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Full Length Research Paper

Effects of space flight factors on genetic diversity of *Buchloe dactyloides* seeds

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The objective of this research was to investigate the effects of space flight factors on *Buchloe dactyloides* "Jingyin No.3" seeds. After the retrieval, basic turf characters of plants were tested. Among the 100 plants tested, 21 showed great change on phenotype characters, including leaf blade length and width, height, stem diameter, number of tillers, number and length of stolon, length of stolon inter node, leaf color and extent of leaf turning yellow. 33 primers were screened in inter-simple sequence repeats (ISSR) analysis to evaluate DNA variation between mutations and their ground controls. Results show that 15.6 reliable bands were generated by 7 primers, of which 12.9 (80.9%) were polymorphic. Based on the study, we can conclude that the space flight factors could induce inheritable mutagenic changes on *B. dactyloides* seeds, and do further research to demonstrate these changes in genetic material of the mutants.

Key words: Genetic diversity, *Buchloe dactyloides*, spaceflight, inter-simple sequence repeats.

INTRODUCTION

Genetic diversity is an important precursor in studies of many species because the amount and distribution are likely to affect the evolutionary potential of species and/or populations (Futuyma, 1986).

In recent years, the number of molecular assays available for application in this area has increased dramatically. Genetic variation of wide *Buchloe dactyloides* accessions and nature populations had been evaluated by randomly amplified polymorphic DNA (RAPD) (Huff and Smouse, 1993; Wu, 1991), microsatellites simple sequence repeats, (SSRs), inter-simple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) markers (Budak and Shearman, 2004a, b).

Effects of space flight factors on the plant have been a hot topic of investigation since the beginning of human space exploration. Within the past few decades, spaceflight mutation has already been applied successfully to the crops including rice (Ma et al., 2007; Li et al., 2007), wheat, cotton, tomato (Nechitailo et al., 2005), tobacco (Zheng et al., 2004), *Arabidopsis* (Paul et al., 2005) and maize (Mei et al., 1994, 1998) with a consequence that over 20 new cultivars have already been released (Wang et al., 2003, 2004).

For example, "Ganzaoxian 47", with significantly strong resistance to rice blast, was selected from progenies of spaceflight rice seeds (Li et al., 2001, 2002b); a male sterile mutant of maize which was useful to maize breeding was selected from the spaceflight induced seeds (Li et al., 2002a); "Teyouhang No.1" and "IIYouhang No.1" were two super-high-yielding hybrid rice obtained by recoverable satellite-flown, whose protein contents were 1.9 and 2.0% higher than that of the control ("IIYouming86") (Xie et al., 2004).

To obtain new germplasms by the recoverable satellite launch, a great number of phenotypes have been observed and compared between the spaceflight mutants and ground control. It showed the space treatment could promote these traits such as height, leaf blade width, number of leaves, germination percentage of seed, seedling emergence and growth in field. Yu et al. (2007) found that in rice flown on recoverable satellites, the height of the mutants was 31% taller than that of control. Wang et al. (2004) reported germination potential and germination percentage had been promoted by 17.1 and 4.8%. Soga et al. (2002) reported that elongation growth of *Arabidopsis* hypocotyls was stimulated under

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microgravity conditions in space, which was similar to the results for wheat coleoptiles, lettuce hypocotyls, and garden-cress hypocotyls grown in space (Halstead and Dutcher, 1987). Besides the phenotype observation, Fourier Transform Infrared Spectroscopy (FTIR), (Yang et al., 2006), the detection technology of peroxidase isoenzyme pattern (Liu, 1999) and molecular markers, including AFLP (Pu et al., 2006), RAPD (Mei et al., 1998; Ren et al., 2009), SCAR (Wang et al., 2008), SSR (Zhou et al., 2001; Fang et al., 2010) and so on, were also used to space-flown varieties and ground control.

ISSR has been described as a powerful technique to evaluate genetic diversity among closely related species and to detect similarities between and within species levels (Davila et al., 1998; Ghariani et al., 2003; Moreno et al., 1998; Pasakinskiene et al., 2000; Zietkiewicz et al., 1994). *Buchloe dactyloides*, as one of the oldest gramineae grasses in North America, which has been primarily used as the forage for almost 100 years, also plays an important role in landscaping and environmental sustenance in North China (Riordan et al., 1998). But the study on genetic diversity of *B. dactyloides* populations after seeds have been flown on satellite by ISSR molecular markers has never been reported.

