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The Use of ISSR Marker for Analyzing the Genetic Diversity of Nigerian Date Palms (*Phoenix dactylifera* L.)

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) is a major fruit crop of arid climate region in countries of the Middle East and North Africa. DNA markers are powerful tools that can provide information on the relatedness among date palms that are difficult to distinguish morphologically. The present study was aimed at the analysis of genetic diversity that exists among and within 10 date palms ((RCP1 (tissue culture palm), Medjoole, R4P2, R5P7, R6P8, R7P6, R8P1, R8P4, R8P6 and R8P13) grown at the Nigerian Institute for Oil palm Research (NIFOR) experimental Substation Dutse, Jigawa State using Inter-Simple Sequence Repeat (ISSR) markers (14A, 44B, HB-08, HB-10, HB-12 and HB-14). DNA was extracted from the 10 date palm leaf samples using DNeasy Mini Kit, PCR amplification was done using ISSR markers and amplification products were analysed by electrophoresis in 2 % agarose gel stained with SYBR Green and photographed under UV light. Among the six ISSR markers used to cluster the 10 date palms, 44B, HB-10 and HB-12 gave the highest variation percentage polymorphism. Genetic distance and relationship were estimated among the date palm varieties. The highest similarity coefficient value (0.84) was observed between R5P7 and R8P6 which seems to be the nearest two varieties and can be closely regrouped. The similarity coefficient value of 0.00 was obtained between RCP1 and Medjoole date palms, indicating that these were among themselves similar but distinct from the other date palms. The result from this work showed evidence of divergence among the date palms grown in Nigeria.

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious perennial long-lived monocotyledon plant that belongs to Arecaceae family. Date palm cultivation in Nigeria is done for the date fruit and is a major economic development of the people in the Northern states of Nigeria. Date is a major fruit crop of the arid climate region in the countries of the Middle East and North Africa. The total production of date palm fruit ranges between 2.5 and 4 million tons per year in the world[1].

Date palms are generally propagated by separating the offshoots produced by individual trees. This help to maintain the genetic integrity of date palm cultivars. The offshoots used for propagation are produced in limited numbers during the date palm's life span[2]. *In vitro* propagation is very important as an option to complement conventional methods of generating planting materials. It is a viable method of producing large numbers of date palm planting materials. The advantages offered by *in vitro* multiplication include the avenue it provides of multiplying a single productive individual plant. For date palm which is dioecious, this feature is extremely useful[2]. Date palms have always been clonally propagated to ensure the identity and uniformity of the cultivars. Discrimination among closely related cultivars and clones is often extremely difficult[3].

However, understanding genetic structure of date palm at the regional level is elementary for efficient use of these variable resources and better up keep of date palm populations[4] as has already been demonstrated in several other crops[5]. Morphological markers of date palm are mainly based on fruit characteristics (shape, weight, colour, skin aspect, consistency and texture) and morphology of leaves and spines, have been used to describe many varieties. However, using morphometric characters alone for genetic diversity among closely related cultivars is often unreliable, especially because of the influence of environmental conditions[6].

Biochemical markers (isozymes and proteins) have proven to be effective in the identification of date palm varieties. However, these give limited information and are usually indirect approach for detecting genomic variation. The molecular markers have proven to be useful tools for identification and phylogenetic analysis of different plant species and cultivars[7]. The genetic improvement of a crop species depends on the ability to select promising plant material. To facilitate the selection process, molecular markers that are associated with important traits can be used as selection tools[8]. Characterization and analysis of the available genetic diversity therefore, constitute indispensable step with regard to the development of breeding strategies[7]. Worldwide, many markers have been used to identify almond genotypes such as Random Amplified Polymorphic DNA (RAPD) is possibly the simplest test of all recently applied DNA-based test for date palm identification[9]. Amplified Fragment Length Polymorphism (AFLP) provides an effective, rapid and economical tool for detecting a larger number of polymorphic genetic markers that are highly reliable and reproducible and are able to be genotyped automatically. The AFLP technique has been used extensively to detect genetic polymorphisms, evaluate and characterize breed resources, construct genetic maps and identify genes[9].

