Cell Research (1997), 7, 99-106

RAPD analysis of natural populations of *Acanthopanax* brachypus[1]

YAN HUA JUN, SI LAN DAI, NAI HU WU[2]
Institute of Developmental Biology, Chinese Academy of Sciences, Beijing 100080

ABSTRACT

Random Amplified Polymorphic DNA (RAPD) analysis is a new technology of molecular marking which has proved very powerful in detecting genetic diversity at the level of population. The genomic DNAs used in our experiment were extracted from fresh leaves taken from 59 individuals sampled from three natural populations in Yan An, Shanxi Province. Through more than 2,000 PCRs, deep-going RAPD analysis was carried out on DNA samples from 49 inviduals. The percentage of polymorphic RAPD loci found in these three populations were respectively 27.2 %, 18.6 % and 5.4 %; the average genetic distances within population, 0.055, 0.036 and 0.008; the average genetic distances between populations (I-II), (I-III) and (II-III), 0.105, 0.096 and 0.060. The genetic diversity of A. brachypus within and between populations was found, for the first time, to be rather poor, thus revealing innate factors as the cause contributing to its endangered status. In addition, our work also provides basic materials for elucidating the underlying cause of its endangerment and for its protection biology.

Key words: *Acanthopanax brachypus; genetic diversity; random amplified polymorphic DNA (RAPD) analysis.*

INTRODUCTION

In this study, we focused on an endangered species of pharmaceutical plant, Acan-

1. This paper is specially dedicated to Professor Lu Ji SHI on the 80th anniversary of his birthday
2. The author to whom correspondence is to be addressed

thopanax brachypus L., which is declining in number and has a restricted distribution. Molecular biological technology has revolutionalized the evolutionary biological research on microevolution and macroevolution. Microevolution refers to the change in genetic composition of the individuals within a species at the level of population. This may be expressed in terms of intraspecific genetic diversity, i.e. the total sum of genetic variations among populations or among different individuals of the same population. These variations are important reserve for evolution, which determine the potential for future evolution of the species. The richer the genetic variation of a species is, the greater its adaptability to environmental changes, and the greater its chances for survival and evolution. Conversely, the loss of genetic diversity of a species means its partial extinction. One of the most important fields of evolutionary biology is the study, using molecular biological methods, of the types, distribution, origin, adaptability value, and dissemination mechanisms of genetic variation of the natural populations of a species. The objective is to bring to light the mechanisms and motive force of genetic variation occurring under natural conditions, and the modes and pathways of speciation. Endangered species are now one of the foci in evolutional study. At the same time that protection of biological diversity becomes one of the great environmental problems that receives world wide attention, protection of endangered species becomes the focus of protection of biological diversity which takes, as its first and foremost task, the protection of as much of the existing genetic variations of species as possible. Thus, assessment of the level of intraspecific genetic variation is very important to the preservation and management of species. And the study of the genetic variation of natural popultaions of A. brachypus and its evolutional trends holds important implications for the investigation into the molecular mechanism underlying its decline, the prediction of its fate, and the formulation of the appropriate strategy for its long-term protection[1].

The acquisition of lots of genetic markers is a prerequisite for the study of genetic variation of populations. In recent years, the development of molecular biology has helped establish a series of technologies for the direct detection of DNA diversity which, with its incomparable superiority with respect to the isoenzyme technology, shows great potential for study of genetic diversity in populations. Random Amplified Polymorphic DNA (RAPD) analysis is a new development starting from 1990 and, simple in operation and free from radioactive pollution, it is particularly suitable for study of genetic variation at population level[2-4]. And we first established the procedure for the direct molecular marking of polymorphic DNA of A. brachypus, and then carried out RAPD analysis of genetic variation of natural popultions of it at molecular level using the procedure established.