To uncover the molecular nature of mutation induced by space flight factors, some of *B. dactyloides* plants with morphological changes were chosen for ISSR analysis in the present study. Our objectives were to detect polymorphisms between mutation plants and ground controls or within mutated individuals based on ISSR.

MATERIALS AND METHODS

Plant materials

Dry seeds of *B. dactyloides* were provided by the Grassland Research Institute, China Agricultural University.

Space flight treatment

The seeds were grouped into two portions: One was kept at 7°C for ground control; the other was prepared for space flight. The seeds were carried into space aboard on a recoverable seed breeding satellite “Practice 8” for a 15 days flight from September 9 to 24 in 2006. The flight conditions were as follows: The distances for perigee and apogee were 180 and 469 km, respectively; orbit obliquity was 63°, and cabin temperatures at flight stage were 7.21-20.72°C. Heavy particle rate was 4.44 particles/cm 2 days. Average space radiation dose for plant seeds at linear energy transfer (LET) space was 4.79 mGy.

Field observation

A single seed from each collected singled spike was planted into an individual pot in the greenhouse, with an approximate temperature 22°C and 16 h photoperiod. Then both space flight and ground control seedlings were planted in the field. Field observation was carried out to evaluate morphological changes of plants developed from flight seeds. Ten types of basic turf characters were observed such as leaf blade length and width, height, stem diameter, number of tillers, number and length of stolon, length of stolon inter node, leaf color and extent of leaf turning yellow. 21 *B. dactyloides* individuals with great changes in phenotype were selected among the 100 spaceflight *B. dactyloides* single plants. Leaves of the 21 mutations and 21 plants from ground control were collected for DNA extraction.

DNA extraction and ISSR polymerase chain reaction (PCR) amplification

Fresh young leaves were powdered in liquid nitrogen and genomic DNA was extracted using the CTAB method described by Doyle (1991). The isolated genomic DNA was diluted to 10 ng/ml and stored at -20°C for ISSR amplification. After purification, DNA samples were quantified using Gene-Quant spectrophotometer (Pharmacia) and their quality was evaluated by electrophoresis on 0.8% agarose gel according to Sambrook et al. (1989).

In a preliminary study, 33 primers provided by University of British Columbia (UBC) were screened for PCR amplification (Table 1). Finally, 7 ISSR primers that generated clear, reproducible banding patterns were selected for further analysis.

The effects of Mg^{2+}, dNTP, DNA templates, primers and DNA polymerase on the amplification were tested, and the determined optimal reaction system of ISSR for *B. dactyloides* was as follows: 1×Taq polymerase buffer, 0.3 mmol/L dNTP, 1.0U Taq DNA polymerase, 0.6 µmol/L Primers, 1.0 mmol/L Mg^{2+}, 50 ng of template DNA in the total 25 µl reaction volume. Amplification was performed in a PTC thermocycler (Bio-Rad, USA) under the following cycle profile: an initial step of 3 min at 94°C, followed by 35 cycles, each one including 30 s at 94°C for denaturation, 30 s at 48 to 60°C (depending on the used primer) for annealing and 1 min at 72°C for elongation. A 10 min step at 72°C is programmed as a final extension. The amplification products were separated through electrophoresis on 12.0% polyacrylamide gel electrophoresis (PAGE) and stained with silver staining as described by Bassam et al. (1991).

Data analysis

Firstly, significant analysis and cluster analysis were conducted to the field study data using the SAS8.2 software. Moreover, unequivocally scorable and consistently reproducible amplified ISSR bands were scored as present (1) and absent (0), each of which was treated as an independent character regardless of its intensity. Smeared and weak bands were excluded. Fragments of the same molecular weight were considered as the same locus. The following genetic diversity parameters were calculated: the percentage of polymorphic bands (PPB), the polymorphic information content (PIC), total genetic diversity of materials i and j (N_{ij}), genetic diversity of material i (Ni), genetic diversity of material j (Nj), Nei’s (1973) coefficient of genetic differentiation among populations ($GS = \frac{2N_{ij}}{N_i + N_j}$), genetic distance (GD=1-GS). A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on the matrix of Nei’s (1978) unbiased genetic similarity coefficients (NTSYS-pc version 2.11x, Rolf, 2000) (Table 1).