Consequently, to understand the genetic relationship among and within the date palm varieties, Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Inter-simple sequence repeat (ISSRs) and Amplified Fragment Length Polymorphism (AFLPs) markers have been used widely and efficiently to analyze genetic diversity within and among date palm cultivars in many middle east countries such as Egypt, Oman, Morocco, Saudi Arabia, Tunisia and Sudan[9]. ISSR

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markers are very simple, rapid, inexpensive and are highly reproducible because of their primer length and to the high stringency achieved by the annealing temperature. ISSR is believed to be one of the most efficient techniques that always reveal high polymorphism and determine genetic diversity in date palm. ISSR markers have been used in discriminating date palm cultivars and have been introduced as a useful tool in several DNA marker studies[10, 11, 12, 13, 14, 15].

ISSR was employed by Zahedi *et al*.[16] and Karim *et al*.[14] to investigate the phylogenetic relationship among a set of Tunisian date palm cultivars and reported ISSR as an informative markers. Similarly, ISSR technique was proven efficient for determining molecular phylogeny of date palm cultivars grown in Saudi Arabia and Egypt[17]. The aim of this study therefore, was to use ISSR markers to determine genetic diversity among date palms grown in Nigerian Institute for Oil Palm Research Experimental Substation Dutse, Jigawa State, Nigeria.

Materials and Methods

Plant Material

Fresh leaves from ten randomly selected date palms (male and female) (RCP1 (tissue culture palm), Medjoole, R4P2, R5P7, R6P8, R7P6, R8P1, R8P4, R8P6 and R8P13), raised at the NIFOR date palm experimental substation Dutse Jigawa state Nigeria were used for the study.

DNA extraction

DNA Isolation was carried out using DNeasy Mini Kit (QIAGEN) according to manufacturer's instruction. An aliquot of 200 mg leaf sample was cut into small pieces and ground using liquid nitrogen to a fine powder, then the powder was transferred to an eppendorf tube. Aliquot of 400 µl of buffer API and 4 µl of RNase stock solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated for 10 min at 65 ºC in a water bath and mixed 2-3 times during incubation by inverting the tube. Aliquot of 130 µl of buffer AP_2 was added to the lysate, mixed and incubated for 5 min on ice. The lysate was applied to the QIA Shredder Spin column sitting in a 2 ml collection tube and centrifuged for 2 min at maximum speed (10,000 rpm) at 4 ºC. The supernatant was transferred to a new tube without disturbing the cell-debris pellet of which 450 µl of lysate was transferred to a fresh tube. Then, 0.5 volume of buffer AP_3 and 1 volume of ethanol (96-100%) were added to the cleared lysate and mixed. Then 650 µl of the mixture was transferred through DNeasy Mini spin column setting in a 2 ml eppendorf collection tube. It was then centrifuged for 1 min at 8,000 rpm at 4 ºC and the flow-through was then discarded. DNeasy column was then placed in a new 2 ml collection tube. Buffer AW (500 µl) was added to the DNeasy column and centrifuged for 1 min at 8,000 rpm at 4 ºC. Then 500 µl buffer AW was added to DNeasy column and centrifuged for 2 min at maximum speed (10,000 rpm) to dry the column membrane. The DNeasy column was then transferred to a 1.5 ml eppendorf tube and 100 µl of preheated (65 ºC) buffer AE was pipetted on it to elute the bound DNA directly out from the DNeasy column membrane. It was then incubated for 5 min at room temperature and centrifuged for 1 min at 8000 rpm to elute the DNA. The DNA was stored at 4 ºC for further use.

ISSR-PCR analysis and electrophoresis

ISSR-PCR reactions were performed using six primers (Table 1) according to Williams *et al*.[18]. The polymerase chain reaction (PCR) mixture (30 µl) consisted of 2.5 mM dNTPs, 25 mM MgCl, 10 pmol primer, 1U Taq DNA polymerase (Bioron, Germany) and 25 ng genomic DNA. The ISSR program was performed as 1 cycle of 94 ºC for 4 minutes followed by 45 cycles of 94 ºC for 1 minute and 57 ºC for 1 minute and a final extension step of 72 ºC for 10 minutes.

