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Determination of genetic variation of Rhanterium epapposum in Kuwait desert using RAPD and SRAP DNA-based markers

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ABSTRACT

Rhanterium epapposum (*R. epapposum*) Olive is a perennial shrub found in the northern part of Kuwait with unique climate and land conditions such as drought and salt tolerance. Due to several reasons, this species is on the verge of extinction. *R. epapposum* is one of the desert forage plants upon which camel and sheep graze. Bedouins also use *R. epapposum* as a source of fuel. Thus, it is necessary to evaluate the genetic diversity within and among population of germplasm to determine and understand the extent of genetic variation that exists in the species. This evaluation was performed in an effort to reduce or stop the genetic erosion. Twenty-four Random Amplified Polymorphic DNA (RAPD) and 23 Sequence Related Amplified Polymorphisms (SRAPs) markers were used to amplify DNA fragments from 18 different samples collected from five major locations in Kuwait. Results indicated that there were 64 alleles produced by RAPD, while 129 alleles were produced by SRAP. With slightly different samples in each cluster, the cultivars were grouped into four distinct clusters by coefficients of similarity that were generated with RAPD, SRAP, and the combined data of both. With the absence of any pedigree information, this study indicated that these DNA-based markers could be used to obtain efficient, accurate, and high throughput fingerprinting, revealing significant variation among the existing locations that can be explored in order to preserve the species.

Keywords: Genetic diversity; RAPD; Rhanterium epapposum; SRAP.

INTRODUCTION

Kuwait is situated at the northwestern corner of the Arabian Gulf. It occupies an area of 18,000 km^2 (Brown & Al-Mazrooei, 2003). Kuwait is an arid country with extremely dry hot summers and an average maximum temperature of 46°C. Winter is cool, with temperature ranging from 4 to 10°C. Rainfall is erratic with large temporal fluctuations ranging from 115 to 120 mm/yr (Omar *et al.*, 2009).

Kuwait's native vegetation is a valuable source of novel genes that can be used and exploited for the betterment of agricultural and environmental systems in Kuwait (Abo El-Nil *et al.*, 2000). These novel genes represent a transition between sem-idesert and desert vegetation, offering a valuable gene pool and plant material for drought and salt-tolerance research (Omar & Bhat, 2008). *Rhanterium epapposum (R. epapposum*) Oliv (common name Arfaj), the national flower of Kuwait (Halwagy *et al.*, 1982), is found in deep, sandy soils and shallow substrates, where the plant is sometimes accompanied by *Haloxylon salicornicum* (Halwagy *et al.*, 1982).

Arfaj-a bushy shrub with a height of 50 to 100 cm-consists of a network of scattered branches. Arfaj plants quickly produce small, pale green, thorny leaves after rainfall in winter. During summer, the leaves fall and the branches become bare and lignified with living fiber (Omar & Bhat, 2008). The plant starts flowering between April and May, producing bright yellow flowers of 1.5 cm width (Omar *et al.*, 2000). This perennial shrub is widely distributed in the southern part of Kuwait and is often confined to protected areas (Halwagy & Halwagy, 1974). The Arfaj plant is one of the desert forage plants, where camel and sheep graze on. Bedouins also use the Arfaj plant as a source of fuel. The importance of R. epapposum is the ability to tolerate adverse conditions such as soil pollution, drought and salt, as well as its potential use in desert and urban revegetation, national greenery programs, and protecting soil from erosion.

The *R. epapposum* community is currently suffering severe degradation and is facing the danger of extinction. The percentage of distribution of the *R. epapposum* community decreased significantly from 30.6 % in 1974, to 2.1 % in 2001 due to overgrazing, off-road driving, camping, and construction activities (Brown, 2003). This degradation indicates that this community has considerably retreated from the rangelands of Kuwait. Presently, the R. epapposum community is confined to protected areas such as municipal waste treatment plants, military air bases, military camps, and some restricted oil fields (Omar *et al.*, 2001). It is of paramount importance in these protected areas that *R. epapposum* and other plants in Kuwait are conserved for future generation. Thus, it is necessary to evaluate genetic diversity within and among the population of germplasm to determine and understand the extent of genetic variation. The evaluation would produce the information that is crucial in establishing an effective and efficient conservation practices for endangered plants such as the *R. epapposum* (FAO, 1998; Engles *et al.*, 2002). In addition, understanding genetic diversity would provide predictive estimates of genetic variation within a species to facilitate breeding material selection and further genetic improvement (Qi *et al.*, 2008).

