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Diversity analysis supported phenotypic traits and RAPD based markers in collections of Roselle

Omalsaad. S. Mohammed[1], Anwar E. Aboubakr[1]

Gazala M. Alhdad[1]

[1]Botany Department, Faculty of Science, Sirt University, Sirte Libya

E-mail address: omalsaad.83@su.edu.ly

الملخص

لتقييم التنوع الجيني بين اصناف الكركديه (Hibiscus sabdariffa) تم دراسة صفات مورفولوجية وجينية محددة لغرض تجميع الاصناف المختلفة في أنماط وراثية مميزة، من خلال إجراء مقارنة بين الطرق المورفولوجية والجزيئية، أظهرت النتائج بناءً على الوصف المورفولوجي في التحليل العنقودي قيمة تباين منخفضة بين الاصناف ودعمت هذه النتائج بالتحليل الجيني. التوصيف الجزيئي الذي تم اجرائه على ثمانية اصناف من النباتات وذلك باستخدام اثنين من البادئات Chl 1 و Ch14بطريقة الحمض النووي العشوائي متعدد الأشكال المضخم .(RAPD) لقد اظهرت البادئات تضخيم 42 جزءًا: منها 28 كانت متعددة الأشكال مما يشير إلى أن اصناف الكركديه قريبة وراثيًا. فأعطى Chl1 أعلى تنوع وراثي ومحتوى معلومات متعدد الأشكال بنسبة (72%). دليل التشابه يتراوح بين 0.62–0.9 مما يظهر تقارب المدى الجيني، وكانت درجة التشابه للصنف 8 هو الأدنى حيث صُنف في مجموعة منفصلة عن جميع الاصناف الأخرى. بالإضافة إلى ذلك، فقد تم الحصول أيضًا على درجة تشابه مرتفعة بين الأصناف UKM1 and UKM2، وكان الصنف 3 له قيمة تشابه عالية مع الصنف 6. وكانت المسافة الجينية قريبة جدًا بين الأصناف. وبالتالي، فان دلالة RAPD لديها القدرة على تحديد الأصناف وتوصيف التنوعات الجينية داخل الأصناف.

Abstract

In order to evaluate the genetic diversity among Roselle (*Hibiscus sabdariffa*) accessions, a specified morphological and genetic

markers were used to group the various accessions into distinct genotypes comparing between morphological and molecular methods were conducted. The groupings of accessions based on morphological description in cluster analysis were showed low variance value which was supported by genetic analysis. Molecular characterization was undertaken on eight accessions using two primers (Chl1 and Chl4) and the random amplified polymorphic DNA (RAPD) method. Two primers were able to amplify 42 fragments of which 28 were polymorphic indicating that Roselle accessions are genetically close. Chl1 gave the highest genetic diversity and polymorphic information content (72%). The similarity index ranged from 0.62-0.91, which revealed a close range of genetic identification. A similarity coefficient of accession 8 was the lowest where this accession was classed into a separated group. In addition, high similarity index was also obtained among some of the accessions; UKM1 was highly similar to UKM2 and accession 3 had high similarity value with accession 6. The genetic distance was very close within the varieties. Thus, these RAPD markers have the potential for identification of varieties and characterization of genetic variation within the varieties.

Key words: Roselle, morphology, Genetic diversity and RAPD-PCR.

Introduction

There are nearly 300 species of hibiscus around the world. *Hibiscus sabdariffa L.*, also called Roselle, is a member of the Malvaceae family. The origin of this species is believed to be native of tropical Africa (Tounkara *et al*., 2011). This subtropical plant is an annual herbaceous shrub with a height of up to 2.5 m; tetraploid species characterized by smooth, cylindrical red stems, reddish veins and alternate 7.5 - 12.5 cm long green leaves (Mohamed *et al*. 2007; Hussein *et al*. 2010). Roselle plant, foremost the calyx, has long been used in traditional medicine as It is a fully source of anti-