Visualisation of amplification products and data analysis

Reproducible bands visualized on agarose gels were scored using a binary code in a data matrix (1 and 0) for their present and absent respectively for the six primers. Fragments with same mobility were considered as identical, irrespective of the intensity of the fragment. Data were then computed with SPSS-10 program to produce a genetic distance matrix using Dice similarity coefficients[19]. A dendrogram was generated by cluster analysis using unweighted pair group method of the arithmetic averages (UPGMA).

Results and Discussion

In this work, the use of ISSR markers to determine genetic diversity among date palm grown in the NIFOR Experimental Substation, Dutse, Jigawa State was studied. The 6 primers used for ISSR analysis produce reproducible and scorable bands (Plates 1a -f). Amplification profiles were scored for the presence of polymorphism among the genotypes of the studied date palms. A total number of 310 ISSR bands were generated (Table 2). The maximum number of fragments was 10 bands, which was produced by HB-14 primer with 40 % polymorphism. The minimum number of fragments was 6 bands produced by primers 44B, HB-08 and HB-12 with 83.3 %, 66.6 % and 83.3 % polymorphism respectively (Table 2). The average was 7 bands per primer. Primers HB-10 generated the highest polymorphism (85.71 %) followed by primers 44B and HB-12 (83.33 %). The lowest polymorphism was generated from primer HB-14 (40 %).

The level of polymorphism among the studied date palms is important for the reliability of a molecular marker technique. And this is necessary to discriminate the genomic DNA of the 10 date palms studied (Figure 1). ISSR marker is a molecular marker based on inter-tandem repeats of short DNA sequences. These inter repeats are highly polymorphic, even among closely related genotypes, due to the lack of functional constrains in these non-functioning regions. Cluster analysis conducted to generate a dendrogram using ISSR markers (Figure 1) illustrated the relationships among the ten studied date palms. R4P2 and R5P7were separated from the rest of the studied date palms at a distance of 24.5and14.0 with R8P13 and Medjoole at a distance of 19.5 and 12.0 respectively in one sub-group. R5P7 and R6P8 showed that they are closely related at a distance of 10. R8P4 and R7P6 showed genetic similarity separated at a distance of 8.0. RCP1 (tissue culture date palm) and R8P1 also showed genetic similarity separated at a distance of 6.0. Tissue culture palm and Medjoole are closely related and separated at a distance of 2.0 with R8P4 and R8P6 also showed they are closely related and separated at a distance of 1.0 (Figure 1, Table 3). The cluster analysis shows that Tissue Culture palm and Medjoole date palm are the same date palms.

The ISSR profile for the six primers showed an average similarity matrix of 0.00-0.84(Table 3). R8P6 and R5P7 have the highest similarity coefficient value(0.84) which can be regrouped because they are near. Medjoole and Tissue culture date palms have a similarity coefficient value of 0.00 indicating that they are similar but distinct from the other date palms. A high level of similarity was also observed in the other date palms. This present study has helped to design ISSR technology to enlarge the number of molecular markers for genetic analysis of the Nigerian date palms varieties. Our result showed evidence of genetic diversity and interrelationship between the studied date palms. This study could also help to conserve in Nigeria where older date palms are endangered and need to be conserved. ISSR analysis showed a low level of polymorphism among the 10 date palms used for the study. The results obtained demonstrated the efficiency of ISSR markers for genetic variation among date palms grown in the same location in Nigeria. The results also revealed that the overall polymorphism among date palm genotypes identified by ISSR markers suggest that ISSR markers are very effective for assessing the molecular polymorphism of date palms grown in the same location.

Emoghene *et al*.[20] in the study of molecular characterization of some date palms in Nigeria using RAPD markers, reported that RAPD method is an alternative strategy to access phylogenetic relationship between sets of date palm varieties and Karim *et al*.[14] reported the efficacy of ISSR technology in determing genetic diversity. Also Hamza *et al*.[21] reported that molecular studies have proved the efficiency of molecular markers in assessing genetic diversity between date palm genotypes. Similar results were obtained by Ahmed *et al*[22].

