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Genetic Identification of Chinese Drug Materials in Yams (*Dioscorea* spp.) by RAPD Analysis

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ABSTRACT

The botanical origins of Chinese drug materials in yams were examined among eighteen cultivarities/ species of *Dioscorea* by randomly amplified polymorphic DNA (RAPD). DNA isolated from fresh leaves were used as a template in polymerase chain reactions (PCR) using thirty random decamer primers. Eighteen primers produced 237 polymorphic RAPD markers, which were scored for all the lines studied. Four primers including OPP-04, OPP-15, OPAP-04 and OPAP-10, could distinguish all tested samples. Genetic distances and similarities among eighteen cultivarities/species were calculated, and a cluster analysis was used to generate a dendrogram showing a phylogenetic relationship. The studied *Dioscorea* species could be divided into five subgroups by phylogenetic analysis. Genetic distance and similarity analysis showed that line 70W37 was distantly related to 70W47, and line 70W41 was closely related to 70W59. RAPD technique could be successfully applied for the rapid identification and differentiation of *Dioscorea* species.

Key words: botanical origins, yam (*Dioscorea* spp.), phylogenetic analysis

INTRODUCTION

Yam (*Dioscorea* spp.) is one of very important pharmaceutical plants used in the traditional Chinese medicine. In the Chinese pharmacopoeia, Pentsao, the medicinal uses of *Dioscorea* rhizome are prescribed for indigestion, anorexia, diarrhea, and diabetes [(1)]. Yam has many superior characteristics in the rhizome such as a high viscosity, and high contents of polysaccharides, amino acids, protein, vitamins and mineral elements. It also contains dioscin, diosgenin, phytic acid, allantoin, dopamine, batatasin, dioscorea-musilage B, and sterols [(2, 3)]. Diosgenin, a steroidal sapogenin, is one of the important starting materials for the manufacture of sexual hormone drugs including oral contraceptives. In general, yam can be widely applied in the drug industry.

Yam is a perennial herb, distributed in the tropical and subtropical regions. There are more than 600 species in the world, of which 93 species and 9 varieties are found in China, and 14 species and 5 varieties found in Taiwan[(4)]. Since there is a high diversity in phenotypes, including leaf shape, stem length, rhizome shape, rhizome skin color, and flesh color within and among the species of *Dioscorea*, identification according to their morphological characters is difficult. Reports on identification of *Dioscorea* species using the isozyme electrophoresis method and the anatomy method have been published[(5-8)]. Recently, molecular-based techniques, such as the random amplified polymorphic DNA[(9-11)] technique and analysis of DNA by polymerase chain reaction (PCR)[(12-14)] have been recognized as powerful and efficient tools to detect genetic diversity and assess phylogenic relationships. So far, only *Dioscorea alata*, *D. pseudojaponica* and *D. doryophora* in *Dioscorea* species have been studied [(15, 16)]. This present study assessed the extent of genetic diversity and determined the phylogenetic relationships among eighteen lines of *Dioscorea* species by RAPD analysis.

MATERIALS AND METHODS

I. *Plant Materials*

A total of eighteen cultivarities/species of *Dioscorea* were used in this study (Table 1). Plants were cultivated in the fields of the Taiwan Agricultural Research Institute and the National Pingtung University of Science and Technology. Taxonomic statuses and origins of these *Dioscorea* are shown in Table 1. Twelve of eighteen cultivarities/ species originated from Taiwan, four from China, and two from Japan. "W" in the notation represents white flesh of tuber whereas "R" represents red flesh of tuber.

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Table 1. Eighteen cultivarities/ species of *Dioscorea* used in this study

Line notation	Cultivarities/ species	Origin
TNG 1	*Dioscorea alata* L.	Trinidad
TNG 2	*Dioscorea alata* L.	Taiwan
70W34	*Dioscorea alata* L.	China
70W35	*Dioscorea alata* L.	China
70W47	*Dioscorea alata* L.	Taiwan
80W02	*Dioscorea alata* L.	China
70W98	*Dioscorea alata* L.	Taiwan
70W48	*Dioscorea alata* L.	Taiwan
70W42	*Dioscorea alata* L.	Taiwan
70R16	*Dioscorea alata* L. var. *purpurea* (Roxb) M. Pouch.	Taiwan
70R20	*Dioscorea alata* L. var. *purpurea* (Roxb) M. Pouch.	Taiwan
70W41	*Dioscorea batatas* Decne	Japan
70W59	*Dioscorea batatas* Decne	China
70W49	*Dioscorea japonica* Thumb.	Japan
70W43	*Dioscorea japonica* Thumb. var. *pseudojaponica* (Hay.) Yamamoto	Taiwan
70W51	*Dioscorea doryophora* Hance	Taiwan
70W20	*Dioscorea doryophora* Hance	Taiwan
70W37	*Dioscorea bulbifera* L.	Taiwan