Yet to date there is no published research on the genetic diversity and

population structure of *R. epapposum* community in Kuwait or elsewhere. Molecular markers have proved to be a very powerful tool in characterizing genotype, and estimating genetic diversity of plant species. This tool has an advantage of being related directly to DNA level and is not influenced by the environment (Jang & Liu, 2011). Two marker techniques of Random Amplified Polymorphic DNA (RAPD) (Williams *et al*,. 1990) and Sequence Related Amplification Polymorphism (SRAP) (Li & Quiros, 2001) are used to analyze the genetic diversity of R. epapposum species.

Before the advent of molecular techniques, genetic diversity was estimated from pedigree or agronomic and morphological characteristics. However, the disadvantage of pedigree-based estimation is that the breeding process violates many of the assumptions, such as equal genetic contribution of both parents, the unrelatedness of parents with no common ancestral line, and no selection, genetic drift and mutation. Hence, the estimates based on pedigree information are generally inflated and often unrealistic (Almanza-Pinzon *et al*., 2003; Cox *et al*., 1986; Souza & Sorrells, 1989).

The advantage of DNA markers over morphological and isozyme markers is the greater numbers of DNA markers and their general distribution throughout the genome (Paterson *et al*., 1991). The PCR method of generating RAPD markers (Welsh & McClelland, 1990; Williams *et al*., 1990) has been used to construct genetic maps of several plant species. SRAP is a PCR marker system that combines simplicity, reliability, and a moderate throughput ratio (Li & Quiros, 2001). SRAP targets coding sequences and results in the identification of a number of codominant markers. SRAP markers are arbitrarily designed to contain AT- and GC-rich motifs that anneal to intron and exons respectively (Li & Quiros, 2001). When used in BLAST searches, sequenced SRAP amplicons from *Brassica rapa* L. and *Brassica napus* L. (Li & Quiros, 2001) and *Cucurbita moschata* Duchesne ex Poir. Ferriol *et al*., (2003) revealed significant similarities to reported gene sequences deposited in Genebank databases.

The SRAP marker system has been used extensively in genetic diversity analyses (Ferriol *et al*., 2003; Budak *et al*., 2004), but seldom in map construction. Ferriol *et al*., (2003) reported that the information obtained from SRAP markers was more concordant with the morphological variations and the evolutionary history of the morphotypes than the information found with AFLP markers. SRAP technology has been recognized as a new and useful molecular marker system with numerous advantages, including simplicity, convenience, high throughput, numerous codominant markers, high repeatability, and ready-to-sequence and preferentially amplifies open reading frames (Li & Quiros, 2001). As Li & Quiros, (2001) have reported, SRAP has been successfully used for genetic diversity analysis (Ferriol *et al*., 2003, 2004;

Budak *et al*., 2004; Lin *et al*., 2004; Riaz *et al*., 2004) comparative genomics research (Li *et al*., 2003), genetic-map constructions (Li & Quiros 2001; Lin *et al*., 2003), genome transcriptome map constructions (Li *et al*., 2003) and important character gene marker (Li *et al*., 2004; Pan *et al*., 2003). In view of the importance of *R. epapposum* to Kuwait environment and the utility of RAPD and SRAP techniques, the objective of this study was to use DNA-based markers to evaluate the genetic variation among *R. epapposum* samples.

