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Fingerprinting Egyptian Gramineae Species Using Random Amplified Polymorphic DNA (RAPD) and Inter-simple Sequence Repeat (ISSR) Markers

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Abstract: The Egyptian flora can be considered as a national treasure. The North Coast and the Siwa depression contain valuable bio-diversity of Gramineae plants. Breeding programs for crop improvement need to have access to the wealth of genes present in wild germplasms. Two types of molecular markers; random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR), were assayed to determine the genetic diversity of ten gramineae accessions from the North Coast of Egypt. In RAPD analysis, 11 primers initially screened displayed RAPD profiles with polymorphic bands, and many of these bands varied in molecular weight, and intensity. A total of 271 out of 289 bands were polymorphic, consisting of DNA fragments of molecular weights ranging from 212 to 1713 base pairs. The number of polymorphic bands scored per primer ranged from 2 to 8. No bands were found to be unique to the collected gramineae plants using Primers 11, 12 and 20. In ISSR analysis, 11 of the tested ISSR primers generated variable banding patterns. A total of 241 from 260 ISSR bands were polymorphic, among the ten plants. Dendrograms were constructed using UPGMA algorithm based on the similarity index values for gramineae plants. The dendrogram indicated that some plants were grouped together in one cluster. However, some species did not group together and fell in different clusters. The present study shows that RAPD and ISSR analyses are useful for generating candidate specific markers of Gramineae flora in North and North West Coast of Egypt. Genetic characterization not only provides database for genetic biodiversity, but also is a necessity for the protection of Egyptian royalties of the landraces.

Keywords: Gramineae, RAPD markers, ISSR markers, genetic diversity, phylogenetic relationship, Flora in Egypt.

INTRODUCTION

The flora in Egypt can be considered as a national treasure. Gramineae is one of the most important families; including several economic crops such as wheat and barley, as well as wild species which represent a valuable source of biodiversity. Wild Gramineae species growing at the North Coast and the Siwa Oasis are mostly exposed to a biotic stress factors such as drought, salinity and heat, and are adapted to such conditions.

Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations; different markers might reveal different classes of variations[13]. The advent of the polymerase chain reaction (PCR) favored the development of different molecular techniques such as random amplified of polymorphic DNA (RAPD), and inter-simple sequence repeat polymorphic DNA (ISSR), etc[8]. RAPDs proved to be useful as genetic markers in the case of self-pollinating species with a relatively low level of intraspecific polymorphism, such as hexaploid wheat[5] and cultivated barley[15]. ISSR markers, have become good DNA molecular markers for research on populations of the same species, and could be used to assess genetic diversity[11]. ISSRs have been used for cultivar identification in maize[9], potatoes[10], wheat[8], and barley[14].

The objective of this study is to determine the genetic diversity of ten gramineae accessions from the North Coast of Egypt using two types of molecular markers; random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR).

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MATERIALS AND METHODS

This study was done at the Genetics Department, Faculty of Agiculture, Alexandria University and Genetic Engineering & Biotechnology Research Institute, Sadat City, Menofia University.

Plant Materials: In this study ten gramineae species were collected from North and North West of Delta. Samples were identified and classified by the Horticulture Institute, Flora and Phyto taxonomy Researches, The Herbarium, Agricultural Research Center, Ministry of Agriculture, Dokky, Cairo, Egypt[7] (Table, 1).

DNA Extraction: Complete genomic DNA samples were extracted from fresh leaves using Plant Genomic DNA Mini-prep kit (V-gene Biotechnology limited).

RAPD and ISSR analysis

Selection of Primers: Eleven RAPD primers from twenty 10-mer oligonucleotides with arbitrary sequence were screened using three DNA samples and were found to be suitable for the study of all samples (Table, 2). Fifteen primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used in ISSR analysis. Eleven ISSR primers that produced clear and reproducible bands were selected for the amplification of all DNA samples (Table, 3).

PCR Reaction: The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L $MgCl_2$(Promega), 100µM/L of each dNTP (Promega), 66ng/µl Primer and 5 U/µl *Taq* polymerase in a 25µl volume. The amplification protocol was 94 ºC for 5min to pre-denature, followed by 5 cycles of 92 ºC for 30 Sec, 35 ºC for 2min and 72 ºC for 90 sec, followed by 35 cycles of 92 ºC for 30 Sec, 40 ºC for 30 Sec and 72 ºC for 90 Sec, with a final extension at 72 ºC for 5 min, and eventually stored at 4 ºC[4]. The amplified products were electrophoresed in 1% agarose gel with 0.5x TBE buffer. After the gel had been stained with ethidium bromide, band patterns were visualized with a UV transilluminator.

