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GENETIC DIVERSITY OF SIX DIFFERENT EGYPTIAN FORAGE CROPS BY MOLECULAR TOOLS

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ABSTRACT

The aim of the present study was to estimate genetic variation in grass sorghum (sorghum, Tunis grass, Sudan grass) and other forage species (ray grass, pearl millet, guar). Three molecular systems i.e. isozymes-PAGE, seed protein, SDS-PAGE and AFLP were used. The results indicated the presence of high similarity matrix among sorghum and ray grass; pearl millet and Sudan grass (100%) and low similarity matrix among guar and sorghum; guar and Tunis grass (44.4%) in peroxidase analysis, where the total bands was 21 with 100% polymorphism and without any unique bands. This data was different comparing with superoxidase dismutase, protein and AFLP analysis. On the other hand, the combined set data of three molecular system revealed that Tunis grass have similarity matrix in a value of (70.6%) with sorghum and (90.7%) with Sudan grass.

Also, Tunis grass gave similarity matrix of (89.7%), (87.9%) and (71.2%) with ray grass, pearl millet and guar, respectively. In cluster analysis with combined data set, Tunis grass was located in sub cluster 1 with sudan grass and pearl millet in the same group, and in the same cluster of ray grass. The different clusters were found between Tunis grass, sorghum and guar. Tunis grass did not give any unique bands with all analysis but sorghum, guar and ray grass gave unique bands with protein and AFLP analysis.

Keywords: Forage crops, sorghum, diversity, molecular profile, Cluster analysis

INTRODUCTION

Grass sorghums include Sudan grass and Tunis grass are annuals and grow quickly and are generally used for summer pasture. Johnson grass, a perennial grass sorghum, is considered a pest when it is out of control, however, it makes an excellent hay for cattle feed. For human consumption sorghum is used for its grain and a syrup depending on the type grown. Sorghum is considered to be a native to tropical Africa, continues to be a leading cereal grain in the most areas of the continent, and is a major staple food and fodder crop grown worldwide, with an annual average production of 61 million tones over the past decade (FAO, 2005). Moreover, according to FAO, sorghum ranks fifth in world grain production behind wheat, rice, maize, and barley.

Sorghum (*Sorghum bicolor* ssp. *bicolor*) as a traditional cultivars were classified by Harlan and De Wet (1972) into five main races (*bicolor*, *caudatum*, *durra*, *guinea*, kafir) and 10 intermediates (e.g. bicolor-caudatum, durra-kafir), mainly on the basis of spike let and grain morphology. Snowden (1936) defined 7 weedy, 13 wild, and 28 cultivated species and numerous varieties and forms from within this variability. A refinement of Snowden's

classification was developed by Jakuševskij (1969) and is still used in some parts of the world (Fritsch *et al.*, 2001). De Wet and Huckabay's (1967) classification of *S. bicolor* separated the perennial plants as "*S. bicolor* subsp. *halepense*"; from the annual plants of this complex where they were combined into *S. bicolor* subsp. *bicolor*, treating the cultivated members as *S. bicolor* var. *bicolor* and partitioning the wild and weedy relatives into three varieties, *S. bicolor* vars. "*arundinaceum*", "*aethiopicum*", and "*verticilliflorum*" *(Piper, 1915)*. For the same reason as before, these varietals names were also not validly published. To these three varieties, assumed to have been established by De *Wet et al. (1970)* was added a fourth, "*S. bicolor* var. *virgatum* (Hack.) as well as "*S. virgatum* (Hack.) Stapf according to John and Jeff (2007). In Egypt, Tunis grass is a new forage crop and it was identified by the Egyptian flora and Phytotaxonomy Research Department, Agriculture Museum, Dokki, Giza, Egypt. It has a high fresh and dry yield with follow condition, cutting in 120 cm and fertilized 80.2 kg N per hectare for three cuts Abdel-Aziz and Abdel-gwad (2008).