II. *DNA Preparation*

DNA was prepared by grinding one young leaf (1.0 g) in liquid nitrogen, placing the ground tissue into a sealable plastic tube, adding 10 mL of extraction buffer [100 mM Tris-HCl, pH 8.0; 50 mM Na_2EDTA, pH 8.0; 500 mM NaCl; 10 mM mercapto-ethanol] and 1 mL 20% SDS, followed by incubating in a 65˚C water bath for 20 min. Five milliliters of 5M potassium acetate was added to each sample. After incubating on ice for 20 min, samples were centrifuged at 25000 × g, 4˚C for 20 min. The supernatant was transferred to a clean tube, and DNA was precipitated with 10 mL of cold isopropanol and centrifuged at 12000 rpm, 4˚C for 30 min. DNA samples were pelleted, washed in 1 mL 70% ethanol, and dissolved in 300 μL TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) and 5 μL RNase A. DNA samples were extracted by two consecutive phenol-chlroform-isoamyl alcohol (25:24:1) extractions and recovered by precipitation with ethanol containing 0.3 M sodium acetate. DNA was washed twice using 70% ethanol, suspended in distilled water, and quantitated with a spectrophotometer at 260 nm.

III. *RAPD Reactions*

Thirty random 10-base primers were used in this study (Table 2). PCR was carried out in a 25 μL volume containing 10× buffer, 2.5 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 2.0 μM of primer (Operon), 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim), and 5 ng template DNA. Samples were subjected to 35 cycles consisting of denaturation for 1 min at 94˚C, annealing for 1 min at 37˚C, extension for 3 min at 72˚C, and one final extension cycle at 72˚C for 7 min. After completion of the PCR, 10 μL of reaction mixture was loaded into a 1.2% agarose gel, containing 0.5 μg/ mL ethidium bromide for electrophoresis and subsequent visualization by UV transillumination.

IV. *Data Analysis*

The profile of amplified DNA products from each sample was visualized in UV light. Photographs were taken and the profile image was saved on a magnetic disc (Photo-print, Version 98.02, Vilber Lourmat, France). The molecular weight of each amplified band in a gel was calculated and matching analysis was done using Bio-Profile image analysis software (Bio-1D/Bio-gene, Version 97, Vilber Lourmat, France). The presence and absence of amplified bands were denoted by 1 and 0 signals for analysis of Dice genetic distance, similarity coefficient, and dendrogram using NTSYS program.

RESULTS

I. *PCR Primers and RAPD Patterns*

Eighteen primers produced polymorphic products, the remaining twelve primers produced ambiguous patterns that could not be scored reliably. Two hundred forty-nine amplified markers were generated by using the eighteen primers, 237 of which (95.2%) were polymorphic. The number of scored markers produced by each primer ranged from 5 to 21 (Table 2), with an average of about l3 markers per primer. Of eighteen primers, four primers including OPP-04, OPP-15, OPAP-04, and OPAP-10 were able to produce distinct and polymorphic fingerprints. Primer OPP-04 resulted in eleven distinct fingerprints, and primers OPP-15, OPAP-04, and OPAP-10 generated nine and four distinct fingerprints,

Table 2. Nucleotide sequences of the random primers used in the present experiment