oxidants, riboflavin, ascorbic acid and carotene that are nutritionally important beside amino acids and mineral salts (Anjah *et al* .2012). It was recently reported that the seed oil is rich in phytosterols and tocopherols (Hussein *et al*., 2010; Khan.N.H *et al* 2022). Furthermore, a success of any vegetable crop development program depends on genetic variability, genetic advance and characters association with the plant yield (Ibrahim and Hussein 2006, Bhandari *et al*., 2017). Screening of the genetic diversity of the studied plant using various molecular markers has a main importance for the genetic characterization of medicinal plants (Sundaram and Purwar, 2011; Ankrah *et al.* 2019). Random amplification of polymorphic DNA (RAPD) markers are created from the amplification of PCR of genomic DNA fragments have advantages including rapidity, low DNA quantity requirements and the ability to generate various polymorphisms (Williams *et al*., 1990).The knowledge of the genetic diversity of this crop is very important, as it is the main factor towards improving the crop. The advance of several molecular techniques has led plant breeders to estimate genetic diversity on the basis of data generated by different molecular markers, which provide a means of quick analysis of germplasm that often support phenotypic data. The objective of this study was to identify and characterize eight accessions of Roselle using morphological traits and RAPDs to estimate the genetic relationships between the selected genotypes.

Materials and methods

Eight accessions of Roselle (*H. sabdariffa*) were used in this study. Seeds of Roselle accessions were germinated at Faculty of Science and Technology, Universiti Kebangsaan Malaysia. After a week, the geminated seeds were transferred into a small pot and left to grow for a month. After one month from growing samples were planted in the field. The characters of evaluation included phenotypic and

agronomic characteristics (plant height, stem diameter, number of branches per plant, and number of fruits per plant). The parameters were measured to mark if there was noticeable difference between Roselle accessions that have studied.

DNA extraction

DNA was extracted from fresh leaves collected from different varieties by the CTAB method (Doyle and Doyle, 1990**)**. 3 mg of fresh leaves were ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to eppendorf tubes containing 1 ml buffer solution of Cetyl Trimethyl Ammonium Bromide (CTAB), 2 µl β- mercaptoethanol and the homogenate was incubated at 60 C for 4 hrs. The tubes were allowed to cool for 2 mins after incubation then centrifuged at 12000 rpm for 5 mins and the supernatants were transferred into labeled tubes. (200) µl of Chloroform-Isoamyalcohol mixture in the ratio 24:1 was added and mixed gently. The tubes were centrifuged at 12000 rpm for 15 mins and the supernatants transferred into new tubes. 500 µl of ice-cold Isopropanol were added, mixed gently by shaking the tube and incubated in -80ºC for 15mins for DNA precipitation. Pellets formed at the bottom of the tube were dried at room temperature for 10 minutes. A total of 1 mL CTAB wash buffer solution was added into the tube and then incubated at room temperature for 30 mins and centrifuged. Supernatant was discarded and obtained DNA pellet left to dry at room temperature after washing with 70% ethanol. 20-25 µl of Tris-EDTA buffer solution was added into the eppendorf tube containing the dried DNA pellet , then the samples were stored at -20 ºC.. DNA quantifications were specified by visualizing under UV light, after electrophoresis on 0.8% agarose gel. The resuspended DNA was then diluted in TE buffer to 5 µg/µl concentration for use in polymerase chain reaction (PCR) reaction.

Polymerase Chain Reaction (PCR)

PCR amplification was performed in a 25µl reaction volume which contained of 10x buffer, 25 mM MgCl2 (Promega), 10 mM dNTPs (Promega), 20 µM primers, template DNA and 500 U Taq DNA polymerase using Eppendorf Master Cycle. The PCR cycle was carried out as follows: an initial denaturation at 94°C for 3 minutes followed by 40 cycles is composed of 94°C for 60 seconds, 38°C annealing for 1 minute and 72°C for 2 min and a final step of 72°C for 5 min extension. The product was stored at 4°C. Amplified products were separated by electrophoresis on 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination by Gel Doc Apparatus.