In sorghum breeding and genomic resources are less than the other major cereals as rice, wheat, maize and barley according to economic values. However, when interest has focused on the crop due to its drought resistance and small genome size (~760 Mb) compared to close relatives maize (~2500 Mb) and sugarcane (2550 to 4200 Mb). In recent years, the potential of sorghum as a biofuel crop has led to additional investment culminating in the sequencing of the sorghum genome (Bowers *et al.*, 2007). Many molecular marker technologies have been developed and applied to studying patterns of genetic diversity in grass sorghums germplasm collections and in breeding programs (Ferreira, 2005) and Kawar *et al.* (2009). Progress in sorghum characterization of the transcriptome has been paralleled by identification of differential gene expression in response to biotic and a biotic factors, including green bug feeding Park *et al.* (2006). Pratibha Brahmi *et al.* (2004) analyzed the genetic diversity in cultivated guar using allozyme polymorphism and compare it with reported morphological diversity. As well as, Lamy *et al.* (1994) are using pearl millet molecular markers to follow the introgression of genomic segments from the wild progenitors of this crop into several populations based on crosses of wild and cultivated accessions from various parts of western and central Africa. Finally, Ruby Tiwari, *et al.* (2009) established allergenic cross reactivity between the members of the Pooids (*Lolium perenne*, *Phleum pratense*, and *Poa pratensis*) and Chloridoids (*Cynodon dactylon* and *Paspalum notatum*). In the present study, the variation of grass sorghum and other forge species were estimate by molecular tools.

MATERIALS AND METHODS

Plant Materials

This work was carried out in collaboration between the Agricultural Research Center, Field Crops Research Institute, Forage crops research department; National Research Centre, Division of Genetic Engineering and Botany Department, Faculty of Agriculture, Suez Canal University, during two

years 2009 and 2010. Seeds of different six Egyptian forage crops have been obtained from Forage Crops Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. The list of these forage crops is presented in Table I.

Table 1: The common and botanical name, Chromosome number, wild type, season and Egyptian location.

No	Forage crop		Chromosome number Somatic cells polyploidy	Wild type	Season	Egypt location
	Common name	Botanical name				
1	Sorghum	*Sorghum bicolor*	20**	imported	Summer	all
2	Sudan grass	*Sorghum vulgare var. sudanense*	20**	found	Summer	all
3	Tunis grass	*sorghum virgatum*	20**	found	Perennial	north
4	Ray grass	*Lolium multiflorum*	14*	imported	Winter	north
5	Pearl millet	*Pennisetum glaucum*	14#	imported	Summer	all
6	Guar	*Cyamopsis tetragonoloba*	14##	found	Summer	upper

**Klm *et al.*(2005), * Ahloowalia (1965),# Techio et al.(2006) ## Bewal et al.(2009)

Isozyme Analysis

Isozymes extraction from the six cultivars by homogenizing 0.5 g fresh leaves and roots samples in 1 ml extraction buffer (10% glycerol) using a mortar and pestle. The extract was then transferred into clean eppendorf tubes and centrifuged at 10000 rpm for 5 minutes according to *Stegemann et al. (1985b).* The supernatant was transferred to new clean eppendorf tubes and kept at –20 °C until needed for electrophoretic analysis. A volume of 40 µl extract of each sample was mixed with 20 µl sucrose and 10 µl bromophenol blue, then a volume of 50 µl from this mixture was applied to each well. The run was performed at 150 volt until the bromophenol blue dye reached the separating gel and then the voltage was increased to 200 volt. Electrophoresis apparatus was placed inside a refrigerator during running duration. After electrophoresis, the gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions, and then incubated at room temperature in dark for complete staining for about 1 to 2 hours. Gel was placed into this solution and 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear *(Brown, 1978).*

Protein Analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the banding patterns of the six cultivars. Protein fractionation was performed on vertical slab (16.5 cm x 18.5 cm x 0.2 cm) Hoefer E600, Amersham Pharmacia biotech. According to the method of Laemmli *(1970)* as modified by Studier (1973), Sample extraction buffers (Tris-HCl buffer, pH 7.5) *(*Jonathan and Weaden, 1990*)* and Staining solution Coomassie briliant blue-R250 staining solution was well mixed and kept at room temperature in a dark bottle.