MATERIALS AND METHODS

Materials

Fresh leaves from *A. brachypus* were randomly sampled from three natural populations in Hes-

hangyuan (23 individuals), Nanniwan (20) and Linxiao (16) respectively. The individuals sampled were located at least 5 meters apart and the samples were kept in 0 ~ 4 ℃ for transportation.

Reagents

The random primers (OPA and OPB series) were bought from Operon Technologies Inc. The primer sequences are listed in Tab 1. The Taq DNA polymerase, × dNTPR and RNase A were from Sino-Americal Bioengineering Company.

Extraction and purification of genomic DNA

One to two grams of fresh leaves were grounded rapidly in liquid nitrogen and transferred into centrifuge tube (50 ml). After addition of 10 ml of CTAB extracting solution (2 % Cetyl triethyl ammonium bromide, 1.4 *M* NaCl, 20 mM EDTA, 100 m*M* Tris-HCl, 2 % Mercaptoethanol, pre-heated to 65 ℃ and 100 mg of PVP (Polyvinylpyrrolidone), it was incubated under 65 ℃ for one h. After it was cooled to room temperature and equal volume of chloroform-isoamyl alcohol (24:1) was added, it was centrifugalized under 10,000 g for 8 rain. The precipitate was washed with 75 % alcohol and dried. Then 500 μl of TE buffer and RNase were added to a final concentration of 50 μg/ml. After incubation under 37 ℃ for 30 min, it was mixed with equal volume of Cellulose HL 100 before standing for 5 min. After further centrifugation, the supernatant was extracted with equal volume of chloroform-isoamyl alcohol. After addition of 4 *M* NaCl in equal volume and anhydrous alcohol in double volume and standing under room temperature for 20 min, it was centrifugalized once more for 10 min. The DNA precipitate was washed with 70 % alcohol twice, dried with vacuum and dissolved in TE buffer for further use.

PCR

Each 25 μL of the reacting mixture contains 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.001% Gelatin, 0.8 m*M* dNTP, 0.2 μM random primer, 25 ng genomic DNA, and 2 units of Taq DNA polymerase. After addition of 20 μl of liquid paraffin and centrifugalization, it was amplified in a Perkin-Elmer 480 DNA amplifier. After predenaturation under 94 ℃ for 3 min, 45 cycles (94 ℃, 1 min → 34 ℃, 1 min → 72 ℃ , 2 min) were run. Finally it was extended under 72 ℃ for 10 min. The amplification products were separated by electrophoresis in 15 % agarose gel following routine procedure and pictures were taken under UV light.

Data analysis

Only distinct RAPD bands were recorded. The genetic variation between any two individuals were measured in terms of Genetic Distance (D) and Genetic Similarity (S).

$S = 2 N_{xy}/(N_x + N_y)$.

N_{xy}: number of bands shared by both individuals, x and y

N_x: number of bands displayed by only individual x.

N_y: number of bands displayed by only individual y.

$D = I - S$.

The calculation was carried out in a IBM - 486 using a program developed with C language

RESULTS AND ANALYSIS

Extraction and purification of leaf cell total DNA of A. brachypus

In a preparative experiment, it was found that the DNA sample prepared from A. brachypus using the method of Doyle JJ and Doyle JL (1987)[5] contained large

amount of pigments or other secondary metabolites and polysaccharides, thus being unsuitable for further analysis. As shown by our study, (1). addition of higher concentration of mercaptoethanol (5 %) helped removal of pigments; (2). addition of Cellulose HL 100 (product of Serva) might help removal of further pigments without impairing the yield of DNA; (3). Fang et al. reported in 1992 that 1 *M* NaCl might increase the solubility of polysacharrides in alcohol when the latter was used to precipitate DNA, thus helping to decrease the polysaccharides in the precipitate[6], and, after testing NaCl solutions of different concentrations, we found 2 *M* NaCl to be the most effective in removing polysaccharides. In addition, considering the possibility of higher content of polyphenols in the mature leaves we sampled, we added PVP (Polyvinylpyrrolidone) in the extracting buffer.