RESULTS

Morphology observation

After space flight, some characters of the *B. dactyloides*

Table 1. Information of ISSR primers used in the experiment.

Primer	Secquence(5'-3')	T_m[1](℃)	Primer length	nmoles per OD[2]
TP1	$(AG)_8Y$[3]G	56.16	18	4.7
TP2*	$(AG)_8$YT	53.88	18	4.8
TP3*	$(AG)_8$YA	53.88	18	4.6
TP4*	$(GA)_8R$[4]G	56.16	18	4.6
TP5	$(GA)_8$RT	53.88	18	4.6
TP6	$(GT)_8$YC	56.16	18	6.2
TP7	$(AC)_8$RA	53.88	18	5.3
TP8	$(GA)_8$G	54.59	17	4.9
TP9	$(GACA)_4$	51.55	16	5.6
TP10*	$(GA)_8$TC	55.02	18	4.8
TP11	$(AC)_8$GA	55.02	18	5.3
TP12	$(AC)_8$C	54.59	17	5.9
TP13	$(AC)_8$T	52.18	17	5.8
TP14	$(TG)_8$TC	55.02	18	6.2
TP15	$(GGAGA)_3$	53.57	15	5.6
TP16	$(TCC)_5$TG	59.42	17	8.1
TP17*	$(AG)_8$GC	57.30	18	4.7
TP18	$(AG)_8$T	52.18	17	4.9
TP19	$(CT)_8$G	54.59	17	7.9
TP20	$(TC)_8$C	54.59	17	8.2
TP21	$(AC)_8$GT	55.02	18	5.5
TP22	$(TC)_8$G	54.59	17	7.9
TP23	$(AG)_8$TC	55.02	18	4.8
TP24	ACTCGTACT$(AG)_7$	60.17	23	4.00
TP25	TAGATCTGATATCTGAATTCC	52.16	21	4.9
TP26	AGAGTTGGTACGTCTTGATC	55.75	20	5.2
TP27	ACTACGACT$(TG)_7$	60.17	23	4.6
TP28	ACTTCCCCACAGGTTAACACA	58.01	21	4.9
TP29	$(CT)_8$AGA	55.41	19	6.5
TP30	$(GA)_8$GCC	59.72	19	4.6
TP31*	$(AC)_8$GCT	57.56	19	5.3
TP32	CGTAGTCGT$(CA)_7$	61.95	23	4.4
TP33*	AGTCGTAGT$(AC)_7$	60.17	23	4.3

[1] T_m : Melting Temperature; [2] OD: Optical Density; [3] Y: Pyrimidine, ¼ C or T; [4] R: Purine, ¼ A or G. *: These primers marked with "*" were the ISSR primers selected for further study.

accessions changed significantly including the leaf blade width, stem diameter, height, leaf color (SPAD), while the others (leaf blade length, number of tillers, number and length of stolon, length of stolon inter node, extent of leaf turning yellow of *B. dactyloides*) were without significant change (Table 2).

Compared with the ground control, the leaf blade width decreased by 11.60% (P=0.034), that is the leaf became slimmer after been carried with a mean of 1.81 mm. Meanwhile, both the stem diameter (15.63% less than ground control) and the height (7.08% less than ground control) declined after the space flight, with a mean of 0.96 mm (P=0.010) and 26.69 cm (P=0.005) respectively. Moreover, the SPAD value averaged out to 22.52 (P=0.002), higher than ground control, indicating the leaf color darkened significantly (Figure 1).

Selected the mutants among the 21 spaceflight *B. dactyloides* accessions, which had significant difference in leaf blade length and width, stem diameter, height, number of stolon and leaf color (SPAD) than CK.

Leaf blade length of 1 plant increased significantly while that of 6 plants shortened by comparison with CK. It

Table 2. Effects of spaceflight on basic turf characters of *B. dactyloides.*

Characters	SM[1]	CK[2]	P-value[3]	SEM[4]
Leaf blade length(cm)	17.40[a]	18.59[a]	0.370	0.58
Leaf blade width(mm)	1.81[b]	2.02[a]	0.034	0.04
Number of tillers	302[a]	292[a]	0.622	8.92
Stem diameter(mm)	0.96[b]	1.11[a]	0.010	0.03
Height(cm)	26.69[b]	28.58[a]	0.005	0.31
Number of stolon	31.4[a]	29.5[a]	0.531	1.31
Length of stolon inter node(cm)	73.82[a]	78.86[a]	0.245	1.91
Length of stolon	9.2[a]	9.6[a]	0.635	0.36
Leaf color (SPAD[5])	22.52[a]	13.98[b]	0.002	0.99
Extent of leaf turning yellow	3.77[a]	3.46[a]	0.725	0.39