The lower buffer tank was filled with running buffer and attached with upper buffer tank, so that the gels were completely covered. Gels were distained with 350 ml distaining solution. The distaining solution was changed

analysis. Ke ming *et al.* (1995) found that presence or disappearance of some peroxidase and esterase isozyme bands was related to wounding. Some isoperoxidase bands disappeared at the time of vascular tissue formation. Marie and Harold (1971) found that each species pattern was unique, and no single peroxidase band was common to all the species. Based on considerations of interspecific cross compatibility and chromosomal rearrangements the taxonomic division of the genus into three sections was further subdivided to give five groups. Jean *et al.* (1971) have demonstrated that peroxidase was a single polypeptide chain and that subunit association was not involved in the isoenzyme system. On the basis of tryptic peptide maps, it was apparent with the peroxidase isozymes. Isozymes within a group appeared to possess very similar primary structures. Whether more than one gene is involved in their biosynthesis cannot be ascertained at present. The catalytic properties of the isozymes followed an identical pattern.

So, the genetic variation among six species in peroxidase activity due to more than one gene is involved in their biosynthesis and chromosomal rearrangements.

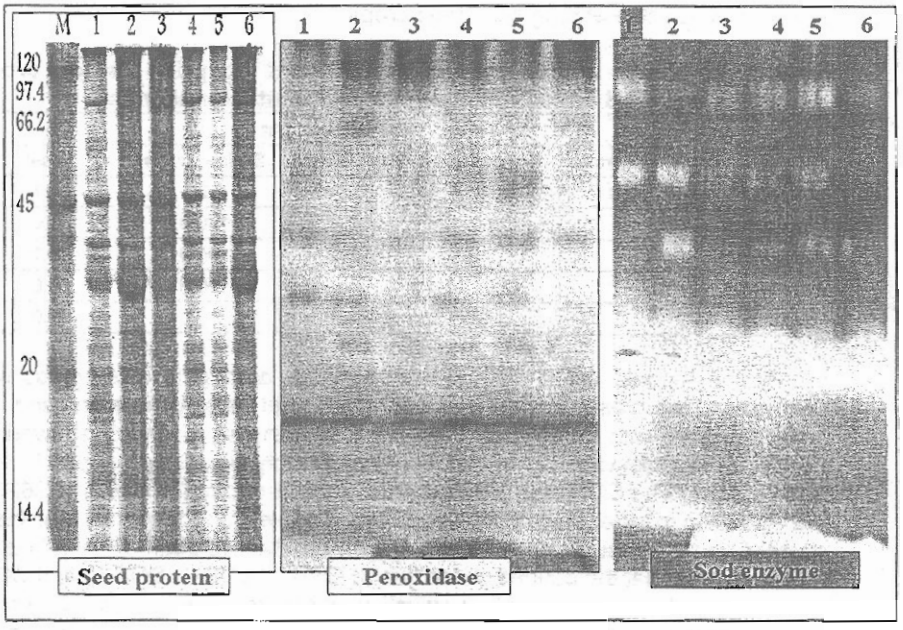

Figure 1: Isozymes (Peroxidase and Total superoxide dismutase (Sod)) and seed protein analysis of six different Egyptian forage crops (lane 1 in seed protein : M. marker; 1- 6 in seed protein , peroxidase, and Sod isozymes sorghum [Tunis grass, Ray grass, Pearl millet, Sudan grass and Guar].

Table 2: Similarity matrix among six different Egyptian forage crops were used based on peroxidase analysis.

