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ASSESSMENT OF GENETIC DIVERSITY AND VARIATION OF *ACER MONO MAX* SEEDLINGS AFTER SPACEFLIGHT

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Abstract

Genetic diversity and variation of *Acer Mono Maxim* seedlings sampled from space-mutated (sm) populations were compared to seedlings from parallel control (ck) ones using molecular markers. RAMP analysis showed that the percentage of polymorphic band, Shannon diversity index and Nei's gene diversity index of the space-mutated populations were higher than those of the control ones, which indicated that genetic variation increased after spaceflight in populations of *Acer Mono Maxim*. By using un-weighted pair group method with arithmetic mean (UPGMA) method, three space-mutated repeats (populations) were clustered together, and control groups clustered separately, which further indicated that there was difference between the space-mutated ones and the control ones, which may be caused by space mutation. Further analysis of genomic inconsistency between the root and leaf samples from the same tree showed that a total variation rate of 6.3% and 1.7% were obtained in ten space-mutated individuals by using RAMP and SSR markers, respectively, however, the variation rate was zero in control ones. It provided that space mutation may be caused the individual variation of *Acer Mono Maxim*.

Key words: *Acer Mono Maxim*; Genetic variation; Space mutation;

Introduction

Bringing plant seeds to space by recoverable satellite and studying their mutation mechanism has become a new breeding method. In recent years, China has brought more than 500 varieties' seeds from over 70 plant species to the space by recoverable satellites, many new varieties were improved and great achievements in space mutation breeding were made. The research method of the mechanism of space mutation has also developed from morphological observation to cellular and molecular level, and greatly progressed (Ding *et al*., 2010; Akhtar *et al*., 2014). Compared with plant space mutation, bringing forest seeds to the space are just at the beginning. Space mutation breeding will reduce the breeding and growing periods of improved tree species, and develop their genetic characteristics more beneficial to human beings (Ma *et al*., 2005). Forest trees, however, have their special features during space mutation study. Most forest trees are reproduced by seeds and are for commercial forest. Their economic characters are mainly regulated by quantitative characters. Because the genotypes of individual seedlings are different, the major issue of space breeding of forest trees is that how to correctly determine the differences of space-mutated individuals are genetic differences among individuals or variations caused by space mutation.

Acer Mono Maxim belongs to Acer of Aceraceae and has colorful leaves and beautiful shape, leaves and fruits. Its leaves turn into red or yellow in autumn. All of these make it an important species to afforest mountains, gardens and roads and for protective forests. It is widely distributed in north and northeast China and Yangtze River basin, and is the most widely distributed Aceraceae in China (Masaki *et al*., 1992). *Acer Mono Maxim* is an important landscape plant. Space mutation may lead genetic variation of *Acer Mono Maxim* in shapes and other aspects so as to provide materials for breeding more beautiful species of *Acer Mono Maxim*.

In this paper, the comparative analysis of genetic diversity and variation among space-mutated and control populations of *Acer Mono Maxim* were presented, based on RAMP molecular markers. Furthermore, the genomic differences between the leaves and roots of the same tree after spaceflight were detected by RAMP and SSR molecular markers. Such studies have been conducted successfully for some of economically important crops too (Shinwari *et al*., 2014; Khan *et al*., 2014). Our objectives were to determine differences in genetic diversity and variation between control and space-mutated seedlings of seeds that endured a 15-d flight, both at the population and individual level.

Material and Methods

The seeds of *Acer Mono Maxim* sampled from Inner Mongolia population were brought to the space by the Satellite Practice VIII on September 9, 2006, traveled 355 hours and over 9,000,000 km at the orbit with an apogee of 463km and perigee of 187km, and returned to the earth on September 24. The seeds of space-mutated *Acer Mono Maxim* and control then were kept under the earth 30cm till April of 2007 at the nursery of Sandstorm Source Forestry Center in Yanqing County, Beijing. Both of them were planted in pots in the April of 2007. The seedlings were taken as the material of this research. The seedlings of space-mutated *Acer Mono Maxim* and control were planted in the yellow soil test field with medium fertility by space of 30cm × 30cm in June 2007.