[1]SM: Spaceflight Mutant; [2]CK: Check; [3]P-value: Probability value, which represents a decreasing index of the reliability of a result; [4] SEM: Standard Error of the Mean, a measure of how far your sample mean is likely to be from the true population mean; [5]SPAD: Soil Plant Analysis Development, which is corresponding to relative chlorophyll concentration.

Figure 1. Change morphological characters. CK and SM represented check (ground control) and spaceflight mutant, respectively. (A) is variety No.15 , whose leaf color is darker than CK. (B) is the leaf blade width change of variety No.16 whose leaf gets slimmer. (C) showed the plant type changed in height; SM is higher than CK.

accounted for 9 of the number of mutants which increased in leaf blade width, but 1 mutant went by contraries. Moreover, 9 plants became slimmer in stem diameter, 11 mutants became shorter in height, 7 mutants

Table 3. The results of ISSR amplication.

Primer	Total bands	Polymorphic bands	PPB[1] (%)	PIC[2]
TP2	24	23	95.8	0.86
TP3	14	11	78.6	0.85
TP4	14	10	71.4	0.82
TP10	17	15	88.2	0.85
TP17	12	9	75.0	0.78
TP31	14	11	78.6	0.87
TP33	14	11	78.6	0.89
Mean	15.6	12.9	80.9	0.85

[1] PPB: Percentage of polymorphic bands; [2] PIC: Polymorphic information content.

had more stolon and 15 mutants had larger SPAD value than CK.

ISSR polymorphism

In the study, the 7 selected primers were applied for PCR amplification of 21 spaceflight *B. dactyloides* accessions (Figure 2), generated 15.6 reliable bands of which 12.9 (80.9%) were polymorphic. It also demonstrated the good discriminatory power of the markers identified (Table 3).

Genetic similarities

In order to draw the relationships among accessions, the genetic distances matrix was computed with neighbour program. The genetic similarities (GS) value of 21 spaceflight accessions varied from 0.364 (No.7 and 12, No.3 and 21, No.12 and 21) to 0.773 (No.7 and 13). Moreover, among the 21 spaceflight *B. dactyloides* accessions, the rate of GS value greater than 0.7, 0.6 to 0.7, 0.5 to 0.6, 0.4 to 0.5 and less than 0.4 accounted for 6.16, 40.28, 42.18, 9.95 and 1.43%, respectively (Table 4).

Cluster analysis

The UPGMA dendrogram based on ISSR obviously revealed the genetic relationships of 21 spaceflight accessions which were been divided into four main clustering groups based on GS value (0.602) (Figure 3). The first cluster (A) included 9 accessions (No.1, 3, 6, 7, 10, 13, 16, 17 and 18), of which the GS value ranged from 0.614 to 0.773. The second cluster (B) included 8 accessions (No.2, 4, 5, 8, 9, 11, 14 and 19), of which the GS value ranged from 0.659 to 0.750. The third cluster (C) included 3 accessions (No.15, 20 and 21), of which the GS value ranged from 0.636 to 0.727. The fourth cluster (D) included 1 accession (No.12).

On the other hand, the cluster analysis based on 6 characters (leaf blade length and width, stem diameter, height, number of stolon and leaf color) with significant difference was also been operated on each single mutants and ground controls accordingly. As a result, the result of cluster analysis based on genetic similarity coefficients did not accord with the one base on phenotype perfectly as a whole.

For example, accessions (No.6 and 16) which had a great GS and a small GD had a major difference in characters including the extent of leaf turning yellow, number and length of stolon while without significant change in number of tillers, stem diameter, length and width of leaf blade. On the contrary, accessions (No.12 and 13) which had a small GS and a great GD had a similar performance in stem diameter, width of leaf blade and leaf color (SPAD).

But there were still few similarities between these two cluster analyses. For instance, accessions (No.15 and 20) in the third cluster(C) (Figure 3) were also be clustered in the same group in the UPGMA-derived dendrogram of *B. dactyloides* materials regarding height and leaf color (SPAD) variation. That is, they had the same significant changes in the two characters. However, the similarity had not been seen in the cluster analysis based on the other four characters.