Proximity Matrix					
Case	Matrix File Input				
	Tunis grass	Ray grass	Pearl millet	Sudan grass	Guar
Sorghum	33.3	100.0	75.0	75.0	40.0
Tunis grass		33.3	75.0	75.0	40.0
Ray grass			75.0	75.0	40.0
Pearl millet				100.0	57.1
Sudan grass					57.1

Superoxide dismutase isozyme (Sod):

The results in total superoxide dismutase (Sod) activity gave 15 bands as a total bands which were divided into 6 bands monomorphic and 9 polymorphic in 60 % polymorphism as shown in Table 7 and Figure 1. In Table 3, it revealed that guar forage crops have the same similarity matrix value (50%) with Tunis grass, ray grass, pearl millet and Sudden grass. This value is lowest value among six forage crops.

Table 3: Similarity matrix among six different Egyptian forage crops were used based on superoxide dismutase analysis.

Case	Matrix File Input				
	Tunis grass	Ray grass	Pearl millet	Sudan grass	Guar
Sorghum	80.0	80.0	80.0	80.0	66.7
Tunis grass		1.000	100.0	100.0	50.0
Ray grass			100.0	100.0	50.0
Pearl millet				100.0	50.0
Sudan grass					50.0

The highest value 100 % was repeated among Sudden grass with Tunis grass, ray grass and pearl millet; pearl millet with tunis grass and ray grass; ray grass with tunis grass. The result of Wang *et al.* (2006) showed an obvious and stable variation in the isozyme phenotypes in two different pearl oyster species. The SOD and EST isozymes from gill and MDH, ME and G6PDH from adductor muscle were species-specific. The electrophoretograms of these isozymes could be used as markers to differentiate the two pearl oysters. Li *et al.* (1995) reported that the banded characters at EST- 1, SOD-1, SOD-2, and SOD-3a loci may be used as biochemical markers to identify the *R. kamoji* chromosomes carrying these loci in a *T. aestivum* ×*R. kamoji* hybridization program. The lowest similarity with the rest of the species was in accord with the morphological studies (Khatamsaz, 1998) and other numerical taxonomic works (Sneath, and Sokal, 1973), (Pooler Simon, 1993) and (Sheidai *et al.*, 2000) So, the considerable molecular diversity which could be found among six forage crops were obvious by isozymes Sod.

Seed protein analysis (SDS-PAGE):

The results show that the protein gave 25 bands in total 111 bands for all forage crops from 113- 11 KDa. In Table 7, Data revealed 66 bands monomorphic, 45 bands polymorphic and 40.5 % polymorphism. The unique bands were found in 28 KDa by ray grass, 63KDa by sorghum and 138 KDa by guar as advantage for this forage crops. In Table 4, high similarity matrix was found to be 94.7% between pearl millet and ray grass, also the same ratio between ray grass and Tunis grass.

Table 4 : Similarity matrix among six different Egyptian forage crops were used based on seed protein analysis.

Proximity Matrix					
Case	Matrix File Input				
	Tunis grass	Ray grass	Pearl millet	Sudan grass	Guar
Sorghum	70.6	66.7	64.7	74.3	83.3
Tunis grass		94.7	94.4	91.9	78.9
Ray grass			94.7	92.3	80.0
Pearl millet				91.9	78.9
Sudan grass					82.1

On other hand, the tunis grass and sorghum have the lowest value of 70.6%. while it gave 91.9% with sudden grass, 94.4% with pearlmillet, 94.7 % with ray grass and 78.9 with guar.

AFLP Analysis:

AFLP genetic relatedness among different six forage crops ;one primer combinations produced a total of 172 scored bands for the 106 polymorphic bands, 66 monomorphic bands and 61.6% polymorphism which reflects the ability of system to be different among materials under study. As well as, the unique bands which appeared in guar and sorghum as follow 290bp, 810bp, 210bp and 660bp, respectively (Figure 2 and Table 7). Moreover, similarity matrix among six different Egyptian forage crops revealed that the highest similarity was 91.2% among tunis grass and ray grass as well as tunis grass and sudden grass followed by sudden grass and ray grass. In this respect, the lowest similarity was 69.1% between guar and pearl millet followed by 70.2% between guar and Tunis grass Table 5.