METHODS

Plant Materials

Twenty-two plant samples of *R. epapposum* were collected from five different locations from the desert of Kuwait (Figure 1) between February and April 2012. The distribution is as follows: Samples from plants 1, 2, 3, and 4 were collected from Sulaibiya; samples from plants 5, 6, 7, and 8 were collected from Nowaiseeb; samples from plants 9, 10, 11, and 12 were collected from Sabah Al-Ahmad Nature Reserve; samples from plants 21 and 22 were collected from Wafra; and the remaining plant samples were collected from Al-Abdaly.

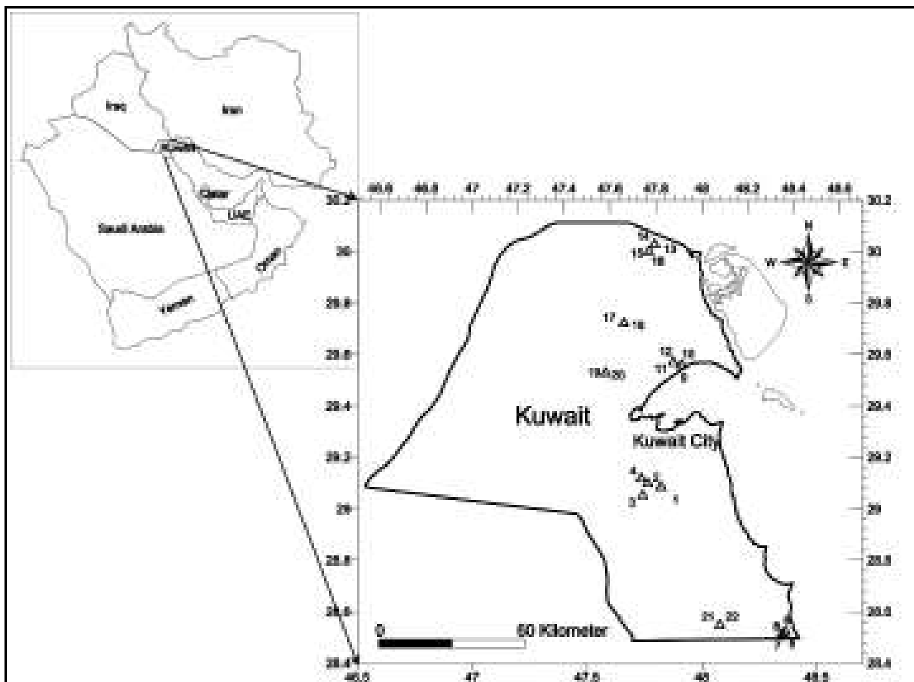

Fig. 1. Locations of sampling stations of *R. epapposum* population used in this study

Each plant consisted of branches that were 80 cm high and with small green leaves. Samples of mostly young leaves, weighing between 500 and 700 g, were collected from each plant. A subsample of 5 g was used for analysis. Prior to DNA extraction, leaves were decontaminated by washing in 5 % detergent (Javex) for 10 minutes, followed by a ten-minute washing with 70 % ethanol and a final extensive washing with sterilized distilled water. The decontaminated leaves were quickly frozen in liquid nitrogen and stored at -80°C until used.

DNA extraction

Genomic DNA of each frozen plant was isolated using liquid nitrogen and 25 ml of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 1 M NaCl, 1 % CTAB, 1 mM 1, 10-phenathroline, and 0.15 % 2-mercaptoethanol). The slurry was incubated at 60°C for one hour, and then mixed with equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation at 12000 rpm, the supernatant was transferred to a new tube. In order to precipitate the DNA, isopropanol was added and the contents were incubated for 30 min.. The pellet was dried, re-suspended in 200 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and 20 μg of RNase, and then incubated at room temperature overnight. The DNA solution was mixed with 20 μl of 8 M ammonium acetate and 400 μl of cold absolute ethanol for 30 min., centrifuged for 10 min., and air-dried at room temperature. The DNA was then resuspended in 200 ml of TE buffer and the DNA concentration was quantified using spectrophotometer (TKO100 Fluorometer, Hoefer Scientific Instruments, San Francisco).