It was found that all the samples prepared using this method could be amplified effectively by PCR and used for RAPD analysis. And with electrophoresis in 0.8 % agarose gel, bands of large molecular weights (usually above 50 kb, indicating little splicing and decomposition of the DNA samples) were demonstrated which could be digested by restriction enzymes, thus indicating their suitablity for RFLP and DNA fingerprinting analysis as well. We also applied this appoach to RAPD study of A. senticosus and Dimocarpus longan Lout. with success (two separate works to be published).

PCR system

A series of experiments was carried out to find out the optimal parameters of experimental condition for PCR based on the procedure reported by Williams et al. (1990)[4]. (1). Of the 3 annealing temperatures tested, amplification was the strongest, resulting in the most distinct bands under 34 ℃; it was weaker under 36 ℃; and no amplification was noted under 42 ℃. (2). Of the 4 DNA template concentrations tested, 12.5 ng, 25 ng and 50 ng made no difference with regard to both the strength of amplication and the banding pattern while, with 100 ng, no distinct band was found. (3). Of the 4 Taq DNA polymerase concentrations (0.5, 1.0, 2.0 and 4.0) tested, distinct bands were noted at 2.0. Our experimental system was finally established using these values as condition parameters.

RAPD analysis of genetic variation of natural populations of A. brachypus

The RAPD loci found

Using the above PCR system, we first used the No. 15 DNA sample taken from Heshangyuan as the template to test the 40 primers of the OPA and OPB kits. Amplification and electrophoresis revealed distinct bands for 22 of the primers tested. Then, using these 22 primers, we did in-depth RAPD analysis of the total leaf cell DNA from 17 individuals form Population I (Fig 1), from 14 individuals form Population II and from 12 individuals form Population III. The results are shown in Tab 1. For 17 of the primers, data were obtained in all the populations.

The number of RAPD loci found for each primer ranged from 1 to 9, with an average of 5.7.

Tab 1. Amplification results for 22 primers

Primers	Sequences	Amplified RAPD loci		
		Population I (17 individuals)	Population I I (14 individuals)	Population I II (12 individuals)
OPA-01	CAGGCCCTTC	7	5	6
OPA-02	TGCCGAACTG	7	7	7
OPA-03	AGTCAGCCAC	6	6	6
OPA-04	AATCGGGCTG	6	6	6
OPA-08	GTGACGTAGG	5	-	-
OPA-09	GGGTAACGCC	6	-	6
OPA-10	GTGATCGCAG	5	4	5
OPA-11	CAATCGCCGT	5	7	6
OPA-13	CAGCACCCAC	1	1	1
OPA-14	TCTGTGCTGG	5	-	-
OPA-16	AGCCAGCGAA	6	6	6
OPA-18	AGGTGACCGT	6	6	6
OPA-19	CAAACGTCGG	5	5	5
OPA-04	GGACTGGAGT	4	4	4
OPA-09	TGGGGGACTC	9	-	-
OPA-10	CTGCTGGGAC	7	7	7
OPA-11	GTAGACCCGT	-	-	1
OPA-13	TTCCCCCGCT	4	4	3
OPA-14	TCCGCTCTGG	4	3	3
OPA-15	GGAGGGTGTT	4	3	3
OPA-17	AGGGAACGAG	8	8	7
OPA-20	GGACCCTTAC	6	5	5

Note: Population I was from Heshangyuan; Population II from Nanniwan; Population III from Linxiao.