Data analysis, RAPD and ISSR data were scored for presence (1), or absence (0) by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. A similarity dendrogram among the ten Gramineae species was produced using UPGMA cluster analysis program.

RESULTS AND DISCUSSION

Results:

RAPD Analysis: Agarose gel electrophoresis for the 11 RAPD-PCR amplified DNA products of gramineae species are shown in figure (1). Among the 20 primers tested, 11 proved useful to characterize the samples (Table, 2). The results revealed that, the 11 useful primers gave 271 amplified bands from a total of 289 bands detected ranging from 212 to 1713 bp. Primer No. 11 had the highest total number and polymorphic number of different PCR bands (56 and 56 bands), respectively, while primer No.13 showed the lowest total number and also polymorphic number of bands (8 and 2 bands), respectively (table, 4). The highest number of common fragments was 12 bands using the primer No.11. The lowest number of common fragments was two bands with primer No.13.

The total specific bands for all species produced with the 11 primers used produced a total of 22 specific bands in the ten species studied. The highest total number of specific bands was five bands using primer No.6. The highest number of specific bands was four with sample No. 27, when primer No. 1 was used. No specific bands were revealed with primers No. 11, 12 and 20 (table, 5).

ISSR Analysis: To quantify ISSR polymorphism, the data obtained were constructed as a matrix of the binary character states. The presence or absence of amplified fragments of a certain size in ISSR patterns was considered as state 1 or 0, respectively. The ISSR primers amplified 13 to 32 bands that ranged in size between 103 and 1000 bp (Fig 2). Among the 15 primers tested, 11 proved useful to characterize the samples, whereas 4 were excluded due to amplification of the same single fragment in other samples or to amplification of few fragments in some samples. The 11 primers chosen for analysis produced a total of 241 polymorphic fragments, ranging from 137 to 1000 bp, among a total of 260 ISSR bands. The highest number of polymorphic loci (32 polymorphic bands) was exhibited with primer S12 and the lowest number (9 polymorphic bands) was with primer S4 (Table, 6). Table (7) shows a total of 32 specific bands among eight of the ten species used in this study.

Phylogenetic Relationship: The relationships within and between groups were estimated by a UPGMA cluster analysis of GS matrices (Figures 3&4). It indicated that all ten Gramineae samples could be distinguished by both RAPD and ISSR markers. Figure (3) is the dendrogram based on RAPD data. Three species (G6, G24 and G27) were less related with the other species, and divergent from each other. Two subgroups were evident for the remaining seven species. The first subgroup contained four species (G5, G8, G26 and G10). The second subgroup consisted of 3 species (G7, G23 and G3). The species were more closely related with each other within the second subgroup more than the first subgroup.

Table 1: Origin of ten wild Gramineae samples from North Coast of Egypt

Code No.	Latin name	Site of collection				
		Km. at Alex.-Matroh costal road	GPS			
G3	*Lolium multiforum* Lam.	30	N	30	53	10
			E	29	22	07
G5	*Bromus diandrus Roth.*	60	N	30	48	07
			E	29	05	34
G6	*Stipa capensis* Thunb	60	N	30	48	07
			E	29	05	34
G7	*Polypogon monspeliensis* (L.) Desf	120	N	31	00	09
			E	28	33	08
G8	*Schismus barbatus* L. Thell	180	N	31	03	12
			E	28	19	28
G10	*Aegilops ventricosa* Tausch	Km. 80 Matroh-El Salom Road	N	31	21	29
			E	26	43	12
G23	*Paspalum paspaloides* (Michx.) Scribn	Alex.-Rashid costal Road	N	31	09	13
			E	29	55	28
G24	*Sorghum virgatum* (Hack). Stapf	Alex.-Rashid costal Rod	N	31	13	05
			E	30	08	01
G26	*Elymus elongates* (Host) Runem. ssp.haifensis (Meld.) Henein & Runem	Alex.-Rashid costal Rod	N	31	09	13
			E	29	55	28
G27	*Cenchrus echinatus* Torr.	Alex.-Rashid costal Rod	N	31	09	13
			E	29	55	28

Table 2: Code and sequence of eleven different random primers (10-mer oligonucleotides) used in the present study.