In June 2009, 120 individual plants were randomly selected, which included 60 space-mutated plants (sm) and 60 control plants (ck). Subsequently each of these two groups were randomly separated into three sample populations respectively, sm1, sm2, and sm3 of the sm group, and ck1, ck2 and ck3 of the ck group. Therefore, 20 plants were included in each of sm1, sm2, sm3, ck1, ck2, and ck3 random sample population and were used for molecular analysis. Young leaves were collected from each of these selected trees for molecular markers analysis.

The leaves and roots of 10 space-mutated seedlings together with 10 control individual seedlings were sampled on June, 2009, and were used for individual mutation detection.

The leaf and root samples were placed into plastic sealable bags and then transported in a foam box containing ice bags and stored at -80°C for genomic DNA extraction.

Extracting genome DNA from the leaves and roots: 200 mg frozen leaves were taken from each sample and grinded into paste under low temperature by little improved CTAB method described by Doyle and Doyle, (Doyle & Doyle, 1987).

The roots were washed by de-ionized water and 75% ethanol solution successively, cut into 0.5cm sections, and then grinded into paste under low temperature by CTAB method. The DNA was tested by 1% agarose gel electrophoretic method, observed and taken photos by gel imaging system. Then pure DNA was selected, diluted to 50ng/μl, and preserved at -20°.

RAMP amplification: DNA samples from leaf and root tissues were amplified with 10 pairs of RAMP primers (Table 1), which are composed of anchor primers GT $(CA)_4$ and GC $(CA)_4$ and five random primers s386, s381, s1181, s485 and s438 described in Williams *et al*. (1990).

The total volume of polymerase chain reaction (PCR) was 25μL, including 2.5μL 10× Buffer (+$MgCl_2$), 2μL dNTP (2.5 mmol/ L), 1μL $GT(CA)_4$ primer (10 pmol/μL), 1μL $GC(CA)_4$ primer (10 pmol/μL), 1μL random primer (10 pmol/μL), 2.0 μL DNA template (50 ng/μL), 0.3μL rTaq polymerase (5 U/μL), and 16.2μL sterile deionized water. The operation procedures of the amplification were as follows: pre-denaturing (94°C, 5min), denaturing (94°C, 1min), annealing (45°C -48°C, 1min), and extending (72°C, 1min) for 40 cycles, and then extending (72°C, 10min). The amplification was conducted in Bio-Rad PCR device. The product was electrophoretically separated in 8% polyacrylamide gel. The bands were statistically analyzed after sliver staining.

SSR amplification: The leaves and roots of the test materials were amplified by PCR with 10 pairs of primers. The product was electrophoretically separated in 8% non-denaturing polyacrylamide gel and 0.5×TBE buffer solution. The bands were observed, taken photos and statistically analyzed after $AgNO_3$ staining. After treated by polyacrylamide gel electrophoresis, polymorphic primers with clear amplifying band were selected and used in all materials. Ten pairs of polymorphic primers were selected (Table 2) (Kikuchi & Shibata, 2008).

The total volume of PCR reaction was 10μL, including 1.5μL 10× Buffer (+$MgCl_2$), 0.3μL dNTP (10 mmol/ L), 1μL F primer (10 pmol/μL), 1μL R primer (10 pmol/μL), 2.0 μL DNA template (50 ng/μL), 0.2μL rTaq polymerase (5 U/μL), and 4μL sterile deionized water. The operation procedures of the amplification were as follows: pre-denaturing (94 , 5min), denaturing (94°C, 45sec), annealing (48°C-58°C, 1min), and extending (72°C, 1min) for 35 cycles, and then extending (72°C, 10min). The amplification was conducted in Bio-Rad PCR device. The product was electrophoretically separated in 8% polyacrylamide gel. The variable bands were observed on the film to statistically analyze the variation rate.