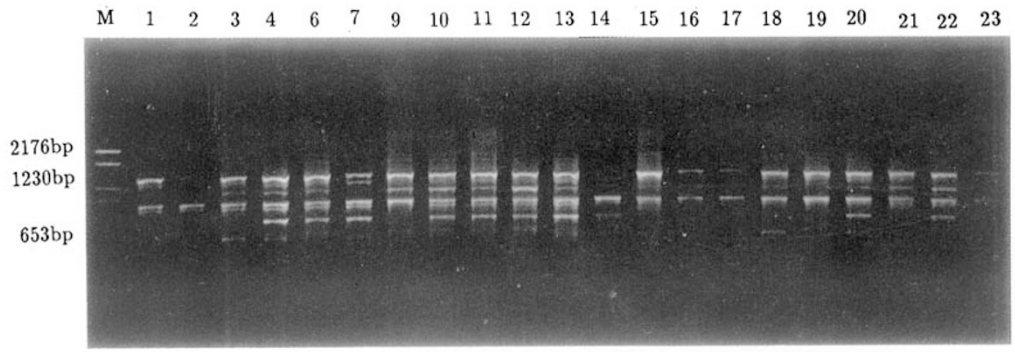

Fig 1. Electrophoresis patterns of products amplified using OPA,01 as primer (the polymorphic RAPD markers)
Note: The upper numerals are sample numbers.
M: Molecular weight markers (Boeringer DNA MolecUar Marker VI)

Tab 2. Polymorphic DNA loci

	Total RAPD loci	Polymorphic RAPD loci	Percentage (%)
Population I	116	31	26.7
Population II	87	16	18.4
Population III	93	5	5.4

As shown in Tab 2, the percentage of polymorphic RAPD loci found among the total loci varied from 31/116 (26.7 %) in Population I through 16/87 (18.4 %) in Population II to 5/93 (5.4 %) in Population III.

Genetic simularity and distance within population

Using the method as described in Date analysis method, both the genetic similarity and distances were calculated and the results are shown in the Tab 3 and 4.

Tab 3. Genetic similarity and distances within each population

	Population I	Population II	Population III
S	0.945	0.964	0.992
S*max*	0.981	1.000	1.000
S*min*	0.911	0.934	0.972
D	0.055	0.036	0.008

Note: S - genetic similarity; S*max* - maximum genetic similarity S*min* - minimum genetic similarity; d - genetic distance.

Tab 4. Genetic similarity and distances between populations

	Population (I-II)	Population (I-III)	Population (II-III)
S	0.895	0.904	0.940
S*max*	0.935	0.940	0.979
S*min*	0.853	0.870	0.894
D	0.105	0.096	0.060

As shown in Tab 3, the genetic similarity is the highest and the genetic distance, the lowest in Population III where the fewest polymorphic RAPD loci were found. The average genetic distances of the three populations are respectively 0.055, 0.036 and 0.008. As compared with the values obtained from those of natural populations of other plants so far reported, they are rather low, indicating the paucity of genetic diversity of A. brachypus.

The genetic similarity and distances among populations

The genetic similarity and distance between each pair of individuals drawn separately from different populations were calculated and the results are shown in the

Tab 4. The genetic distances (I- II), (I- III) and (II- III) are respectively 0.105, 0.096 and 0.060. Thus the inter-population genetic variation is also very low in *A. brachypus*.

DISCUSSION

Extraction of total DNA

As a result of the diversity of plant species and the complexity of the secondary metabolites of plants, no general procedure for extracting DNA has been developed so far. The modified CTAB method[7] developed by us has helped us to obtain high quality genomic DNA of *A. brachypus* and has proved effective in removing such impurities as pigments and polysaccharides. It is believed that the method is also applicable to plants with similar characteristics. For instance, we have successfully applied this method for the isolation and purification of total leaf DNA of A. senticosus and *Dimocarpus* longan Lour. (works to be published). To study DNA variation at population level requires preparation of large amount of samples. Thus, simple and rapid methods that require only small amount of materials are needed. The method we developed and used here is simple, it doesn't require the use of chromatography or cesium chloride gradient centrifugation, and only a small amount of material is needed, so that it is particularly suitable for genetic analysis at population level.