No.	OligoName:	SEQUENCE
1	Z-01	5'-TCT GTG CCA C-3'
2	Z-06	5'-GTC CCG TTC A-3'
3	Z-08	5'-GGG TGG GTA A-3'
4	Z-11	5'-CTC AGT CGC A-3'
5	Z-12	5'-TCA ACG GGA C-3'
6	Z-13	5'-GAC TAA GCC C-3'
7	Z-16	5'-TCC CCA TCA C-3'
8	Z-17	5'-CCT TCC CAC T-3'
9	Z-18	5'-AGG GTC TGT G-3'
10	Z-19	5'-GTG CGA GCA A-3'
11	Z-20	5'-ACT TTG GCG G-3'

Table 3: Code and sequence of eleven different ISSR primers used in the present study.

No.	OligoName	Code	SEQUENCE
1	ISSR 844A	S2	5'-CTC TCT CTC TCT CTC TAC-3'
2	ISSR 844B	S3	5'-CTC TCT CTC TCT CTC TGC-3'
3	ISSR 17898A	S4	5'- CAC ACA CAC ACA AC -3'

Table 3: Continued

4	ISSR 17898B	S5	5'- CAC ACA CAC ACA GT -3'
5	ISSR 17899A	S6	5'- CAC ACA CAC ACA AG-3'
6	ISSR 17899B	S7	5'- CAC ACA CAC ACA GG-3'
7	ISSR HB-9	S9	5'- GTG TGT GTG TGT GG -3'
8	ISSR HB-10	S10	5'- GAG AGA GAG AGA CC -3'
9	ISSR HB-11	S11	5'- GTG TGT GTG TGT CC -3'
10	ISSR HB-12	S12	5'- CAC CAC CAC GC -3'
11	ISSR HB-15	S15	5'- GTG GTG GTG GC -3'

Table 4: The total number of polymorphic bands for each of the 10 gramineae species, using 11 RAPD primers

No. of primer	Total No. of bands										Total bands	Polymorphic bands
	G3	G5	G6	G7	G8	G10	G23	G24	G26	G27		
Z01	2	2	3	4	1	1	2	5	1	6	27	24
Z06	3	1	5	2	3	4	2	7	3	2	31	31
Z08	5	1	6	3	3	1	3	4	2	3	30	29
Z11	4	7	4	7	6	8	8	3	7	2	56	56
Z12	1	2	4	5	2	4	2	3	4	1	28	26
Z13	1	1	2	1	1	1	0	1	1	1	8	2
Z16	0	0	1	2	0	2	1	3	4	0	13	11
Z17	2	1	3	3	0	0	6	3	2	3	23	22
Z18	5	2	4	2	3	5	4	4	0	6	35	35
Z19	0	0	3	0	1	2	1	2	1	4	14	11
Z20	3	2	3	2	2	3	2	3	4	0	24	24
Total	25	16	37	31	22	31	31	38	29	28	289	271

Table 5: The Molecular weight and total number of specific polymorphic bands in each of the Gramineae species, using 11 RAPD primers

No. primer	Molecular weight of specific bands										Total specific bands in each primer
	G3	G5	G6	G7	G8	G10	G23	G24	G26	G27	
Z01	-	-	-	-	-	-	-	-	-	1250 1138 982 807	4
Z06	1056	-	-	-	-	307	-	1489 1416 1230	-	-	5
Z08	-	-	1713	--	-	-	-	-	-	-	1
Z11	-	-	-	-	-	-	-	-	-	-	0
Z12	-	-	-	-	-	-	-	-	-	-	0
Z13	-	-	884	-	-	-	-	415	597	-	3
Z16	-	-	-	900	-	-	-	-	382	-	2
Z17	-	-	400	-	-	-	-	681	-	-	2
Z18	900	-	-	-	288	-	-	-	-	-	2
Z19	-	-	815	-	696	-	-	-	-	1168	3
Z20	-	-	-	-	-	-	-	-	-	-	0
Total	2	0	4	1	2	1	0	5	2	5	22