Code of primers	sequence	GC content (%)	Numbers of bands polymorphic	Numbers of amplified bands (amplified samples/total samples)
OPP-01	GTAGCACTCC	60	7	8(14/18)
OPP-02	TCGGCACGAC	70	10	11(12/18)
OPP-03	GTGATACGCC	60	15	15(12/18)
OPP-04	GTGTCTCAGG	60	13	13(16/18)
OPP-05	CCCCGGTAAG	70	21	21(17/18)
OPP-08	ACATCGCCCA	60	18	19(18/18)
OPP-10	TCCCGCCTAC	70	8	8(10/18)
OPP-12	AAGGGCGAGT	60	11	11(18/18)
OPP-15	GGAAGCCAAC	70	17	17(16/18)
OPP-17	TGACCCGCCT	60	6	6(16/18)
OPP-18	GGCTTGGCCT	70	5	5(8/18)
OPP-19	GGGAAGGACA	60	18	18(17/18)
OPAP-01	AACTGGCCCC	70	14	19(18/18)
OPAP-04	CTCTTGGGCT	60	16	18(17/18)
OPAP-05	GACTTCAGGG	60	11	13(16/18)
OPAP-06	GTCACGTCTC	60	18	18(18/18)
OPAP-08	ACCCCCACAC	70	20	20(18/18)
OPAP-10	CTGGCTTCTC	60	9	9(17/18)
Total			237	249

respectively. By comparing the polymorphic fingerprints, eighteen cultivarities/species of *Dioscorea* could be clearly differentiated (Figure 1).

II. *Genetic Distance and Similarities*

Genetic distance and similarities among eighteen cultivarities/ species were calculated. A dendrogram derived from all the species was generated using NTSYS program. Lines 70W41 and 70W59, which originated from Japan and China, respectively, showed the smallest genetic distance (0.184) and the largest similarity (0.831). In contrast, lines 70W47 and 70W37, which originated from Taiwan, showed the largest genetic distance and the smallest similarity (Table 3). Therefore, the analysis of genetic distance and similarities showed that line 70W47 was distantly related to 70W37, and line 70W41 was closely related to 70W59.

III. *Phylogenetic Tree*

A dendrogram, based on the genetic distance from RAPD data, is shown in Figure 2. In the phylogenetic tree, *Dioscorea* species were divided into five subgroups based on the genetic distance. The first subgroup I including lines TNG1, TNG2, 70W34, 70W47, and 70R16, belonged to *D. alata*, except 70R16, namely *D. alata* var. *purpurea*. The second subgroup II included lines 70W41, 70W59, 70W49, 70W43, and 70W51. Lines 70W41and 70R59 belonged to *D. batatas*, lines 70W59 and 70W49 belonged to *D. japanica*, and line 70W51 belong to *D. doryophora*. The fourth subgroup IV including lines 70W35, 80W02, 70W42, 70W98, 70W48, and 70R20, belonged to *D. alata*, except 70R16 belonged to *D. alata* var. *purpurea.* Subgroup III and V contained 70W20 (*D. doryophora*) and 70W37 (*D. bulbifera*), respectively.

DISCUSSION

Molecular markers are fundamental tools for plant biologists. RAPD markers are suitable for determining similarities among inbreds, establishing phylogenies, fingerprinting varieties, tagging desirable genes, and mapping plant genomes. RAPD markers are obtained by PCR amplification of random DNA segments from single arbitrary primers [9-11]. Arbitrary primers used in the present study were 10 bp in size; they had a GC content of 60% to 70 % and did not contain palindromic sequence. Williams *et al.* 1990[17] were the first to use this technique on plants and gave the name RAPD. In our previous study, twelve cultivarities/species of *Dioscorea* were clearly distinguished using anatomical studies and a microscopic index table[18]. The relationships among eighteen lines of *Dioscorea* could be definitely determined by anatomical studies. In recent years, molecular-based techniques such as RAPD analysis have been established and applied to construct phylogenetic dendrograms of closely related species, to investigate intraspecies relationships, and to identify particular varieties of the crops[15, 16, 18]. Their value and convenience have been widely recognized and these techniques are commonly put to practical use.

Polymorphism detected by RAPD was determined by the different DNA sequence of the sites, which the primer bound, or by the insertions and deletions occurring between these sites. In the present study, high polymorphisms among eighteen cultivarities/species of *Dioscorea* studied were obtained using eighteen primers. Of the 249 amplified prod-

Table 3. The genetic distance and similarity among eighteen cultivarities/ species of *Dioscorea*, using NTSYS program