Data analysis

The analysis of variance (ANOVA) was used to study the significant differences between the treatments and test LSD (0.05) at the level of significance 5%. To compare averages of transactions. We used CoStat version 6.4 Software in the analysis of variance and used SPSS version 21 in cluster analysis. Data of RAPD markers were recorded as presence (1) or absence (0) of band products from the examination of photographic. The specific bands are useful for identifying different accessions were named with a primer. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created to measure genetic distance and similarity using of NTSYS-PC (Numerical taxonomy and multivariate analysis system program) Version 2.10e (Rohlf, 2000). The dendrogram was generated by using a distance matrix using the unweighed pair group method with arithmetic average (UPGMA) subprogram of NTSYS-PC.

Results

Morphological characterization of Roselle accessions

All 8 selected Roselle accessions as shown in the table 1 were characterized morphologically. The study focused on the variance in morphological characters (stem color, flower color and fruit color) and agronomic characters (plant height, stem diameter, number of branches per plant and the number of fruits per plant). According to the table1, the morphological difference among accessions was narrow.

Table1: Morphological traits of eight accessions of Roselle

Accessions	Origin	Stem color	flower color	Fruit color
Accession 3	Sudan	Reddish	Red	Red
Accession 6	Malaysia	Reddish	Red	Red
Accession 8	Sudan	Reddish	Yellow	Red
Accession 12	Sudan	Red	Yellow	Red
Accession 21	Arabian Peninsula	Dark red	Red	Dark red
UKMR1	Malaysia	Smooth reddish	Red	Red
UKMR2	Malaysia	Smooth reddish	Light red	Dark red
Nigeria	-	Red	Red	Dark red

In the study of the number of fruits for each plant, ACC 3 and ACC 6 had the highest means (133.66 and 124.66) respectively which showed no significant differences between them (P = 0.451). In addition, the highest mean length of plants was in ACC8 (157.5 Cm) followed by ACC12 (132.6 Cm) and ACC.21 (132.3 Cm). In contrast, UKM1 showed the lowest mean of height of plant (70.83 Cm). There are no significant variance between ACC12 and ACC21. Moreover, the mean number of branches for each plant was also tested; the result indicated that there are significant variance

between ACC8 and ACC3 (Table 2), Whereas ACC12 and ACC21 accessions showed no significant differences. UKM1 and Nigeria had the lowest value of the number of branches (4 and 4.66). The mean of the stem diameter ranged from 4.26 Cm to 6.83 Cm.

Table2: **Analysis of variance (mean) of four quantitative characters for Roselle accessions**

Accession	Plant height (Cm)	N. of branches	Stem diameter (Cm)	N. of fruits
ACC.3	125.5^{b}	10.83^{ab}	5.5^{ab}	133.66^{a}
ACC.6	113.5^{b}	9^{abc}	5.53^{ab}	124.66^{a}
ACC.8	157.5^{a}	12.16^{a}	6.83^{a}	98^{b}
ACC.12	132.6^{b}	7.5^{bc}	6.45^{a}	88^{b}
ACC.21	132.3^{b}	6.16^{c}	6.55^{a}	58.16^{c}
UKM1	70.83^{d}	4^{c}	5.56^{ab}	27.3^{d}
UKM2	96.5^{c}	5^{c}	5.71^{ab}	53^{c}
NIGERIA	78.16^{cd}	4.66^{c}	4.26^{b}	16^{d}

*Numbers that follow with different letters within the same column indicate significant differences between accessions at the level 5% using (LSD 0.05)

Cluster analysis

The studied agro-morphological indicators were used to establish the cluster between the varieties and studied morphological phenotypes. Figure (1) showed the accessions that were linked together in each step of the cluster analysis. According to the dendrogram, accessions 12 and 21 are grouped in the same cluster. In addition, accessions 3 and 6 were also found within one cluster. The results showed also grouping of sub-clusters of plants UKM, UKM2 and Nigeria within one cluster. Accession 8 was discrete in subgroup.