Band record and data analysis: Taking the band with molecular weight of 500-2000bp made by Marker DL2000 as standard band, if there was a band, record it as "1", if not, record it as "0". The following indicators of genetic diversity were obtained by software PopGene1.32: (1) number of polymorphic band (*A*) and percentage of polymorphic band (*P*); (2) observed number of alleles (*Ao*), effective number of alleles (*Ae*); (3) Nei's gene diversity index (*h*) and Shannon's information index (*I*); (4) Nei's genetic distance (*D*) and genetic identity coefficient (*GS*), and clustering by UPGMA method using *D* or *GS*; and (5) calculating the coefficient of gene differentiation among populations within species by Nei's (Nei 1973) gene diversity method ($Gst = Dst / H_T$, where $H_T = Hs + D_{ST}$, H_T is total gene diversity, *Hs* is the gene diversity of the population, *Dst* is the gene diversity among populations. The expected heterozygosity (H_e) and observed heterozygosity (H_O) were calculated by software AFLP-SURV1.0. After homogeneity of sample variances was verified using a Levene's test, an Independent-Samples T Test was used to compare means between sm and ck group, using the SPSS16.0 software.

It was expected that the DNA extracted from leaves and roots of the same tree would show identical fragment sizes. For each single tree, the fragment that the leaf and root have in common at a particular locus was regarded as the 'wild-type', and any discrepant fragment was regard as variable band and interpreted as a result of a mutational event. The total amplified fragments and variable bands that were clear and reliable were recorded. Variation rate (%) = total variable bands/total amplified fragments × 100%.

Results

Assessment of genetic diversity: Using ten pairs of primers, 360 bands were obtained in 120 individuals of six populations (three space-mutated repeats and three control ones) by RAMP amplification. 256 polymorphic loci were got in six populations while amplifying with ten pairs of primers, with an average of 25.6 loci for each pair of primers and with a polymorphic band rate of 71.11%.

Table 1. Primer name, primer sequences and annealing temperature used for RAMP analysis.

Primer name	Primer sequences	Annealing temperature (Ta)
S386+RAMP1R	5′GAGGGAAGAG+GT$(CA)_4$	45
S438+RAMP1R	5′GGTGAGGTCA+GT$(CA)_4$	48
S381+RAMP1R	5′GGCATGACCT+GT$(CA)_4$	46
S1181+RAMP1R	5′GGCAGGTGGA+GT$(CA)_4$	45
S485+RAMP1R	5′CCGCGTCTTG+GT$(CA)_4$	45
S386+RAMP2F	5′GAGGGAAGAG+GC$(CA)_4$	46
S438+RAMP2F	5′GGTGAGGTCA+GC$(CA)_4$	48
S381+RAMP2F	5′GGCATGACCT+GC$(CA)_4$	46
S1181+RAMP2F	5′GGCAGGTGGA+GC$(CA)_4$	46
S485+RAMP2F	5′CCGCGTCTTG+GC$(CA)_4$	45

Table 2. Primer name primer sequences and annealing temperature used for SSR analysis

Gene locus	Name	Primer sequences (5′–3′)	*Ta*	Repeat sequences	Section size	*A*	H_O	*He*
Am748	AB303358	F:6-FAM-CCCTTGAACCCGACTATTT R: GGATTGGTAAGAGGGTACATACTA	58°C	$(AG)_{15}$	295	3	0.147	0.140
Am909	AB303360	F: GACACAAGTATGGACGGTGATTTC R: HEX-GGCCAACTTTGAGATAAGC	58°C	$(AG)_{18}A(AG)_4$	258	7	0.618	0.663
Am116	AB303350	F: AACGCTACCGACTTCGCCAACT R:6-FAM-TGGAGGTCAAGTGCTGGAAACAA	58°C	$(CT)_{20}$	258	18	0.882	0.887
Am668	AB303356	F: NED-AACAACTCGGGCACTTCTC R: TGTTATTTTTACTCCCAAAGGTCT	60°C	$(AG)_3AA(AG)_{18}$ GG$(AG)_4$	214	22	0.912	0.945
Am340	AB303353	F: CGGAGCCAACTTGAGAGTAGAG R: NED-ATTGAAGGTCCTTAATCCACGTC	58°C	$(AG)_{22}$	189	23	0.824	0.939
Am258	AB303352	F: CCGGTGCATCTATCTCCAT R: HEX-CATCCATAAAGTAAAAATTGAGGG	58°C	$(CT)_{17}$	181	13	0.794	0.876
Am096	AB303348	F: HEX-TAAGCTTCATACGCCATCAACCT R: GGCATCACCAAATCCAGACAC	58°C	$(CT)_{22}$	180	16	0.647	0.906
Am118	AB303351	F: GAGGGAGGAGGCTGAGAAGA R: HEX-TATCAAAGAAGCCAAGGAAGGTG	58°C	$(CT)_{16}$	171	15	0.971	0.897
Am742	AB303357	F: EX-AGAACAGGCGGAGAGTTTCGAGTC R: CCCGACGACAACCACCCAT	58°C	$(AG)_{17}$	163	9	0.853	0.822
Am775	AB303359	F: NED-AATCCACAACCACAGCCGCATCAG R: GGTGGCGACGGCAGCTAGGGTTAG	58°C	$(CT)_{19}$	151	12	0.824	0.860