The dendrogram would explain the differences among the six different Egyptian forage crops which had the same sprite in protein and AFLP systems as shown in Fig. 3.

The dendrogram based on AFLP, Figure 3, one primers divided into two main clusters distribution of the six different Egyptian forage crops, sorghum and Guar were placed in the cluster 1, while, the second cluster involved the rest of Egyptian forage crops. The second cluster subdivided further into subclusters. The first subcluster included sudden grass only. The second subcluster included Tunis grass, Ray grass and Pearl millet.

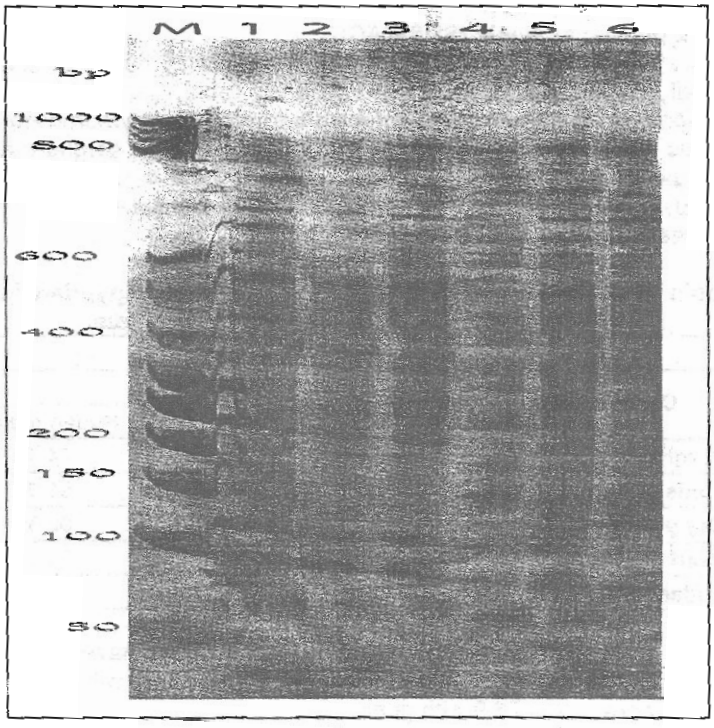

Figure 2: Analysis of six different Egyptian forage crops by AFLP; lane 1 in AFLP: M. marker;1- 6 in AFLP gel: sorghum, Tunis grass, Ray grass, Pearl millet, Sudan grass and Guar.

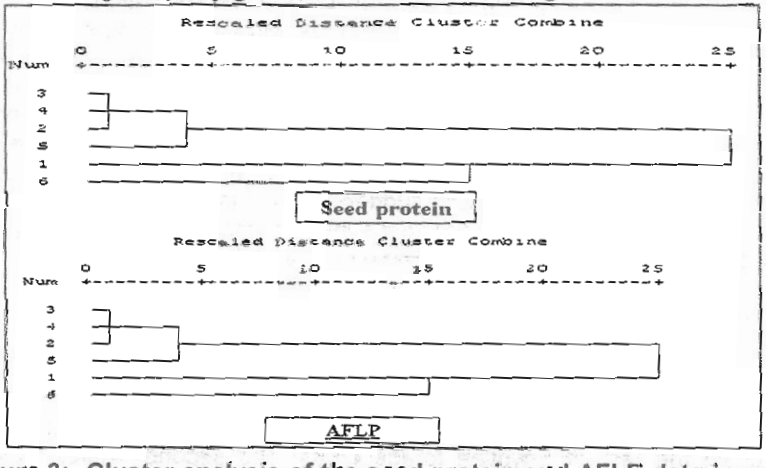

Figure 3: Cluster analysis of the seed protein and AFLP data in case of the of six different Egyptian forage crops; 1- 6: Sorghum, Tunis grass, Ray grass, Pearl millet, Sudan grass and Guar.