In order to find out the relationship between the phenotype and genetic ISSR data, correlation analysis was conducted, and we calculated that the related coefficient was only 0.1874. It revealed the results of morphological analysis has been greatly influenced on external environment and anthropic factor. Consequently, the phenotype and ISSR data of *B. dactyloides* had no definitely corresponding relations.

DISCUSSION

Space inducement, as a new developed method, has been applied broadly in the research of plenty of crops to find out the truth about what change it brings about, comparing the spaceflight group with the ground control and explain the mechanisms inside.

Firstly, significant morphology mutations were clearly

Table 4. GS value of the 21 spaceflight *B. dactyloides* accessions based on ISSR.

S/N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1																				
2	0.682	1																			
3	0.659	0.553	1																		
4	0.659	0.704	0.545	1																	
5	0.523	0.659	0.5	0.682	1																
6	0.705	0.659	0.682	0.636	0.591	1															
7	0.659	0.659	0.591	0.591	0.682	0.636	1														
8	0.636	0.682	0.477	0.614	0.614	0.659	0.614	1													
9	0.5	0.636	0.523	0.659	0.659	0.659	0.614	0.545	1												
10	0.636	0.636	0.614	0.523	0.568	0.705	0.75	0.682	0.636	1											
11	0.523	0.75	0.409	0.682	0.591	0.682	0.682	0.705	0.659	0.705	1										
12	0.432	0.568	0.545	0.545	0.591	0.5	0.364	0.477	0.523	0.432	0.5	1									
13	0.705	0.659	0.591	0.636	0.682	0.682	0.773	0.568	0.659	0.659	0.591	0.445	1								
14	0.545	0.682	0.477	0.614	0.523	0.523	0.614	0.545	0.591	0.5	0.659	0.477	0.568	1							
15	0.568	0.523	0.591	0.636	0.545	0.545	0.5	0.614	0.614	0.568	0.591	0.591	0.636	0.523	1						
16	0.591	0.591	0.659	0.659	0.568	0.75	0.614	0.545	0.636	0.5	0.614	0.523	0.659	0.682	0.568	1					
17	0.682	0.636	0.522	0.659	0.477	0.659	0.568	0.5	0.545	0.5	0.568	0.432	0.659	0.591	0.568	0.636	1				
18	0.568	0.659	0.591	0.682	0.682	0.682	0.682	0.659	0.568	0.705	0.636	0.409	0.591	0.614	0.5	0.659	0.614	1			
19	0.545	0.636	0.477	0.568	0.568	0.614	0.614	0.682	0.545	0.455	0.659	0.477	0.568	0.545	0.432	0.545	0.545	0.477	1		
20	0.523	0.568	0.409	0.591	0.682	0.5	0.682	0.523	0.659	0.614	0.682	0.5	0.727	0.523	0.727	0.568	0.568	0.455	0.523	1	
21	0.523	0.523	0.364	0.636	0.591	0.5	0.5	0.523	0.614	0.477	0.545	0.364	0.545	0.614	0.636	0.477	0.614	0.591	0.477	0.636	1

demonstrated happened to space-flown *B. dactyloides* seeds in our study including the leaf blade width, stem diameter, height, leaf color (SPAD) and so on (Figure 1). As a result, 21 spaceflight *B. dactyloides* mutants were selected. A great number of examples which revealed the effects of space flight factors filled the academic world. According to Fan et al. (2005) study, 32 height mutants, 27 short heading date mutants and 20 more-tillering mutants were obtained in the subsequent SP_3 and SP_4 generation of rice which experienced the space flight of “Shenzhou 4”. Fan et al. (2010) revealed that height, leave color (SPAD), number and size of leave increased under space flight condition, and finally got 4 mutants in all. These mutants are mostly of outstanding performance in growth, reproduction and resistance which have a positive influence in space-breeding research.