PCR amplification

Twenty-four random RAPD primers (OPB07, OPB08, OPB10, OPB12, OPB15, OPC01, OPC02, OP C05, OPC06, OPC11, OPC15, OPC16, OPD02, OPD03, OPD04, OPD05, OPD07, OPD10, OPD15, OPE01, OPE02, OPE03, OPE04, and OPE16) were purchased from Operon Technologies Inc. (Almada, CA, USA). The SRAP primer combination IDs (15, 18, 30, 35, 38, 41, 44, 51, 65, 71, 75, 76, 80, 90, 95, 96, 110, 118, 147, 172, 198, and 200) were obtained from Dweikat *et al*., (2010). The PCR reaction mixture (25μl total) consisted of 50 mM KCl and 10 mM Tris-HCl (pH 8.8), 1.5 mM (2.0 mM for RAPD primers) MgCl2, 125 mM of dNTP, 50 ng of RAPD primer and 50 ng of SRAP primer, 1.0 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.5 mg.ml^{-1} bovine serum albumin (BSA), and 25 ng of genomic DNA.

Amplification was performed in an MJ Research PTC100 (Programmable Thermal Controller, MJ Research) using a program suitable for each marker system. The program for the SRAP markers consisted of initial denaturation for

one minute at 94°C, followed by 32 cycles of 30 s at 94°C, 50 s at 47°C, 50 s at 72°C, and final extension for five minutes at 72°C. The program for RAPD was basically the same, except that the annealing temperature was reduced to 37°C. The amplified reaction mixtures were stored at 4°C until they were gel-fractionated on 12 % (w/v) polyacrylamide gel (37 acrylamide:1 bis-acrylamide) in a TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate,1 mM EDTA) using vertical gel apparatus (Hoefer Scientific Instruments, SE600) at 300 V for three hours. The buffer temperature was controlled by a circulating bath with the temperature set to 20°C. Gels were stained in ethidium bromide (1 mg/ml) for 20 min., destained in deionized water for one hour, and then photographed with the Gel Doc 2000 (Bio-Rad). The PCRs were performed 3 times. Those that were included in the linkage analysis were only the markers that were highly reproducible and based on clear presence or absence of polymorphism without any intensity variation.

Data scoring

Each band was considered a single locus/allele and was scored as present (1) or absent (0), each of which was treated as an independent character. Genetic diversity analyses were conducted on the basis of the scores.

Statistical analysis

The marker scores were used to estimate Jaccard's similarity coefficients using R/prabclus (R Development Core Team 2012). A dendrogram was constructed based on Jaccards dissimilarity coefficient (Jaccard, 1908) using each marker data for all plant samples following the Unweighted Pair Group Method (UPGMA) (Sokal & Michener, 1958). Polymorphism Information Content (PIC) values were calculated as in Anderson *et al.* (1993), assuming homologous alleles. Polymorphic information content for a locus is calculated as:

$$PIC = 1 - \sum Pij^2$$

Pij is the relative frequency of the jth allele of the ith locus, summed over all the alleles for individual marker locus over all lines.

The combined data for both markers were also utilized for further clustering. The similarity data were also used to conduct the principal coordinate analysis (PCoA) and to generate the multidimensional scaling (Zuur *et al.*, 2007).

RESULTS AND DISCUSSION

Knowledge of genetic diversity among adapted individuals of a species has a

considerable impact on the improvement and survival of the species. Such information could be obtained from pedigree analysis morphological traits or by using molecular markers. In this study, 24 RAPD and 23 SRAP randomly selected markers were used to characterize and evaluate the genetic diversity among 22 plant samples collected from five distinct locations in the Kuwait desert (Figure 1). Using SRAP analysis, all the primers produced DNA fragments with 23 markers, giving clear polymorphism with 74 polymorphic alleles. The percentage of marker polymorphism was 47.3 and the number of individual polymorphic fragments ranged between 53 in sample from plants # 18 and 37 in sample from plant # 5. PIC values ranged from 0.16 to 0.83. Since there was no pedigree or any genetic information available for the species, phenotypic variation present in the species would be very limited. DNA-based markers would be the best and only method to estimate, the relatedness among the collected samples.