Table 5: Similarity matrix among six different Egyptian forage crops were used based on AFLP analysis.

Case	Matrix File Input				
	Tunis grass	Ray grass	Pearl millet	Sudan grass	Guar
Sorghum	73.7	73.3	65.5	80.0	80.0
Tunis grass		91.2	88.5	91.2	70.2
Ray grass			83.6	90.0	73.3
Pearl millet				87.3	69.1
Sudan grass					76.7

Combined data: The data in Table 6 and Figure 4 showed the genetic relationships among six different Egyptian forage crops as follow:

Table 6: Similarity matrix among six different Egyptian forage crops were used based on peroxidase, SOD, seed protein and AFLP analysis.

Proximity Matrix					
Case	Matrix File Input				
	Tunis grass	Ray grass	Pearl millet	Sudan grass	Guar
Sorghum	70.6	72.9	66.7	77.8	78.8
Tunis grass		89.7	90.2	90.7	71.2
Ray grass			87.9	90.3	73.4
Pearl millet				90.7	71.2
Sudan grass					76.4

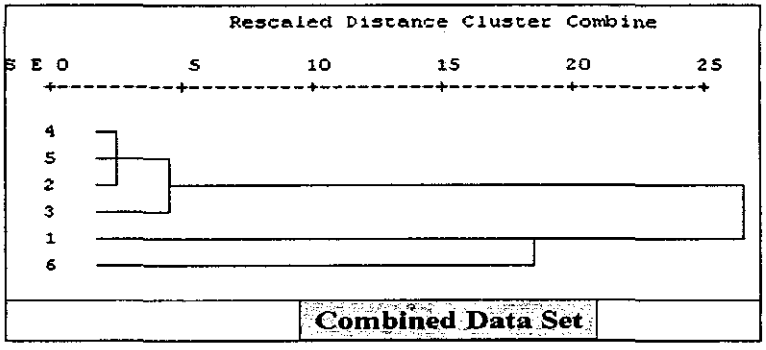

Fig. 4: Cluster analysis of the based on peroxidase, SOD, seed protein and AFLP analysis data in case of the of six different Egyptian forage crops;1- 6: 1=Sorghum, 2=Tunis grass, 3=Ray grass, 4 = Pearl millet, 5= Sudan grass and 6= Guar.

The similarity matrix among six different Egyptian forage crops were revealed a high similarity of 90.7% among Sudan grass and tunis grass as well as Sudan grass and pearlmillet followed by 90.3 % for Sudan grass and ray grass. While, the lowest similarity of 70.6% was found between Tunis

grass and sorghum followed by 71.2% between guar and Tunis grass as seen in Table 6. The dendrogram based on all systems in this study shown Figure 4, it divided the crops into two main clusters distribution of the six different Egyptian forage crops, sorghum and guar were placed in the cluster 1, while, the second cluster involved the rest of Egyptian forage crops. The second cluster subdivided further into subclusters. The first subcluster includes Sudan grass only and the subcluster two was distributed Tunis grass, Ray grass and Pearl millet. This could agree with the Indian gene centre possesses a rich genetic diversity in native grasses and legumes. There are reports of 245 genera and 1,256 species of Gramineae of which about 21 genera and 139 species are endemic. One-third of Indian grasses are considered to have fodder value. Most of the grasses belong to the tribes Andropogoneae (30%), Paniceae (15%), and Eragrosteae (9%). Similarly, out of about 400 species of 60 genera of Leguminosae, 21 genera are reported to be useful as forage. The main centers of genetic diversity are peninsular India (for tropical types) and North-Eastern Region (for sub-tropical types) besides some micro-centres for certain species (forage and grasses, 2008). The data in Table 7 illustrate the efficient of marker systems species diversity. AFLP system give the highest total number of bands 172 followed by protein 111 bands as total bands; while the lowest total bands in isozymes SOD followed by peroxidase enzyme 11 and 15 bands respectively. The polymorphic bands were highest in the AFLP followed by protein and Peroxidase with values, 105, 45 and 21 respectively. While, the polymorphism percentage were highest in peroxidase with value 100 % followed by AFLP and isozymes SOD with values 61.6 and 60, respectively.