In the work of Haider *et al*.[23], they reported close relationships among the cultivars they studied and that genetic polymorphism among them was narrow using RAPD and ISSR markers. The Dice similarity coefficient

Table 1: The nucleotide sequences of primers used for ISSR – PCR analysis

Marker	Primer code	Sequence 5′--- 3′	Annealing Temp. ^{0}C/Sec.
ISSR	14A	5′ CTC TCT CTC TCT CTC TTG 3′	57/60
	44B	5′ CTC TCT CTC TCT CTC TAG 3′	57/60
	HB – 08	5′ GAG AGA GAG AGA GG 3′	57/60
	HB – 10	5′ GAG AGA GAG AGA CC 3′	57/60
	HB – 12	5′ CAC CAC CAC GC 3′	57/60
	HB – 14	5′ GTC GTG GTGs GC 3′	57/60

Table 2: ISSR– PCR amplification products of DNA extracted from leaves of ten date palm cultivars using six ISSR primers

Primer	Sequence 5′--- 3′	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism (%)	Total number of band amplified
14A	5′ CTC TCT CTC TCT CTC TTG 3′	7	3	4	57.14	50
44B	5′ CTC TCT CTC TCT CTC TAG 3′	6	1	5	83.33	49
HB – 08	5′ GAG AGA GAG AGA GG 3′	6	2	4	66.66	43
HB – 10	5′ GAG AGA GAG AGA CC 3′	7	1	6	85.71	53
HB – 12	5′ CAC CAC CAC GC 3′	6	1	5	83.33	38
HB – 14	5′ GTC GTG GTG GC 3′	10	6	4	40.00	77
Total		42	14	28	416.17	310
Mean					69.36	

Table 3: Similarity Index of ISSR analysis

Date palms	1	2	3	4	5	6	7	8	9	10
1 RCP1	1.00									
2Medjoole	0.04	1.00								
3 R4P2	0.44	0.48	1.00							
4 R5P7	0.36	0.36	0.83	1.00						
5 R6P8	0.31	0.28	0.36	0.24	1.00					
6 R7P6	0.09	0.23	0.55	0.31	0.23	1.00				
7 R8P1	0.42	0.21	0.76	0.41	0.33	0.16	1.00			
8 R8P4	0.36	0.34	0.67	0.43	0.23	0.18	0.28	1.00		
9 R8P6	0.31	0.28	0.60	0.84	0.28	0.23	0.21	0.00	1.00	
10 R8P13	0.33	0.38	1.00	0.73	0.75	0.58	0.43	0.45	0.38	1.00