Secondly, based on the study, we can conclude that the spaceflight *B. dactyloides* mutants changed greatly on several characters and expressed high polymorphism by ISSR with the PPB 80.9% by 7 primers after having been carried to the outer space. It verified these changes not only from appearance but also in the inheritable genotype. Similar results were reported in *Cistanche deserticola* that 9 out of the 94 ISSR primers were showed high polymorphism, and a total of 118 bands were generated, of which 80 were polymorphic (Wu et al., 2011). The degree of polymorphism exhibited by ISSR banding patterns in their study is considerably high (82.20%) compared to that of the RAPDs reported by Cui et al. (2004) (47.37%) and by Dang et al. (2003) respectively. What is more, in our study,

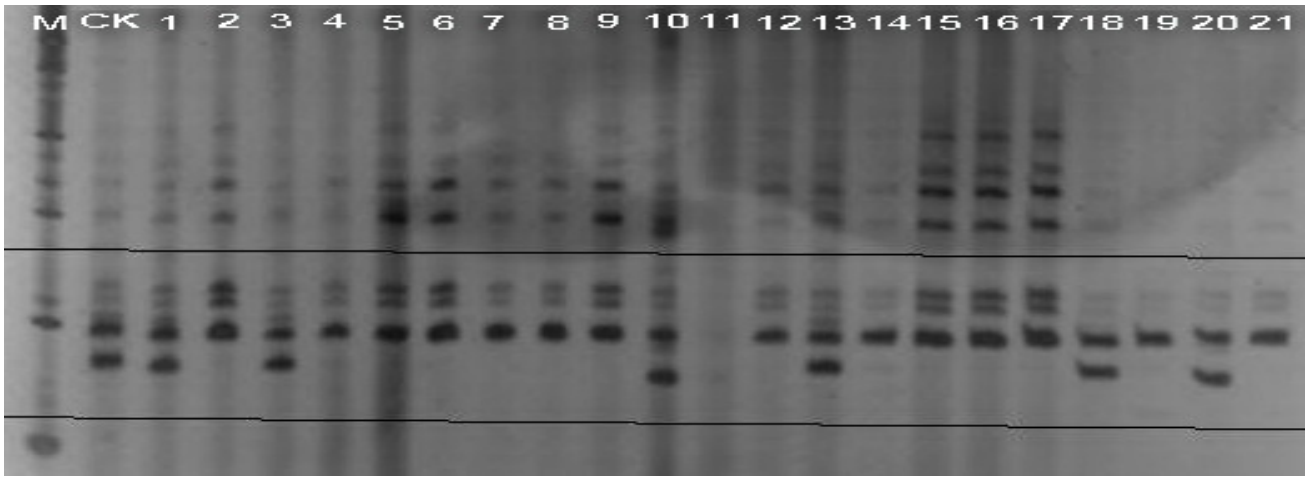

Figure 2. PCR amplication directed by primer TP3. M: 2000 bp DNA ladder; CK: Check; Lanes 1-21: The 21 spaceflight *B. dactyloides* accessions.