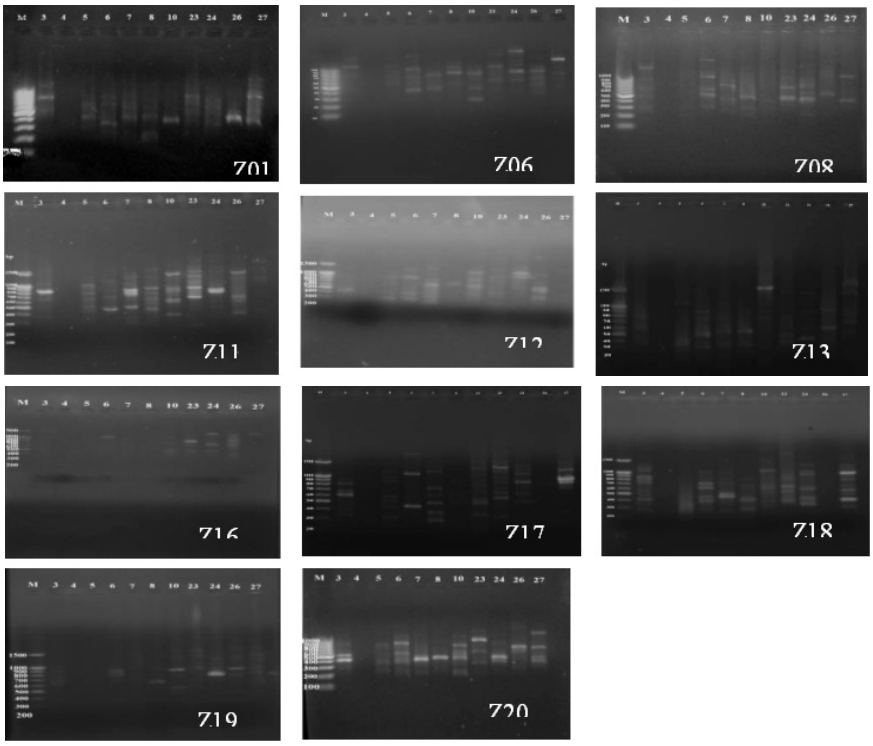

Fig. 1: RAPD profiles of ten Gramineae species (G3, G5, G6, G7, G8, G10, G23, G24, G26 and G27).

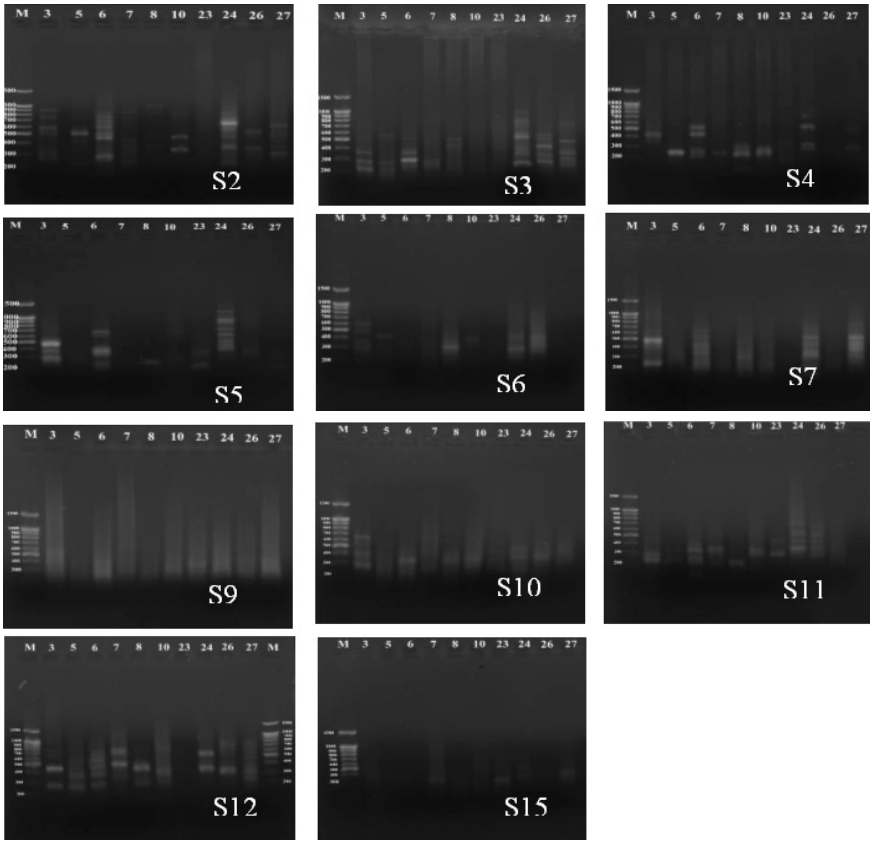

Fig. 2: ISSR profiles of ten Gramineae species (G3, G5, G6, G7, G8, G10, G23, G24, G26 and G27).