At the group level, the space-mutated (sm) group showed more polymorphic bands (132) and a slightly higher percentage of polymorphic band (72.22%) in comparison with controls (ck, 124, 69.84%) (Table 3). The parameters of Observed number of alleles (*Ao*), Effective number of alleles (*Ae*), Nei's gene diversity index (*h*) and Shannon's information index (*I*) were statistically analyzed and compared. The results (Table 3) showed that the *h*, *I*, *Ao*, *Ae, Ho* and *He* of the space-mutated populations (*h*=0.3185 ± 0.0463; *I*=0.4580 ± 0.0662; *Ao*=1.7222 ± 0.1005; *Ae*=1.6485 ± 0.0947; *Ho*=0.3314 ± 0.0397; *He*=0.3896 ± 0.0454) were higher than those of the control ones (*h*=0.2908 ± 0.0267; *I*=0.4214 ± 0.0386; *Ao*=1.7000 ± 0.0764; *Ae*=1.5153 ± 0.0968; *Ho*=0.3292 ± 0.0352; *He*=0.3810 ± 0.0244) (Table 3).

Population genetic identity coefficient and genetic distance: To further analyze the genetic differentiation among populations, the genetic identity coefficient and genetic distance of the individuals in the populations were calculated with 360 amplified bands produced using 10 pairs of RAMP primers (Table 4). The result showed that the values of GS and D among the six populations varied between 0.8724 and 0.9375 and between 0.0645 and 0.1365 respectively, which demonstrated that the identity among populations was higher and the genetic distance was smaller; of the three space-mutated populations and three control ones, the population genetic identity coefficients were higher and the genetic distances were smaller, while comparing the space-mutated populations and the control ones, the population genetic identity coefficients were smaller and the genetic distances were larger.

Population cluster analysis: By UPGMA method, cluster analysis was made using the genetic distance matrix (Pang *et al*., 1995) obtained by RAMP molecule marker data calculation. The genetic distances of the six populations varied between 0.0645 and 0.1365 (Table 4). Using the amplified result of ten pairs of primers and the genetic distance matrix among populations, the cluster analysis of the six populations was conducted by UPGMA method with software dps3.01. The result (Fig. 1) showed that the three space-mutated populations clustered together and the three control ones cluster, the cluster diagram illustrated that the space-mutated group were well separated from the ground-based control group, which indicated that there was difference between the space-mutated populations and the control ones.

Table 3. Genetic parameters of *Acer Mono Maxim* based on RAMP markers.