Furthermore, the unique bands in Table 7 of sorghum, ray-grass and guar from all species had the unique bands which could differ from one specie to others. The isozymes in both peroxidase and SOD could not gave the unique band. The protein and AFLP could appeared unique bands. Sorghum species had two bands in AFLP with molecular weight 210 and 660 bp, as well as bands in protein which appeared in 63 kDa. On the other hand, guar was appeared to have three bands with values 810 and 290 in AFLP system and 138 kDa with protein system. Finally ray-grass gave one unique band in protein system which has 29 kDa.

Table 7: Levels of polymorphism and unique varieties-specific bands and status of it based on peroxidase, SOD, seed protein SDS-PAGE and AFLP analysis.

No.	Total bands	Polymorphic band	Monomorphic band	Polymorphism %	Unique bands	
					Genotypes	MW
POX	21	21	0	100	-	-
SOD	15	9	6	60	-	-
Protein	111	45	66	40.5	Ray grass	29 kDa
					Guar	138 kDa
					Sorghum	63 kDa
AFLP	172	106	66	61.6	Guar	290bp.
					Guar	810bp.
					Sorghum	210bp.
					Sorghum	660bp.

MW=molecular weight, (-)= unique bands not found

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تقدير درجة التباين الوراثي بين ستة محاصيل مصرية للعلف بإستخدام التقنية الجزيئية

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تهدف هذه الدراسة لتقدير التباين الوراثي لبعض أنواع السورجم (السورجم، حشيشة الفرس ، حشيشة السودان،) وغيرها من أنواع حشائش العلف مثل (حشيشة راي ، الدخن ، الجوار). بإستخدام ثلاثة أنظمة من التحليلات الجزيئية ، البروتين في البذور ، المشابهات الانزيمية و AFLP . ولقد أشارت النتائج إلى أن ارتفاع التشابه بين السورجم وحشيشة الراي ؛ الدخن والسودان العشب بنسبة (١٠٠ ٪) وانخفاض نسبة التشابه بين الجواروالسورجم الرفيعة ؛ الجوار وحشيشة الفرس إلى (٤٤.٤ ٪) في تحليل البيروكسيديز.

حيث كان عدد الحزم ٢١ مع نسبة تعدد مظهري ١٠٠ ٪ ودون أي حزم فريدة أو مميزة من نوعها. وكانت هذه البيانات غير متوافقة مع نتائج تحليل إنزيم ديسموتاز superoxidase والبروتين وتحليل AFLP.

من ناحية أخرى ، كشفت مجموعة البيانات المجمعة لثلاثة أنظمة جزيئية أن حشيشة الفرس تتشابه وراثياً بنسب (٧٠.٦ ٪) مع السورجم و (٩٠.٧ ٪) حشيشة السودان.

أيضا حشيشة الفرس أعطت تشابه وراثي (٨٩.٧ ٪) ، (٨٧.٩ ٪) و (٧١.٢ ٪) مع حشيشة الراي ، الدخن والجوار على التوالي. في التحليل المجمع مع مجموعة البيانات المشتركة ، ويقع حشيشة الفرس في المجموعة الفرعية ١ العشب مع حشيشة السودان والدخن في نفس المجموعة ، وفي المجموعة نفسها الراي جراس. وقد أدرجت مجموعات مختلفة بين تونس الذرة الرفيعة والحشائش والجوار. و لم تعطي حشيشة الفرس أي حزم فريدة من نوعها مع كل تحليل ولكن السورجم ، والجوار والعشب راي اعطيت الحزم الفريدة الأتية من نوعها مع البروتين وتحليل AFLP.

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