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.257	0.287	0.569	0.369	0.645	0.573	0.686	0.387	0.625	0.453	0.429	0.619	0.501	0.519	0.378	0.694	1.024
2	0.769		0.386	0.540	0.494	0.604	0.455	0.490	0.450	0.570	0.572	0.499	0.496	0.619	0.688	0.490	0.647	0.978
3	0.750	0.673		0.528	0.200	0.718	0.479	0.565	0.329	0.530	0.405	0.432	0.716	0.505	0.548	0.450	0.668	1.120
4	0.542	0.571	0.557		0.582	0.599	0.570	0.441	0.656	0.594	0.671	0.729	0.617	0.719	0.883	0.747	0.645	0.832
5	0.689	0.601	0.818	0.522		0.780	0.589	0.620	0.303	0.527	0.426	0.438	0.805	0.486	0.581	0.416	0.848	1.265
6	0.479	0.515	0.435	0.544	0.402		0.396	0.408	0.691	0.475	0.789	0.753	0.342	0.837	0.937	0.804	0.786	0.743
7	0.547	0.627	0.593	0.565	0.526	0.661		0.316	0.550	0.537	0.589	0.613	0.396	0.767	0.739	0.604	0.725	0.880
8	0.479	0.597	0.532	0.643	0.497	0.660	0.727		0.654	0.567	0.743	0.679	0.348	0.740	0.827	0.721	0.788	0.863
9	0.679	0.633	0.719	0.494	0.737	0.452	0.557	0.491		0.433	0.431	0.461	0.689	0.492	0.618	0.466	0.737	1.148
10	0.525	0.563	0.571	0.549	0.569	0.603	0.584	0.563	0.634		0.682	0.732	0.588	0.866	0.896	0.746	0.654	0.989
11	0.634	0.558	0.667	0.480	0.653	0.402	0.529	0.443	0.649	0.489		0.184	0.702	0.302	0.328	0.345	0.495	0.986
12	0.651	0.603	0.649	0.459	0.644	0.426	0.523	0.479	0.631	0.469	0.831		0.690	0.342	0.352	0.306	0.475	0.994
13	0.500	0.583	0.446	0.537	0.402	0.709	0.667	0.705	0.463	0.545	0.449	0.463		0.832	0.790	0.654	0.650	0.796
14	0.603	0.527	0.602	0.450	0.615	0.375	0.437	0.436	0.609	0.401	0.738	0.708	0.387		0.328	0.456	0.581	1.081
15	0.595	0.500	0.578	0.395	0.558	0.355	0.462	0.415	0.539	0.400	0.719	0.703	0.420	0.717		0.410	0.651	1.080
16	0.685	0.609	0.637	0.452	0.658	0.405	0.529	0.461	0.627	0.464	0.707	0.736	0.481	0.631	0.664		0.633	1.085
17	0.482	0.517	0.489	0.525	0.404	0.449	0.484	0.454	0.459	0.519	0.579	0.598	0.518	0.523	0.503	0.512		0.664
18	0.294	0.325	0.260	0.409	0.221	0.466	0.383	0.400	0.257	0.337	0.295	0.300	0.436	0.261	0.277	0.275	0.478	

1. TNG 1, *D. alata* L.; 2. TNG 2 , *D. alata* L.; 3. 70W34 , *D. alata* L.; 4. 70W35 , *D. alata* L.; 5. 70W47, *D. alata* L.; 6. 80W02 , *D. alata* L.; 7. 70W98, *D. alata* L.; 8. 70W48, *D. alata* L.; 9. 70R16, *D. alata* L. var. *purpurea* (Roxb) M. Pouch.; 10. 70R20, *D. alata* L.var. *purpurea* (Roxb) M. Pouch.; 11. 70W41, *D. batatas* Decne; 12. 70W59, *D. batatas* Decne; 13. 70W42 , *D. alata* L.; 14. 70W49, *D. japonica* Thumb.; 15. 70W43, *D. japonica* Thumb. var. *pseudojaponica* (Hay.) Yamamoto; 16. 70W51, *D. doryophora* Hance; 17. 70W20, *D. doryophora* Hance; 18. 70W37, *D. bulbifera* L.

ucts, 237 were polymorphic. Results showed that a high degree of genetic variability existed within intraspecies of *Dioscorea*, which originated from four countries. Eighteen species of *Dioscorea* were classified into five subgroups on the basis of RAPD data, and could be clearly distinguished by RAPD analysis by combination of four primers.