Group	Sample size	*A*	*P(%)*	*Ao*	*Ae*	*h*	*I*	*Ho*	*He*
sm	60	132	72.22	1.7222±0.1005	1.6485±0.0947	0.3185±0.0463	0.4580±0.0662	0.3314±0.0397	0.3896±0.0454
ck	60	124	69.84	1.7000±0.0764	1.5153±0.0968	0.2908±0.0267	0.4214±0.0386	0.3292±0.0352	0.3810±0.0244

A, number of polymorphic band; *P*, percentage of polymorphic band; *Ao*, observed number of alleles; *Ae*, effective number of alleles; *h*, Nei's gene diversity; *I*, Shannon's information index; H_e, expected heterozygosity; and H_O, observed heterozygosity; standard deviations are shown in parentheses; * indicated significantly different compared ck at 0.05 probability level

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Population	CK1	SM1	CK 2	SM 2	CK 3	SM 3
CK 1	****	0.8943	0.9116	0.9065	0.9121	0.9104
SM 1	0.1117	****	0.9004	0.9245	0.8724	0.9198
CK 2	0.0926	0.1049	****	0.8970	0.9291	0.8971
SM 2	0.0982	0.0785	0.1086	****	0.8880	0.9375
CK 3	0.0920	0.1365	0.0735	0.1188	****	0.9043
SM 3	0.0938	0.0836	0.1086	0.0645	0.1006	****

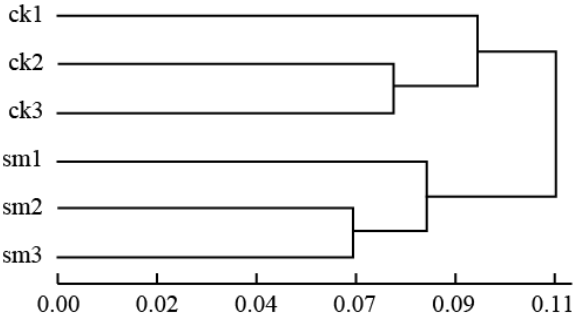

Fig. 1. Dendrogram of the space-mutated populations and the control ones by UPGMA method.

Analysis of individual variation rate

RAMP marker analysis: 6792 amplified bands were obtained in the leaves and roots of ten control individuals with three repeats by RAMP molecule marker using ten pairs of primers. However, there was no difference between the roots and leaves of each individual. 6326 amplified bands were obtained in the leaves and roots of ten space-mutated individuals, of which 399 different gene bands were found, with a total variation rate of 6.3% (Table 5).

SSR marker analysis: The polymorphism of space-mutated individuals was high through PCR amplification in the leaves and roots using ten pairs of SSR primers. 1452 amplified bands were obtained in the leaves and roots of ten control individuals with three repeats using ten pairs of primers, and no difference was found. 1329 amplified bands were obtained in the leaves and roots of ten space-mutated individuals, of which 23 different bands were found, with a total variation rate of 1.7% (Table 6).

Discussion

Many new species of plants has been bred by space mutation. However, for the convenience of testing and obtaining mutated genotypes, all of them are obtained with single gene materials. Molecule marker technology is an effective method for the genetic test of plants (Bretó *et al*., 1993, Kaemmer *et al*., 2006, Guilford *et al*., 1997, Sun *et al*., 1997, Davila *et al*., 1999, Gupta & Rustgi 2004, Omrani-Sabbaghi *et al*., 2007), and has been used to test space-mutated plants. In the molecule test to the genome DNA of space-mutated sweet pepper, Xie *et al*. (2010) conducted amplification analysis to the genome DNA using RAPD technology, and completed sequencing to some different bands. The result indicates that the genome DNA of the descendants SP4 of space-mutated sweet pepper shows variations compared to other ground control ones, and eight polymorphic loci are found.

However, most forest tree individuals are heterozygote. Taking seeds obtained by open pollination as the material of space-mutation, it is difficult to decide whether the differences of different genotypes are their own differences or are caused by mutation while testing the genetic differences using molecule marker method, because the genotypes of different seedlings are different. There are few researches and effective test methods on this field. Comparing and analyzing the difference of the genetic structures of the mutated population and the control ones by using research methods of population genetics to determine the result of space-mutation may be an effective method. The genetic variation of the seedling populations of space-mutated *Acer Mono Maxim* was tested by RAMP molecule marker technology in this paper. And the result shows that, 256 polymorphic loci were obtained in the six populations while amplifying with ten pairs of primers, with an average of 25.6 loci for each pair of primers and a polymorphic loci rate of 71.11%. This reflected higher polymorphism in the *Acer Mono Maxim* populations with RAMP maker. This result was similar to that of the Liu (2009). In their research, the genetic diversity of 31 Acer plants was studied by SRAP molecular marker. 186 amplified bands and 183 polymorphic loci were obtained by using 11 pairs of primers. The polymorphic loci rate reached 98.04%, which showed that Acer plants had rich genetic diversity. However, RAMP molecular marker analyses showed a slightly higher level of genetic diversity in the space-mutated group compared to the control group. Through comparing the genetic identity