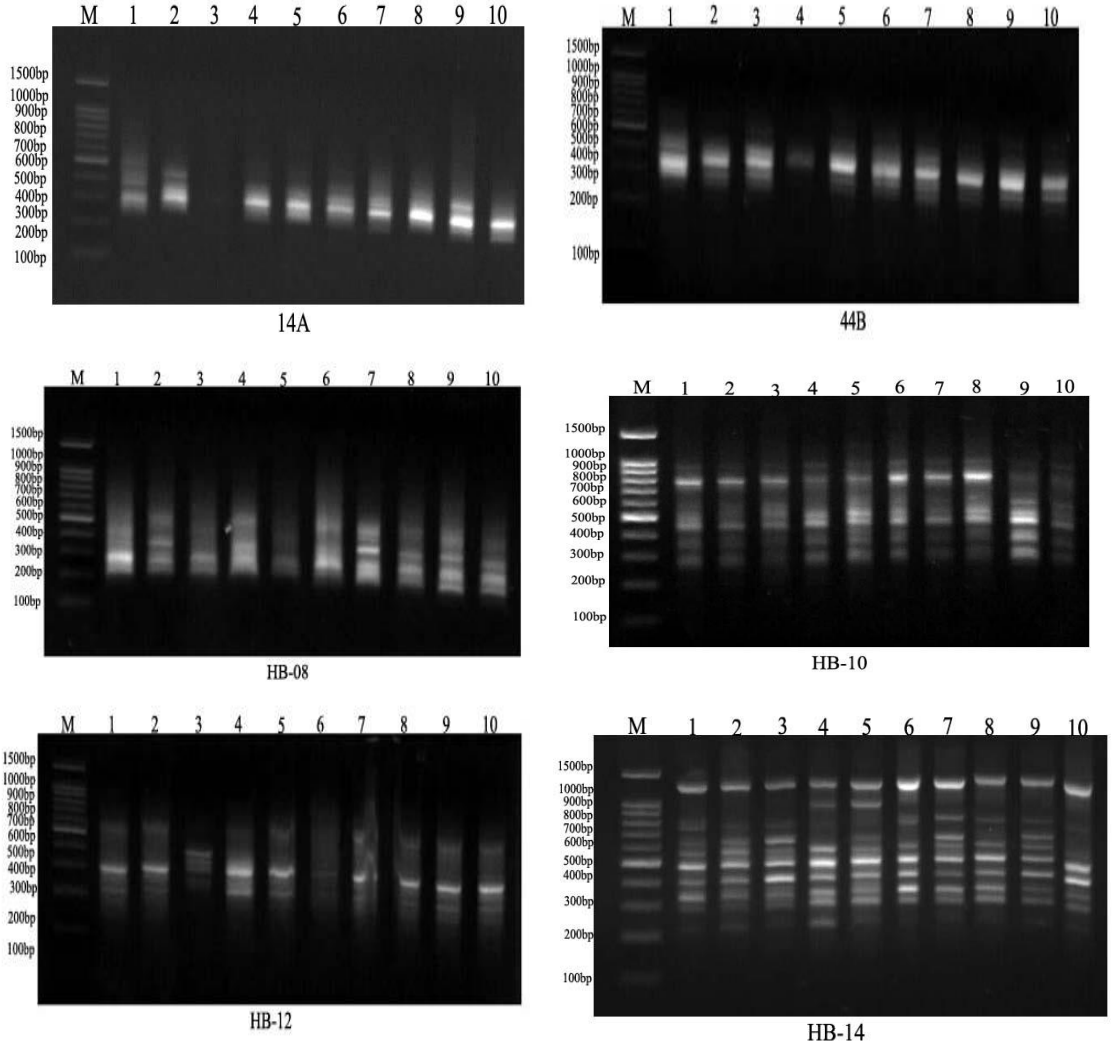

Plate 1: ISSR profile of ten date palm cultivars amplified with six different ISSR primers. (a) Primer 14A, (b) Primer 44B, (c) HB-08, (d) HB-10, (e) HB-12, (f) HB-14. M: 100bp ladder marker. Lanes 1 through 10 refer to date palm cultivars: RCP1 (tissue culture palm), Medjoole, R4P2, R5P7, R6P8, R7P6, R8P1, R8P4, R8P6 and R8P13.