Eleven lines of *Dioscorea alata* were divided into two subgroups (I and IV) by phylogenetic analysis and success-

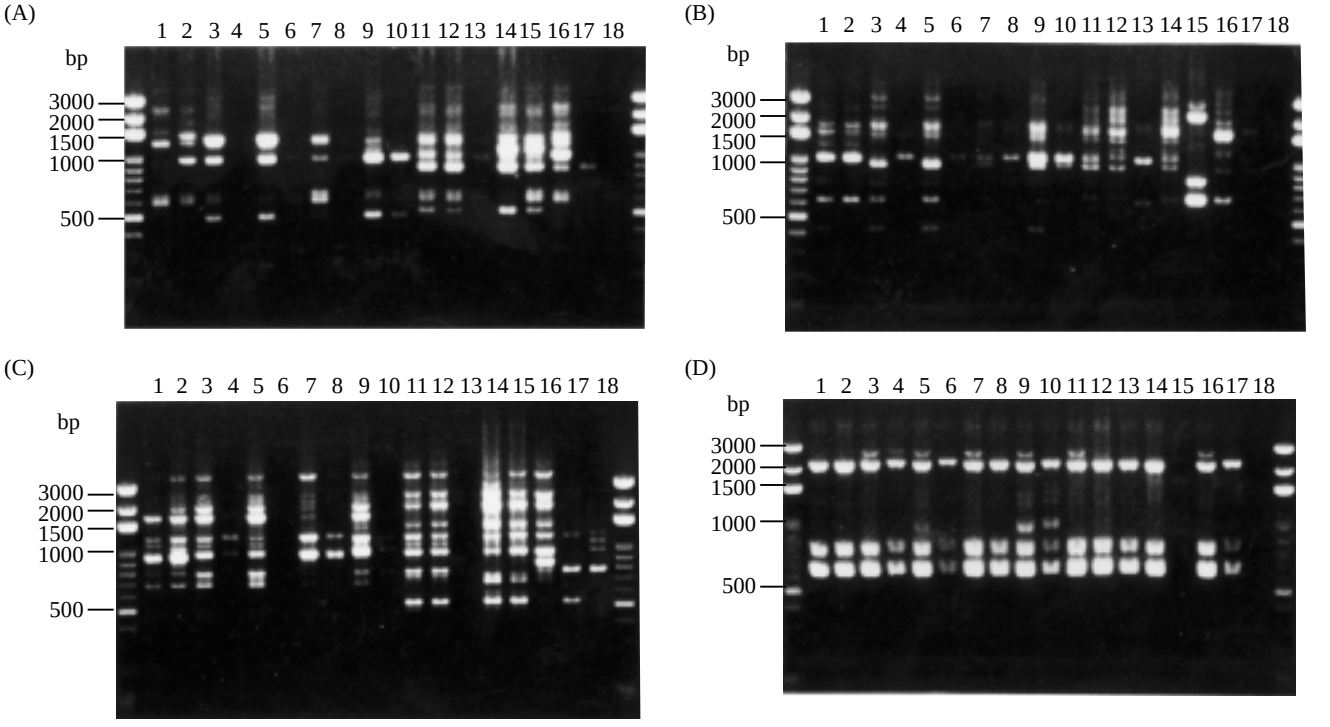

Figure 1. RAPD patterns obtained from eighteen cultivarities of *Dioscorea* with primers (A) OPP-04, (B) OPP-15, (C)OPAP-04, and (D)OPAP-10. Primer OPP-04 used in this study generated eleven different RAPD patterns while primers OPP-15 and OPAP-04 as well as primer OPAP-10 resulted in nine and four distinct RAPD patterns, respectively. DNA molecular weight marker (Bio 100 DNA ladder^{TM}) is indicated as bp. 1,TNG1; 2,TNG2; 3, 20W34; 4, 70W35; 5, 70W47; 6, 80W02; 7, 70W98; 8, 70W48; 9, 70R16; 10, 70R20; 11, 70W41; 12, 70W59; 13, 70W42; 14, 70W49; 15, 70W43; 16, 70W51; 17, 70W20; 18, 70W37.