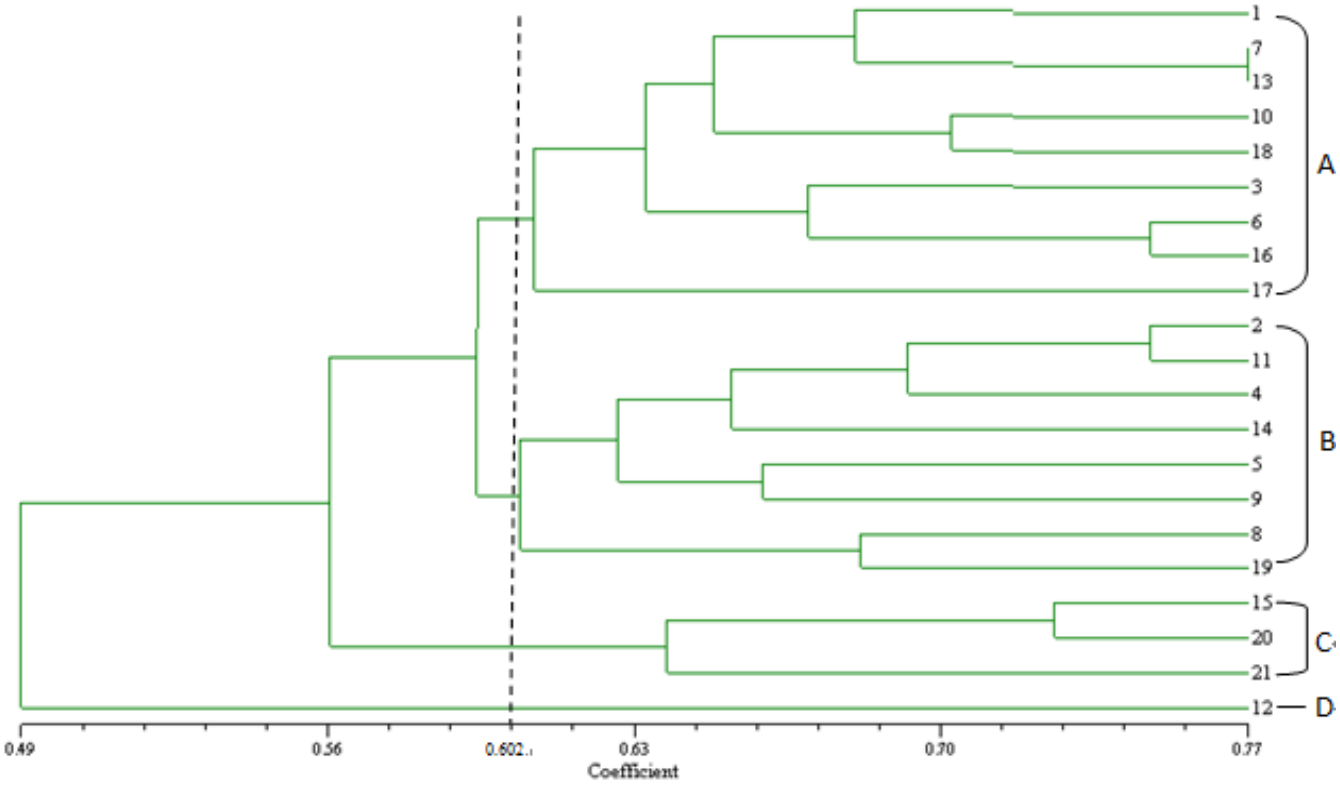

Figure 3. UPGMA-derived dendrogram of 21 spaceflight *B. dactyloides* materials based on genetic similarity coefficients.

got higher average PIC (0.85) than that of polymorphism analysis in spaceflight alfalfa (0.6366) by using SSR molecular (Fan et al., 2010). Therefore, ISSR markers were definitely been regarded as an effective tool to reveal the genetic diversity of spaceflight *B. dactyloides* mutants.

In addition, it should be noted that, in our study, the result of PCR amplication showed part of mutants had less bands than the ground control, which implied that spaceflight mutants had occur deletion under the space radiation environment (Figure 2). This probably is connected with the mutative traits. Reports indicated that cosmic radiation could bring about breakage or deletion of chromosome DNA in the same time microgravity

disturbed the DNA repair pathway (Gartenbach et al., 1994). Vibration and linear acceleration, which occurred at the launch and return of satellite, also caused chromosome aberrations, and brought the breakage of large fragment of genomic DNA with the control (Anikeeva et al., 1979; Vaulina and Kostina, 1975). So, a lot of chromosome rearrangement during the carry of *B. dactyloides* seeds had occurred such as duplication, translocation, inversion, insertion and deletion. The same conclusions were reached in studies on molecular analysis of space flight mutants of rice, kidney bean, tomato (Zhou et al., 2001; Zhang Jian et al., 2000; Lu Jinying et al., 2005).

Thirdly, the result of cluster analysis based on genetic similarity coefficients did not accord with the one base on phenotype perfectly. That is, both the morphological and genetic variation had a considerably complicated relationship. An agreement was reached with the similar conclusion dawn by Fan et al., (2010). She found that alfalfa materials C-01, C-28, D-49, A-07 all with high DNA polymorphism while the former three had obvious change in phenotype and the latter one (A-07) was not seen any variation. Consequently, it revealed that cosmic radiation could bring about great mutations that indicated the great prospect of providing brand-new excellent germplasms for breeding but there is no connection between the phenotype and DNA polymorphism.

Further, they had already been used in grasses, for example: alfalfa (*Medicago sativa* L.) and yellow tall fescue (*Festuca arundinacea*) (Fang et al., 2010; Hu et al., 2004). Both of them had something in common that they came to the conclusions based on their studies of consecutive generations from SP1 to further selected progenies. Therefore, to understand the mutagenic effects of space flight on plants and lay a solid foundation of breeding, it is better to conduct long-term research for reasonable data and unshakable evidence. More work is being conducted to select the stable mutants with favorable traits and provide a valuable material in developing new cultivars.

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