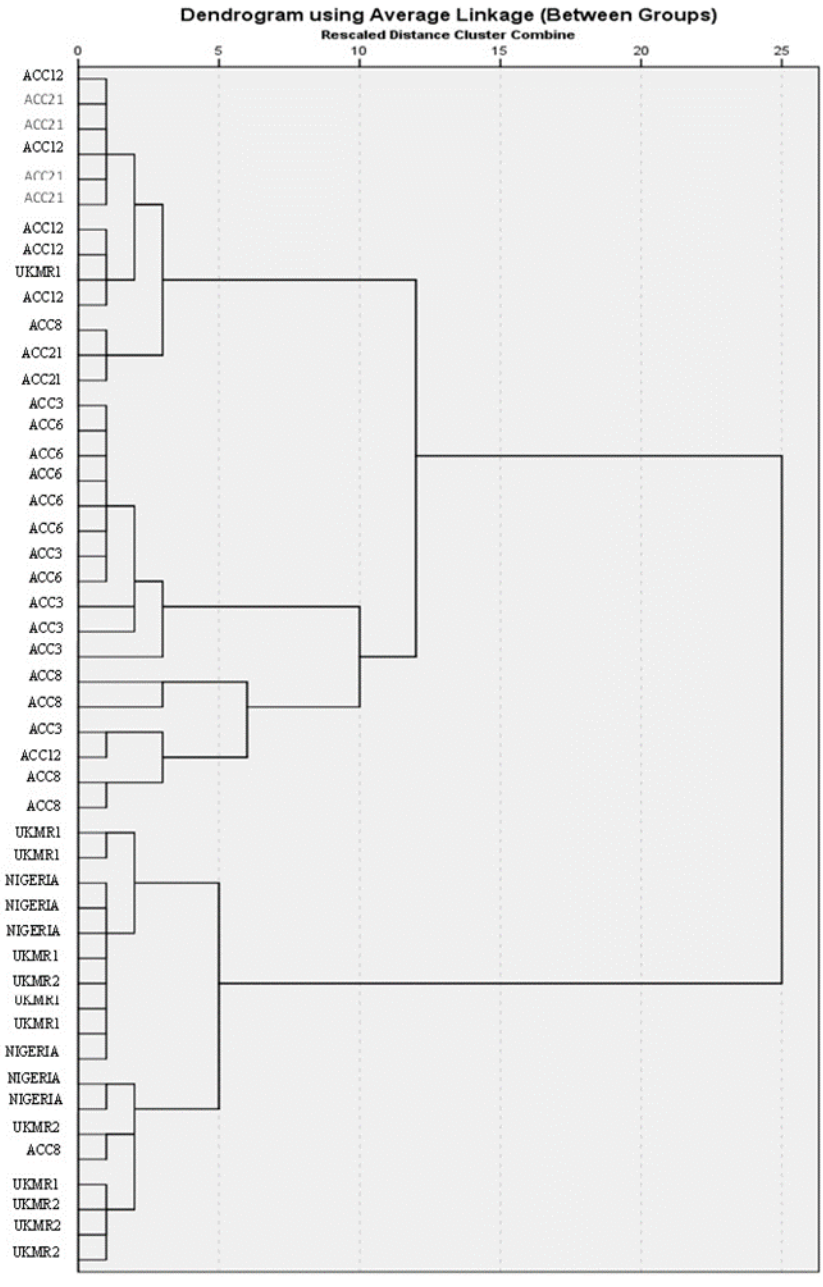


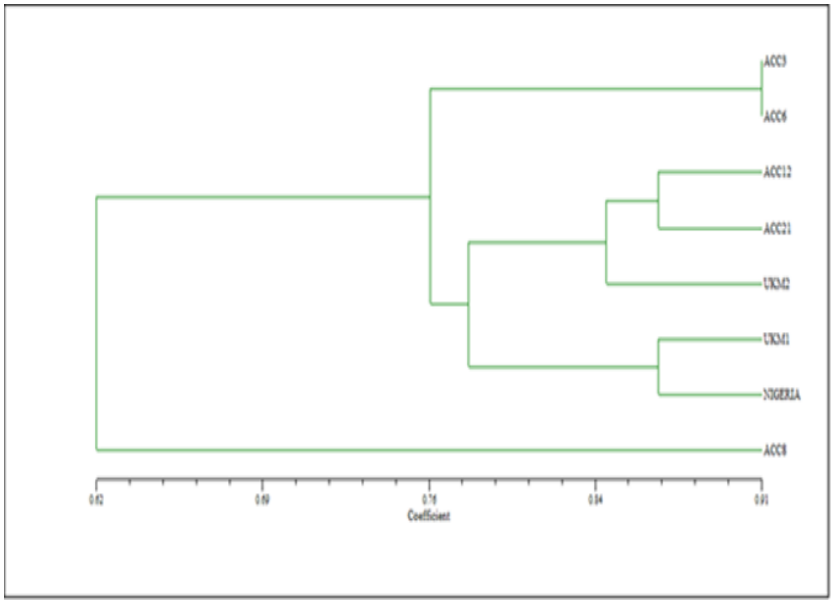
Figure 1: Dendrogram showing clustering of eight accession of Roselle

Genetic polymorphism among Roselle accessions

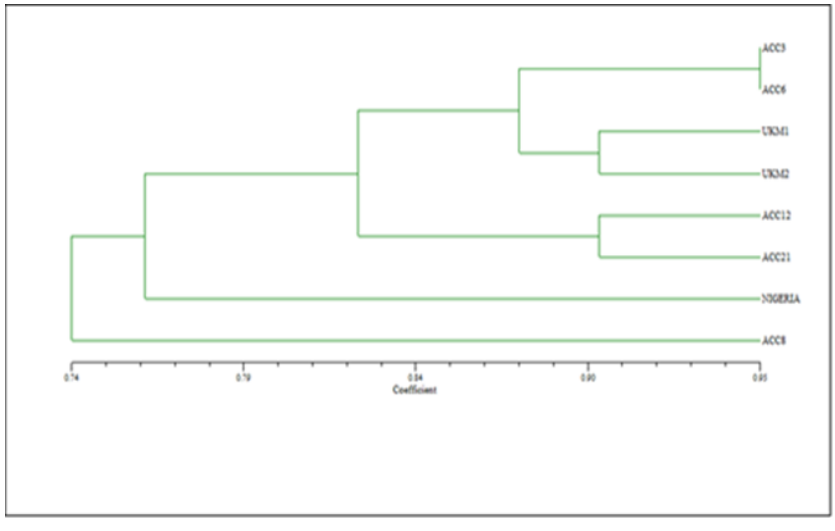
The amplification fragments varied from primer to another, where the primer chl1 showed the highest fragments (22 fragments), the primer chl4 produced (20 fragments) (Table 3). The polymorphism percentage was 72 in the chl1 primer whereas the chl4 primer revealed polymorphism 60. The cluster analysis was conducted according to chloroplast marker of 8 accessions (figure 2). A maximum similarity value of 0.91 was observed between accession 3 and accession 6, which detected in chl1, whereas accession 8 showed lowest similarity index (0.62). Accessions 12 and 21 showed close similarity with 0.85 correspondence value. In case of the second primer chl4 depicted that accession 8 has the lowest genetic similarity (0.74) as compared to the rest of accessions, whereas Nigeria accession showed relative close similarity value (0.76). The high similarity value was between two accessions 3 and 6 (95). Moreover, accessions UKM1 and UKM2 showed high similarity index (90). These results showed that genetic diversity was very low among all accessions of Roselle.