Genetic variation level of A. brachypus and the implication for its protection

The distribution of A. brachypus is restricted, with both the number of populations and that of the individuals within each population being very small. Our analysis has also revealed a very low genetic variation level. Chalmers et al. did RAPD analysis on 8 populations of *Gliricidia* sepium, giving the following genetic similarity values: 0.7220, 0.7822, 0.7798, 0.8098, 0.8245, 0.8499, 0.9298 and 0.9417[8]. Our analysis of 3 populations of A. senticosus has given the values: 0.759, 0.637 and 0.612. As compared with them, the values obtained in this study (0.945, 0.964 and 0.992) are considerably higher, indicating the low genetic variation level of A. barchypus.

Low genetic diversity is a common feature characterizing plants with restricted distribution and it is thus no surprise that *A. brachypus* with its restricted distribution was found to have such a low genetic variation level. As Hamrick and Godt[10] reported based on their isoenzyme study of large amount of plant materials, marked correlation was found between plants with restricted distribution and low genetic variation level.

Gustafsson and Gustafsson[11] have done RAPD analysis on 8 populations of an endangered species, *Vicia pisiformis*, and found a very low average genetic variation level with only 4 % polymorphic loci noted in the 216 RAPD loci. Similarly, only 17.5 % of the RAPD loci were found to be polymorphic in the 3 populations of A.

brachypus in average. So the low genetic variation level of *A. brachypus* may very well be one of the chief reasons for its decline.

Intraspecific genetic diversity is an essential element of modern concept of species, upon which a species depends for its survival and evolution. It is known that genetic variation is of paramont important to the survival of endangered species. A certain amount of genetic variation is the prerequisite for the adaptative potential and consequently the evolutionary potential of a species. On the other hand, lack of sufficient genetic variation sets grave restriction on the adaptative power of a spcies and limits expansion of its populations, bringing with it greater risk of extinction. It is thus obvious that the assessment of genetic variation level of a species is very important to its protection and management and, hopefully, the messages brought up by our study may shed light on the formulation of a rational and effective approach to the protection of A. brachypus.

ACKNOWLEDGEMENT

This work was supported by National Foundation of Natural Sciences

REFERENCES

[1] ZHOU Li-wei, WU Nai-hu. Research advances about genetic diversity of endangered species in higher plants. Progress in biotechnology (In Chinese) 1995; **15 (4)**:22-5.

[2] Welsh J, Mc Clelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucl Acids Res 1990; **18**:7213-8.

[3] Williams JGK et al. DNA polymorphisms amplified by arbitrary primers. Nucl Acids Res 1990; **18**:6531-5.

[4] Hardy H, Balick M, Schierwater. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. Molecular Ecology 1992; **1**:55-63.

[5] Chalmers KJ et al. Detection of genetic variation between and within populations of *Gliricidia sepium* and *Gliricidia* maculata using RAPD markers. Heredity 1992; **69**:465-73.

[6] Fang Guoweiet al. A qucik and inexpensive method for removing polysaccharides from plant genomic DNA. Biotech 1992; **13**:52-6.

[7] Doyle J J, Doyll JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bul 1987; **19**:11-5.

[8] Chalmer KJ et al. Evaluation of the extent of genetic variation on mahoganies (Meliaceae) using RAPD markers. Ther Appl Genet 1994; **89**:504-8.

[9] DAI Si-lan, WU Nai-hu. RAPD analysis of *Acanthopanax* senticsus for its genetic diversity. Progress in Natural Sciences, (In Chinese) (to be published).

[10] Hamrick JL, Godt MJW. Plant population Genetics: Breeding and Genetic Resources. In: Brown AHD et al (eds) Sunderland, Mass: Sinauer 1990:43-6.

[11] Gustafsson L, Gustafsson P. Low genetic variation in Swedish populations of the rare species Vicia pisiformis (Fabaceae) revealed with rflp (rDNA) and RAPD. P1 Syst Evol 1994; **189**:133-48.

Received 31-3-1997. Revised 23-5-1997. Accepted 2-6-1997.