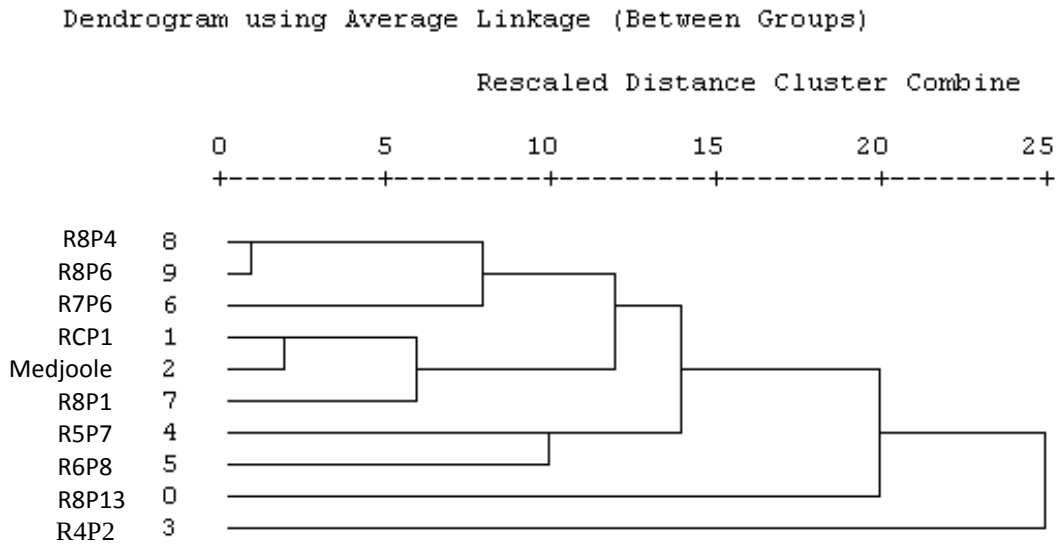

Figure 1: Dendrogram of nine Nigerian and one foreign date palm cultivars based on Dice genetic similarity coefficient using ISSR data.

matrix was used to draw a relationship among the date palms. The genetic variation between the studied date palms is as shown in Figure 1. RCP1 (tissue cultured palm) and medjoole were very closely related to each other which suggests that they could be considered same date palms. While R4P2 was in separate far group compared to the rest of the date palms. R7P6, R8P1, R8P4 and R8P6 may be regrouped together (Figure 1). R8P4 and R8P6 date palms have the lowest similarity (0.00) while the highest similarity (0.84) was observed between R5P7 and R8P6. Genetic similarity was observed between RCP1 (tissue cultured palm) and medjoole and R8P4 and R8P6. Also R4P2 and R8P6 have the highest genetic distance among the date palms. The result obtained from the ISSR analysis is in agreement with Sedra *et al*.[24] who reported morphologically that similar varieties are associated with the fruit quality, also stated that cultivars which originated from Algeria and Iraq did not show a far relation from Tunisian ecotypes, suggesting a narrow genetic diversity of date palms. Similarly, the study gave evidence of diversity and interrelatedness among the Nigerian date palms studied.

Conclusion

The ISSR markers have helped to determine genetic diversity among the studied date palms. It can be concluded that all date palm ecotypes are interrelated in spite of their agronomic divergence.

Conflict of interest

The authors declare no conflict of interest

Authors' declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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References

1. Asemota O, Eke CR, Emoghene BO, Aisueni NO, Adetunji OA, Gidado RM., Solomon BO. Investigation of somatic embryogenesis for *in vitro* cultures of date palm (*Phoenix dactylifera* L.). Acta Hort. 2010; 882:225 – 231.
2. Ahmed TA, Al-Qaradawi AY. Molecular phylogeny of Qatari date palm genotypes using simple sequence repeats markers. Biotechnol. 2008; 8(1): 126 – 131.
3. Akkak A, Scariota V, TorelloMarinoni D, Boccacci P, Beltramo C, Botta R. Development and evaluation of microsatellite markers in *Phoenix dactylifera* L. and their transferability to other *Phoenix* species. Biologia Plantarum. 2009; 53(1): 164-166.
4. Arabnezhad H, Bahar M, Mohammadi HR, Latifian M. Development, characterization and use of microsatellite markers for germplasm analysis in date palm (*Phoenix dactylifera* L.). Scientia Horticulturae. 2012; 134: 150-156.
5. Shinwari S, Mumtaz AS, Rabbani MA, Akbar F, Shinwari ZK. Genetic divergence inTaramira (*Eruca sativa* L.) Germplasm based on quantitative and qualitative characters. Pak J Bot. 2013; 45(SI): 375-381.
6. Khanam S, Sham A, Bennetzen JL, Aly MAM. Analysis of molecular marker-based characterization and genetic variation in date palm (*Phoenix dactylifera* L.). AJCS. 2012; 6(8): 1236-1244.
7. Srivashtav VS, Solanki VH, Patel HK, Kansara RV. Molecular Characterization of date palm (*Phoenix dactylifera*) using combined marker analysis grown in Kutch region of India. J Cell Tiss Res. 2014; 14(1): 4083-4088.
8. Eissa EA, Abdel-Razek AB, El-Sharabasy SF, Rizk RM. Morphological and molecular characterization of soft date palm (*Phoenix dactylifera* L.) cultivars in Egypt. Egypt J Genet Cytol. 2009; 38: 269 – 284.
9. Ibrahim IA, Hashem MH, Hemeida AA, Hassan MM, Maksoud AIA. Characterization of genetic diversity of Date palm (*Phoenix dactylifera L.*) cultivars collected from New valley governorate (El-Kharga and Dakhleh) based on Morphological variability and molecular markers. Life Sci J. 2014; 11 (11):879-889.
10. Hussein EHA, Adawy SS, Ismai SEM, El-Itriby Arab J Biotech HA. Molecular characterization of some Egyptian date palm germplasm using RAPD and ISSR markers.. 2004; 8(1): 83 – 98.
11. Adawy SS, Hussein EHA, El-Khishin D, Saker MM, Mohamed AA, El-Itriby HA. Genotyping Egyptian date palm cultivars using RAPD, ISSR, AFLP markers and estimation of genetic