Similarity index values obtained from the polymorphic data were used to estimate the genetic relatedness among plant samples. A dendrogram based on the similarity values produced from the SRAP was constructed using the UPGMA cluster in order to illustrate the association between the plant samples used in this study (Figure 2a). The dendrogram revealed four major clusters of *Rhanterium epapposum* samples. First cluster included sample from plant # 2 alone; the second cluster included samples from plants # 5 and 6; the third cluster included samples from plants # 21 and 22; and the fourth cluster included the remaining plant samples. Interestingly, grouping according to the coefficients of the RAPD resulted in four clusters as follows: samples from plants # 5 and 6 in one cluster; sample from plant # 1 in another cluster; samples from plants # 3, 9, and 10 were grouped in separate clusters; and the remaining plant samples were grouped in one cluster (Figure 2b. Data obtained from both RAPD and SRAP were combined and utilized to generate the diversity grouping.

Based on the results shown in Figure 2c, the 18 samples selected for analysis were grouped into four clusters with similar grouping to the SRAP data where samples from plants # 21 and 22 had their own cluster; so did samples from plants # 15 and 16 (Figure 2b and c). In all three dendrograms, samples from plants # 21 and 22 were present in the same group of the dendrogram. The same was true for samples from plants # 5 and 6. The main causes for this diversity grouping (Figure 2) could be attributed to the difference in the distances of sampling between locations (Figure 1), which may have caused isolation barrier in each location.

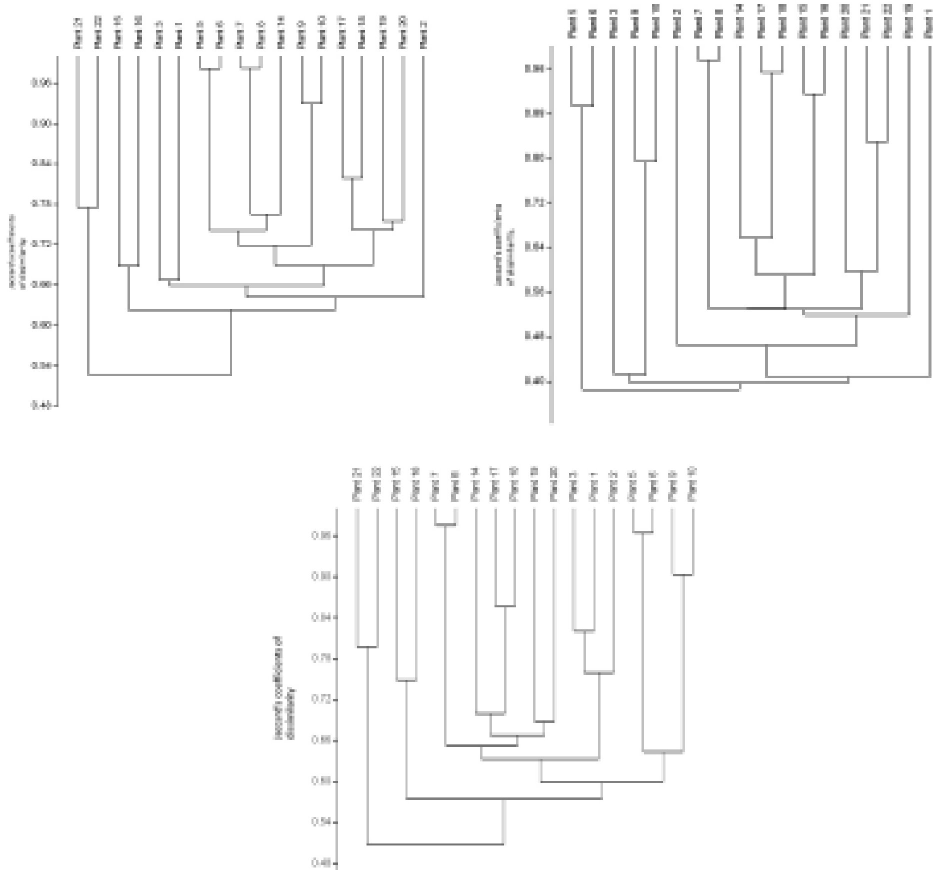

Fig. 2. Dendrogram of 18 samples of *R. epapposum* collected from five locations from Kuwait based on RAPD markers (A) (top left), SRAP (B) (top right), and the combination of SRAP and RAPD (C) (bottom). Values in the *X*-axis correspond to Jaccard's coefficients of dissimilarity.