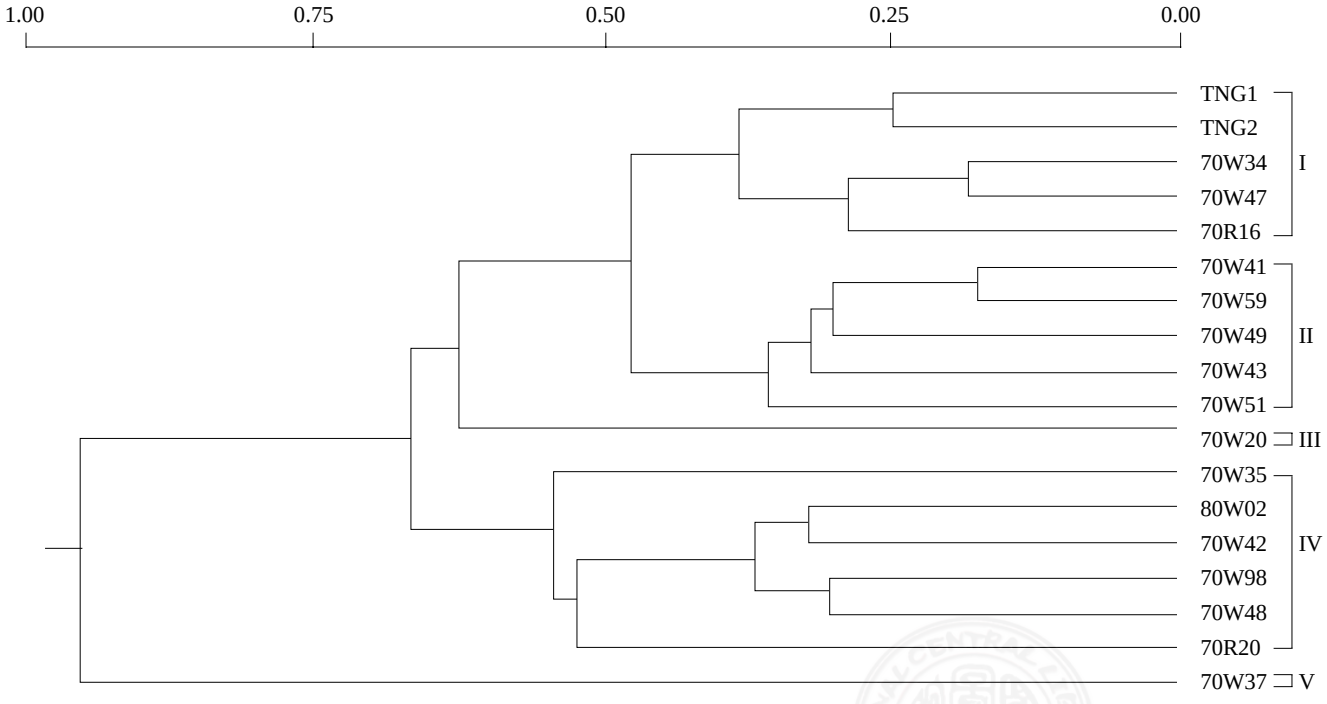

Figure 2. The phylogenetic tree of eighteen species of *Dioscorea* based on genetic distances analyzed with RAPD markers.

fully distinguished by RAPD analysis based on numbers of amplified bands. Although subgroup II, including two *D. batatas*, two *D. japonica*, and one *D. doryophora* (70W51), showing high similarity in morphological characteristic of halberd leaf shape, they could be differentiated with RAPD markers. Lines 70W51 and 70W20 belonged to *D. doryophora*, but were placed into different subgroups (II & III), indicating that high genetic variation occurred in *D. doryophora*. In the present study, results showed RAPD analysis was a rapid and useful approach for distinguishing closely related species in the *Dioscorea* as well as for estimating genetic distance among the species.

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以RAPD法鑑定山藥藥材之基原

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摘　要

以RAPD法檢測薯蕷屬(*Dioscorea*)18個品系山藥藥材的基原。以30個隨機引子進行聚合酶連鎖反應增幅山藥藥材葉子之DNA。30個引子中的18個引子可產生237個基因標誌。其中的四個引子OPP-40,OPP-15,OPAP-04及OPAP-10可用於區分這18個品系之山藥藥材。計算品系間之相似與相異係數及分群分析可建構品系間之基因親緣關係圖，並可將18個品系分為5個次群。基因相似及相異度分析顯示70W37品系與70W47品系差異較大，而70W41品系則與70W59品系較為相似。本研究已成功的應用RAPD法鑑定及區別薯蕷屬的18個品系山藥藥材。

關鍵詞：植物源，山藥，基因親源關係分析