stability among tissue culture derived plants. Arab J Biotech. 2004; 8(1): 99 – 114.
12. Moghaieb REA, Abdel-Hadi AA, Ahmed MRA, Hassan AGM. Genetic diversity and sex determination in date palms (*Phoenix dactylifera* L.) based on DNA markers. Arab J Biotech. 2010; 13(2): 143 – 156.
13. Abdulla M, Gamal O. Investigation on molecular phylogeny of some date palm (*Phoenix dactylifera* L.) cultivars by protein, RAPD and ISSR markers in Saudi Arabia. Australian J Crop Sci. 2010; 4(1): 23 – 28.
14. Karim K, Chokri B, Amel S, Wafa H, Richid H, Nourdine D. Genetic diversity of Tunisian date palm germplasm using ISSR marker. Int J Bot. 2010; 6(2): 182 – 186.
15. Al-Hadidi SH, Al-Agha HN, Ahmed TA. Genetic diversity of date palm in Qatar. Paper presented at Symposium on the Foundation Annual Research Forum. Proceedings of Energy and Environment, Qatar University, Doha, December 2010.
16. Zahedi S, Sakka H, Rhouma A, Ahmed AOM, Marrakchi M, Trifi M. Analysis of Tunisian date palm germplasm using simple sequence repeat primers. Agric J Biotech. 2004; 3(4): 215 – 219.
17. Khierallah HSM, Al-Sammarraie SKI, Mohammed HI. Molecular characterization of some Iraqi Date Palm cultivars using RAPD and ISSR markers. J Asian Sci Res. 2014; 4(9):490-503.
18. Williams JGK, Kubelk AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acid Res. 1990; 18: 6231-6235.
19. Dice L.R., Measures of the amount of ecologic association between species. Ecol. 945; 26: 297-302.
20. Emoghene BO, Asemota O, Idu M, Eke CR. Molecular Characterization of some Date Palms in Nigeria using RAPD markers. J Appl Biol Biotech. 2015; 3(05): 026-030.
21. Hamza H, Benabderrahim MA, Elbekkay M. Investigation of genetic in Tunisian date palm (*Phoenix dactylifera* L.) cultivars using ISSR marker systems and their relation with fruit Characteristics. Turk J Biol. 2012; 30:449-458.
22. Ahmed MM, Soliman SS, Elsayed EH. Molecular identification of Some Egyptian Date Palm males and females varieties (*Phoenix dactylifera* L.) using DNA markers. J Appl Sci Res 2006; 2(5); 270-275.
23. Haider N, Nabulsi I, MirAli N. Phylogenetic relationships among date palm (*Phoenix dactylifera* L.) cultivars in Syria using RAPD and ISSR markers. J Biol Res. 2012; 1(2): 12-24.
24. Sedra MH, Lashermes P, Trouslot P, Combes MC, Hamon S. Identification and genetic diversity analysis of date palm (*Phoenix dactylifera* L.) varieties from Morocco using RAPD markers. Euphytica 1998; 103: 75 – 82.