It should be noted that the samples included in this study represented an almost complete spectrum of the R. epapposum existing in the Kuwait dessert. Similarity index values obtained from the polymorphic data were used to estimate the genetic relatedness among the 18 plant samples (Appendix 1). Results indicated that the genetic similarity coefficient for all plant samples based on RAPD and SRAP markers ranged from 0.979 to 0.540 with an average of 0.69. It was also revealed that samples from plants # 7 and 8 were the most closely related genotypes as they showed the highest similarity index (0.979). This may have been attributed to samples in the geographically close locations of samples from plants # 7 and 8 (Figure 1); while the samples from plants # 6 and 21 were the most distantly related with the lowest index of 0.54. This could be due to the fact that sample from plant # 6 was collected from locations that

were geographically far away from the sampling location of sample from plant # 21 (Figure 1).

Appendix 1: Similarity matrices calculated from allele sharing distance between pairs of individuals based on both RAPD and SRAP analysis.

	Plant 1	Plant 2	Plant 3	Plant 5	Plant 6	Plant 7	Plant 8	Plant 9	Plant 10	Plant 14	Plant 15	Plant 16	Plant 17	Plant 18	Plant 19	Plant 20	Plant 21	Plant 22
Plant 1	1	0.808081	0.873737	0.611111	0.606061	0.686869	0.686869	0.651515	0.686869	0.707071	0.651515	0.636364	0.707071	0.707071	0.686869	0.732323	0.651515	0.621212
Plant 2	0.808081	1	0.843434	0.60101	0.59596	0.676768	0.676768	0.661616	0.686869	0.666667	0.671717	0.626263	0.656566	0.686869	0.727273	0.712121	0.661616	0.671717
Plant 3	0.873737	0.843434	1	0.656566	0.651515	0.70202	0.70202	0.686869	0.712121	0.742424	0.666667	0.631313	0.681818	0.70202	0.722222	0.737374	0.626263	0.636364
Plant 5	0.611111	0.60101	0.656566	1	0.974747	0.691919	0.681818	0.69697	0.722222	0.691919	0.636364	0.611111	0.631313	0.671717	0.661616	0.656566	0.545455	0.59596
Plant 6	0.606061	0.59596	0.651515	0.974747	1	0.686869	0.686869	0.691919	0.727273	0.69697	0.641414	0.606061	0.646465	0.686869	0.666667	0.651515	0.540404	0.60101
Plant 7	0.686869	0.676768	0.70202	0.691919	0.686869	1	0.979798	0.671717	0.686869	0.727273	0.641414	0.626263	0.646465	0.676768	0.686869	0.70202	0.590909	0.621212
Plant 8	0.686869	0.676768	0.70202	0.681818	0.686869	0.979798	1	0.651515	0.666667	0.737374	0.631313	0.616162	0.666667	0.69697	0.69697	0.712121	0.580808	0.621212
Plant 9	0.651515	0.661616	0.686869	0.69697	0.691919	0.671717	0.651515	1	0.934343	0.691919	0.656566	0.641414	0.621212	0.621212	0.651515	0.636364	0.555556	0.606061
Plant 10	0.686869	0.686869	0.712121	0.722222	0.727273	0.686869	0.666667	0.934343	1	0.747475	0.70202	0.666667	0.656566	0.656566	0.676768	0.641414	0.550505	0.590909
Plant 14	0.707071	0.666667	0.742424	0.691919	0.69697	0.727273	0.737374	0.691919	0.747475	1	0.691919	0.666667	0.737374	0.777778	0.727273	0.752525	0.611111	0.651515
Plant 15	0.651515	0.671717	0.666667	0.636364	0.641414	0.641414	0.631313	0.656566	0.70202	0.691919	1	0.833333	0.691919	0.712121	0.681818	0.707071	0.656566	0.636364
Plant 16	0.636364	0.626263	0.631313	0.611111	0.606061	0.626263	0.616162	0.641414	0.666667	0.666667	0.833333	1	0.69697	0.717172	0.656566	0.691919	0.621212	0.611111
Plant 17	0.707071	0.656566	0.681818	0.631313	0.646465	0.646465	0.666667	0.621212	0.656566	0.737374	0.691919	0.69697	1	0.89899	0.717172	0.712121	0.590909	0.631313
Plant 18	0.707071	0.686869	0.70202	0.671717	0.686869	0.676768	0.69697	0.621212	0.656566	0.777778	0.712121	0.717172	0.89899	1	0.777778	0.742424	0.611111	0.651515
Plant 19	0.686869	0.727273	0.722222	0.661616	0.666667	0.686869	0.69697	0.651515	0.676768	0.727273	0.681818	0.656566	0.717172	0.777778	1	0.762626	0.671717	0.691919
Plant 20	0.732323	0.712121	0.737374	0.656566	0.651515	0.70202	0.712121	0.636364	0.641414	0.752525	0.707071	0.691919	0.712121	0.742424	0.762626	1	0.707071	0.727273
Plant 21	0.651515	0.661616	0.626263	0.545455	0.540404	0.590909	0.580808	0.555556	0.550505	0.611111	0.656566	0.621212	0.590909	0.611111	0.671717	0.707071	1	0.888889
Plant 22	0.621212	0.671717	0.636364	0.59596	0.60101	0.621212	0.621212	0.606061	0.590909	0.651515	0.636364	0.611111	0.631313	0.651515	0.691919	0.727273	0.888889	1