coefficients of different populations, it is found that the values of GS vary between 0.8724 and 0.9375 with an average of 0.9070; the genetic identity coefficients of the three space-mutated populations and three control one were higher, while comparing the space-mutated populations and the control ones, the genetic identity coefficients were smaller and the genetic distances were larger. In the genetic distance and population cluster, the genetic distances of the six populations vary between 0.06445 and 0.1365, and the three space-mutated populations cluster together and the three control ones cluster, which further indicated that there was difference between the space-mutated ones and the control ones. This indicated that comparing to the control ones, there were some variations in the space-mutated populations, which may be caused by space mutation.

Considering the inconsistent genetic background of the *Acer Mono Maxim* seeds we studied, it is difficult to test the individual genetic mutations in the seedlings grown from seeds that endured the 15-d flight using conventional methods. Nevertheless, we know that to seedlings, their aboveground parts are developed from the germs of the seeds, while their underground parts are from radicles. After being mutated by radiation, if the seeds mutate genetically, there may be genetic differences in the stems and leaves developing from the germs and the roots from the radicles (Gao *et al*., 2009). In this paper, it tested the seedlings growing from space-mutated seeds by RAMP marker and SSR marker and compared the genetic differences in the leaves and roots. The result showed that there were genetic differences in the aboveground parts and the underground parts of the ten individuals tested by the two methods, the total variation rate of the amplified bands in the leaves and roots treated by RAMP molecular marker reached 6.3%, which was 1.7% higher than that by SSR molecular marker. However, no variation at any investigated locus was found between the leaves and roots of 10 ground-based control trees, within the same tree. Thus, the results proved that there were variations in the space-mutated *Acer Mono Maxim* seed individuals.

The space-mutated populations and control ones with three repeats each came from and were planted and tested in the same environment. The only difference between the 2 groups was that sm group was exposed to the space environment for 15 days, while the ck group was kept on Earth. Considering the profound differences between spaceflight conditions and those on Earth, thus, we suspect that the higher genetic diversity and individual variation observed in the sm *Acer Mono Maxim* were caused by short-term spaceflight of the original seeds or reflect their adaptive response to space environments after growth on Earth.

However, in this paper, it only showed that there are genetic structure changes in the populations and genetic variations in the individuals at the molecular level. Their economy and whether they are helpful or harmful also need further researches. It should comprehensively evaluate the space-mutated materials by setting up contrast experimental forest, with many research methods and from different levels, to more accurately test and select great variants so as to breed new space-mutated forest trees species. In addition, for a better understanding of the mechanism of mutation induced by space flight, further experiments will be necessary to clone and sequence the mutated genome.

Table 5. Amplified bands obtained in the leaves and roots of ten space-mutated individuals and their variation rate.

Space-mutated individuals (leaves and roots)	Amplified bands with ten pairs of primers	Variation bands	Variation rate %
1	744	15	2.00
2	984	9	0.90
3	966	15	1.60
4	820	39	4.70
5	744	75	10.00
6	642	72	11.20
7	708	0	0.00
8	531	69	12.90
9	607	60	9.80
10	546	60	10.90
Total	6326	399	6.30

Table 6. the amplified bands obtained in the leaves and roots using ten pairs of SSR primers and their mutation rate.

Space-mutated individuals (leaves and roots)	Amplified bands with ten pairs of primers	Mutation bands	Mutation rate %
1	130	4	0.90
2	131	1	0.70
3	127	1	0.70
4	122	2	1.60
5	111	3	2.70
6	135	3	2.20
7	138	2	1.40
8	136	4	2.90
9	144	0	0.00
10	153	3	1.90
Total	1329	23	1.70

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