasy	1	0.158	0.243	0.075	0.065
Normal	0.158	1	0.128	0.206	0.068
Zaytony	0.243	0.128	1	0.049	0.140
Bambawi	0.075	0.206	0.049	1	0.243
Tofahy	0.065	0.068	0.140	0.243	1

Table 4. Matrix of genetic dimensions of Sidr cultivars based on six RAPD primers.

DISCUSSION

The results of the molecular characterization showed differences in the number of amplified bands and their molecular weights resulting from amplifying samples of cultivars according to the different primer used, resulting from a difference in the number of complementary sites for that primer or the absence of complementary sites for the primer in the genome of Sidr plant. As in ⁹ genetic diversity studies, using three RAPD primers gave 75 bands, of which 32

(42.67%) were polymorphic and 43 (57.33%) monomorphic. Also, 21 unique bands specific to the cultivar appeared.

Some leaf and fruit morphological traits were found to be cultivar-specific such as leaf area in combination with fruit length. Although all these traits may change with changing climatic conditions and other biotic and abiotic factors, they provide a reliable basis for cultivar identification in Sidr.

The phenotypic variance between cultivars that have a common genetic origin is affected by different factors. For example, inter-individual variance in the degree and location of DNA methylation, a genetic phenomenon widely observed in plants, produces new phenotypic variance that can be inherited steadily through generations¹⁶. The plant's environmental conditions may also affect the phenotypic variance, which leads to changes in the morphological features, which reveals an expression by the environment of new loci and phenotypes¹⁷.

Morphological assessment of current plant genetic resources is essential to managing, using and improving each plant species¹⁸. Wide phenotypic variance within and between *Ziziphus* species is revealed in the present work.

According to¹⁹, genotype and environmental status are among the major factors influencing plant characteristics and may lead to significant variation in fruit dimensions. Improving the weight and length of fruits is one of the most critical objectives of Sidr breeding programs²⁰. Therefore, the current cultivars are a rich source of selection and cross-breeding aimed at improving fruit characteristics in *Ziziphus* species.

The above results show that genetic factors specific to the species explain the discrepancy in the morphological characteristics of leaves and fruits. Also, the environmental impact can indirectly affect the phenotypic traits. It falls under limited levels subject to genetic signals represented by the gene expression processes of the phenotypic characteristics associated with these varieties²¹.

The variance between the leaf characteristics affected distinguishing between the varieties, as¹² studied 8 components of the phenotypic characteristics of Sidr, and the highest percentage of the first component was 96.74%. The results were also in agreement with²², which showed that the features of the fruit's length and diameter significantly impacted the variance between the studied cultivars.

CONCLUSION

Overall, the morphological and molecular analysis indicated that the genetic material under study presented some distinctive features, considering the different traits described in Sidr. However, varieties can be distinguished based on quantitative and qualitative characteristics. Their combination proved to be one of the essential tools to be relied upon in finding genetic differences and determining fingerprints, especially between genders or cultivars of the same species.

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Author Contributions

IMA and AYL conceived of the presented idea. AYL developed the theory and Performed molecular and morphological analyses. MRS and AYL verified the analytical methods. IMA encouraged me to investigate and supervise the findings

of this work. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within this article and its supplementary materials.

Ethics Approval

Not applicable to this paper

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