CONCLUSION

Due to the declining population of *Rhanterium epapposum* community in Kuwait, there was a need to understand and document the extent of genetic variation for this species. In conclusion it was found that, the species has limited genetic diversity and it could be due to the fact that they are geographically closely located. In order to enhance the population of *Rhanterium epapposum*, germplasm collected from the existing population need to be propagated.

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تحديد التنوع الجيني لنبات العرفج الصحراوي باستخدام وسم الحمض النووي لتقنيات RAPD و SRAP

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خلاصة

يمتاز نبات العرفج الذي يتواجد في الجزء الشمالي من دولة الكويت، بصفات فريدة من نوعها كقدرته على تحمل الجفاف والملوحه، كما أنه يمثل أحد الأعلاف الرئيسية لرعي الجمال والأغنام، ومصدراللوقود، وبالتالي فهو على شفا الإنقراض. لذلك هناك حاجة إلى تحديد وفهم مدى التنوع الجيني لأصناف هذا النبات سعياً إلى تقليل ومنع تناقصة المستمر. تهدف هذه الدراسة إلى استخدام وسمات الحمض النووي لتحديد وتقييم عينات نبات العرفج التي تم جمعهامن خمسة مناطق في صحراء الكويت. تم استخدام 24 مسبرا من تقنية SRAP و23 مسبر من تقنية SRAP بهدف تقييم 22 عينه مختلفة تم جمعها. أسفرت النتائج إلى ظهور 64 أليل بواسطة RAPD و129 أليل بواسطة SRAP وقد نتج عن دمج البيانات الناتجة من RAPD وSRAP إلى تقسيم نبات العرفج إلى أربعة مجاميع متميزة مع وجود اختلاف بسيط داخل كل مجموعة. أشارة الدراسة إلى إمكانية إستخدام وسم الحمض النووي للحصول على بصمات وراثية دقيقة ذات جودة عالية تؤدي إلى الكشف عن جود تنوع مهم للنباتات في المواقع المختلفة يمكن استخدامها للحفاظ على النباتات.

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