Table 6: The total number of polymorphic bands for each of the 10 gramineae species, using 11 ISSR primers

No. of primer	Total No. of bands										Total bands	Polymorphic bands
	G3	G5	G6	G7	G8	G10	G23	G24	G26	G27		
Pr. S2	4	1	9	1	1	2	0	8	2	2	30	27
Pr.S3	5	0	5	1	2	0	1	8	6	4	32	30
Pr.S4	1	1	3	1	2	1	0	4	0	0	13	9
Pr.S5	3	0	4	0	1	0	2	7	1	0	18	16
Pr.S6	2	1	0	0	2	1	0	3	4	1	14	11
Pr.S7	3	1	7	0	4	2	0	7	0	4	28	27
Pr.S9	3	0	4	4	2	4	3	4	2	3	29	29
Pr.S10	4	2	2	3	3	2	1	2	2	3	24	23
Pr.S11	3	0	2	1	1	1	2	6	5	3	24	21
Pr.S12	2	4	5	3	2	4	2	2	2	6	32	32
Pr.S15	2	0	0	3	2	0	2	3	2	2	16	16
Total	32	10	41	17	22	17	13	54	26	28	260	241

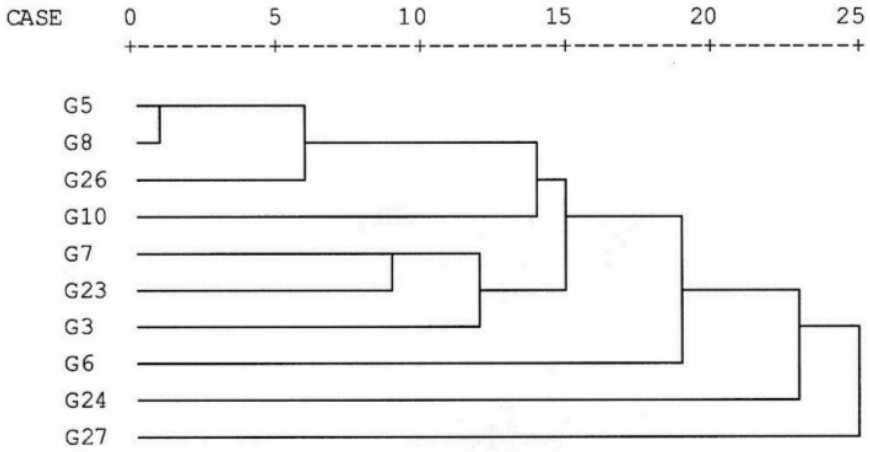

Fig. 3: Dendrogram for the phylogenetics relationships among the ten Gramineae species based on RAPD data.

The presence/absence of the obtained markers was the basis for the creation of ISSR computer databases required for the estimation of genetic distance between species, and their relatedness (Fig. 4).

Three large genetic groups were noted for the investigated gramineae species (Fig. 4). The first group contained five species, four (G5, G7, G8 and G10) of them where from Matroh road. The second one consisted of three species, two were from Alex-Rashid costal road. The third group contained two species, one from Alex-Matroh costal road and the other from Alex-Rashid costal road.

The ISSR data analysis of gramineae species confirmed the close similarity within the group (Fig. 4) comprising species germinated in the same region. Very close relatedness was observed within the first subgroup, whereas the species were more diverse within the second and third subgroups.

Discussion: It is clear that numerous species-specific markers are required for documentation of plant species. This study demonstrates that molecular markers are needed to identify plant species. DNA molecular markers have received considerable attention in recent years. ISSR have been utilized in gramineae plants to identify markers associated with seed size in wheat[1] and fingerprinting in rice[3] as well as in other crops, e.g., in fingerprinting cashew[2].