Table 3: RAPD-PCR fragments generated by chl1, chl4 primers.

primer	Sequences	Total number of bands	Polymorphic bands	%of Polymorphism
C hl1	5' – GAGGCCTACGCCCCA TAGAA – 3'	22	16	72
Chl4	5' - TTCCCGTGTCTTCCG GCTTAC – 3'	20	12	60



(A)



(B)

Figure 2. Dendrogram of 8 Roselle accessions based on (A) Chl1 and (B) chl4 markers

Discussion

Variance analysis

Based on the morphological results stated in table1, there were a slight variety as a most of Roselle accessions had red stem and red fruits. On the other hand, the mean of plant height and number of branches showed significant differences between the accessions. Accession 8 had the highest plant height and number of branches (157.5 Cm, 12.6) respectively. Whereas UKM1 had the lowest value. The strong effect of genotype on plant height and branch number was determined in some Sudan, Egypt and Iran Roselle (var. *sabdariffa*) collections, respectively (Javadzadeh and Saljooghianpour, 2017). In contrast, no significant differences were observed for stem diameter. Clustering of the accessions was indicated the similarity in some accessions according to agro-morphological traits. The results of cluster analysis showed that the accessions 12 and 21 were placed in the same cluster. This indicated a high similarity between them that matches the results showed in the table 2 for morphological traits mainly plant height and stem diameter. Moreover, accessions 3 and 6 had similar trait in most of studied indicators. Accession 8 was separated in sub grouping cluster which is in consistent with the results of variance analysis shown in the table 2. All the correlated characters are important for the breeders because the improvement of one trait may cause improvement in linked traits.

Genetic Diversity of Roselle Accessions using RAPD Markers

The present study included the identification of genetic variation within eight varieties of *Hibiscus sabdariffa*. Information on polymorphic DNA in organelle genomes is needful for evolutionary investigations (Abedian.*et al* 2012; Zhang.*et al*,2012). Although, it is insistent to carry out high output analysis on chloroplast DNA polymorphisms (Zeng *et al* 2010). The tree diagram showed that the

distant within the varieties was not significantly diverge. Our result is closed to previous study (Hanboonsong. *et al*, 2000) who reported a very low genetic diversity (0.02-0.09 dissimilarities) among ninety-four Roselle accessions from Thailand. The close relationships within the cultivars of celery were also reported by using RAPD markers (Yang and Quiros, 1993). In this study, most accessions showed correlation and coherence in morphological and molecular data. The study also presented that most of the accessions showed slight genotypic variations and considerable correlation in the markers, especially in molecular part. Where the range of genetic variation between accessions of Roselle was very close based in two markers from the phylogeny tree in Figure. 2 (A and B), It can be predicted that accessions UKM1,UKM2, accessions 12, accession 21 relatively close while accession 8 showed the lowest similarity. These results correspond with the results of Ibrahim et al. 2013 who mentioned that the genotypic correlation coefficient exceeded the phenotypic correlation coefficient for the most characters of Roselle accessions. However, the study of diversity among the accessions using only the morphological traits is not dependable. This is because most of the quantitative traits are influenced by environmental effect. Subsequently, data obtained from this study showed that phenotypic analysis followed with RAPD molecular marker analysis are very beneficial for indicating relationships among *H. sabdariffa* accessions similar assertions have been recorded by (Seeruttum et al , 2013).

Conclusion

It can be concluded that the RAPD markers provide a more reliable method for identification of varieties than morphological characters. It would allow a more quantitative evaluation of genetic distances between varieties. This investigation of the level and partitioning of genetic variation within the varieties would provide an important input into determining appropriate management of plants strategies.

Furthermore, this approach might be useful in future breeding programs in *Hibiscus sp*. However, other DNA markers like SSR and RFLP should be used to achieve these levels of relationships.

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