Three areas of plant genetic resources conservation can usefully be distinguished; locating and describing the available diversity, developing effective conservation procedures, and identifying materials

Table 7: The Molecular weight and total number of specific polymorphic bands in each of the Gramineae species, using 11 ISSR primers

No. primer	Molecular weight of specific bands										Total specific bands
	G3	G5	G6	G7	G8	G10	G23	G24	G26	G27	in each primer
Pr. S2	799	-	-	-	891	-	-	-	-	-	2
Pr. S3	103	-	-	-	-	-	-	823 689	-	-	3
Pr. S4	-	-	-	-	-	-	-	606	-	-	1
Pr. S5	-	-	-	-	-	-	-	982 875	-	-	2
Pr. S6	378	-	-	-	-	-	-	137	674	-	3
Pr. S7	-	-	675	-	-	-	-	810	-	-	2
Pr. S9	-	-	234	-	-	-	-	-	-	605	2
Pr. S10	-	-	-	-	-	-	-	-	-	-	0
Pr. S11	-	-	-	-	-	-	-	804	-	593 433	3
Pr. S12	-	-	-	-	-	-	-	-	-	-	0
Pr. S15	736 367	-	-	1000 444 329	222	-	275	600 472 387	586 467	486 728	14
Total	5	0	2	3	2	0	1	11	3	5	32

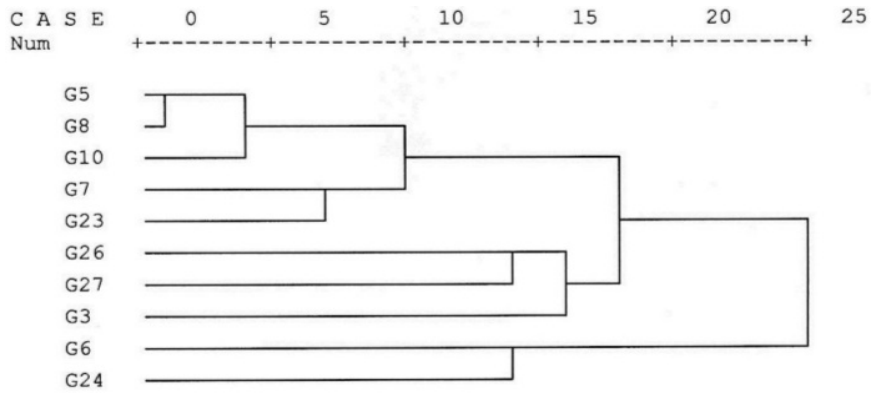

Fig. 4: Dendrogram for the phylogenetics relationships among the ten Gramineae species based on ISSR data.

and methods that are needed by users. Genetic information plays a significant part in determining the effectiveness of work in all of these areas, and molecular markers now provide an excellent way of obtaining large amounts of genetic data to inform the conservation process.

Roodt *et al*[12] identified different cultivars of *Cynodon dactylon* (L.) Pers. RAPD analyses of some-well known cultivars used in South Africa, as well as 10 potential new cultivars, was done. Only five primers were needed to obtain a specific fragment pattern for each cultivar.

The results found in this study were equivalent with that found by Li *et al*,[6]. RAPD and ISSR were applied to detect the level and pattern of genetic diversity of *Monochoria vaginalis* in seven populations from southern China. Among these populations, 116 bands were amplified by 18 RAPD primers, of which 34 bands (29.31%) were polymorphic, and 14 ISSR primers produced 111 bands with 87 polymorphic bands (78.38%). Within each population, a relatively low level of genetic diversity was detected by both RAPD and ISSR analyses. Analysis of molecular variance of the data from the RAPD and ISSR markers detected that the majority of total genetic variation existed among populations (73.50% and 76.70% respectively) and only minor genetic variation within populations (26.50% and 23.30%

respectively). Cluster analysis divided the seven populations into two groups, indicating that the genetic relationships among populations have relatively low correlation with their geographical distribution (Mantel test; r = 0.45 and 0.48 respectively). ISSR markers were more efficient than the RAPD assay. The Mantel test gave r = 0.16, suggesting no correlation between these two molecular markers. The present study demonstrates that genetic characterization using RAPD and ISSR are essential for species identification and provide database for genetic biodiversity as well as for plant genetic resources conservation.

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