Discrimination of wild grapes native to China by RAPD markers

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Summary

A set of 73 types of 18 wild grape species native to China, one interspecific hybrid, 7 *Vitis vinifera* cultivars, the rootstock cultivar SO 4 and one *V. riparia* accession were investigated using the RAPD technique. The screening of 280 decamer oligonucleotides allowed the selection of 20 primers used for the analysis. RAPD fingerprints of 83 grape samples were obtained. 191 bands, intense and easy to score, were chosen as markers. On average 5.7 bands per primer were amplified with an average of 68.7 % polymorphism. The size of amplified bands ranged from 100 to 3000 bp. Discrimination of 83 samples was obtained with one of two primers (OPQ04, OPJ07) combined with one of 5 primers (OPJ01, OPH19, OPP02, OPA15, OPU16). The average number of bands for each sample per primer was 5.7. This revealed a high level of polymorphism among the wild grapes native to China. RAPD markers proved to be useful for identification as they are quick and easy to use.

K e y w o r d s : wild grape accessions, China, RAPD, discrimination.

Introduction

Out of 70 known *Vitis* species more than 27 have their origin in China (Li Shenchen 1985; Niu Lixin 1996). A large number of wild grapes of different *Vitis* species native to China have been collected at the Northwestern Agriculture University. Their morphological traits have been described in detail. Accurate identification of wild grapes native to China is essential for research and viticulture. It is difficult to differentiate between similar phenotypic species especially clones or accessions of one species with different disease resistance genes (Wang Guojin 1986; Chai Juhua 1997; Wang Yuejin 1998).

RAPD (randomly amplified polymorphic DNA) technique is fast and easy, since it does not require any prior knowledge of the sequences of the markers and can be resolved using agarose gels. This technique has already proven its usefulness for the identification of cultivars in numerous plants (Koller 1993; Gogorcena 1994; Aruna 1995). It has also been applied to genetically analyse grapevine cultivars and *Vitis* species (Büscher 1993; Jean-Jaques 1993; Grando 1996) and rootstocks (Xu 1995; This 1997). Considering previous experience, some bands in RAPD profiles are more stable than others (Penner 1993; Xianping Ou 1996). Thus a selection of primers and bands appeared to be a necessary step in order to generate stable markers with this technique. The objectives of the present study were to establish RAPD analysis for Chinese wild grapes, to analyze the usefulness of the RAPD markers for discrimination of Chinese wild grapes and to develop a database for the identification of native, wild grapes of China.

Material and Methods

P l a n t m a t e r i a l : All plant material was obtained from the vineyard of the Northwestern Agricultural University: 73 native clones or accessions of 18 wild grape species and varieties, one interspecific hybrid (Kyoho), 7 *Vitis vinifera* cultivars, one rootstock cultivar (SO 4) and one *V. riparia* accession (No. 2) (Tab. l).

D N A e x t r a c t i o n : Total genomic DNA was isolated from 0.2-0.5 g of frozen young leaf and bud samples on the basis of a CTAB protocol (Sulan Luo 2000).Young leaves and buds (0.2-0.3 g) were ground to a fine powder under liquid nitrogen. The powder was transferred into 500 µl of extraction buffer (2 % CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 20 mM $Na_2S_2O_5$, 3 % β-mercaptoethanol), and then 100 µl of 20 % PVPP (polyvinylpolypyrrolidone) was added. After homogenization, an equal volume of chloroform/octanol (24:1) was added to the tube, then the homogenate was incubated for 30 min at 65 °C and cooled to room temperature. After centrifugation at 12,000 rpm for 10 min, the supernatant was transferred into another tube and nucleic acids were precipitated using 0.6 volumes of isopropanol. The tube was gently agitated for 15 min, and then picked out a threadlike DNA pellet with tips and washed with 70 % ethanol. The dried pellet was dissolved in 500 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 5 µl of RNase (10 $mg \cdot ml^{-1}$) were added to the solution and incubated for 60 min at 37 °C. Nucleic acids were precipitated using two volumes of cold ethanol and resuspended in 50 µl TE buffer. The DNA was quantified on a 0.8 % agarose gel stained with ethidium bromide by visual comparison with known quantities of lambda DNA.

P r i m e r s c r e e n i n g : 280 oligonucleotides from the kit A, B, C, G, H, J, O, P, Q, R, S, U, V, W (Operon Company) were first screened for their ability to amplify using DNA from 10 clones belonging to the initial 10 species in Tab. 1. Polymorphic primers were selected for further analysis.

R A P D a n a l y s i s : The RAPD reaction mixture contained 10 to 20 ng template DNA in a 25 µl reaction vol-

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Table 1

Vitis material used for RAPD analysis

Species	No.	Clone	No.	Clone	No.	Clone
V. vinifera L.	1	Chenin blanc	5	Riesling	8	Ugni blanc
	3	Merlot	6	Cabernet franc		
	4	Cabernet Sauvignon	7	Muscat rose		
Interspecific hybid	2	Kyoho				
	9	SO 4				
V. pseudoretica lata W. T. Wang	10	Guangxi-1	12	Bai-35-1	14	Guangxi –2 ♂
	11	Baihe-13-1	13	Bai -35-1 ♂	15	Shangnan-1
					16	Shangnan –1 ♂
V. quinquangularis Rehd.	17	83-4-67	22	83-4-94 ♂	27	83-4-1
	18	Wei-3	23	83-4-96	28	Shang-24
	19	Wei –3 ♂	24	83-4-96 ♂	29	Dan-2
	20	83-4-49	25	Tai shan-12		
	21	83-4-49 ♂	26	Nanzheng-1		
V. romanetii Rom.	30	Baihe-22	34	Jiangxi -2 ♂	38	Liuba –1
	31	Jiangxi-1	35	Pingli-2	39	Liuba –1 ♂
	32	Jiangxi –1 ♂	36	Pingli-7	40	Pingli-2 ♂
	33	Jiangxi –2	37	Liuba-11		
V. baihensis L. X. Niu	41	Baihe-40				
V. bashanica P.C. He	42	Baihe -41	43	Xunyang-8	44	Baihe -42
V. davidii Foex	45	Jinan-1	47	Fujian-4	49	Xue feng (/ ♂)
	46	Jinan -2 ♂	48	Lueyang-4	50	Tangwei (/ ♂)
V. davidii (Roman) Foex *var. ninqiangensis* L. X. Niu	51	Ningqiang-6				
V. piasekii Maxim	52	Baishui-40	54	Gansu-91	56	Liu-9
	53	Huaxian-1	55	Liu-8	57	Liu-6
V. liubaensis L. X. Niu	58	Langao-2	59	Liu-10		
V. spp. (Qiufuyie)	60	Meixian-6	61	Liu-7		
V. davidii var. *cyanocar*pa (Gang) Sarg	62	Langao-5	63	Zhenan-3		
V. adstricta Hance	64	Taishan-1	66	Anlin-3		
	65	Taishan -1 ♂	67	Anili-2 ♂		
V. amurensis Rupr.	68	Zuoshan-1	71	Zuoshan-2 74003	73	Tonghua-3
	69	Taishan-11	72	Zuoshan 76097	74	Heilongiang ♂
	70	Shuangyou (/ ♂)				
V. qinlingensis P. C. He	75	Pingli-5	76	Lueyang-4		
V. hancockii Hance	77	Jiangxi-3	78	Jiangxi –3 ♂		
V. yeshanensis J. X. Chen	79	Yanshan				
V. vinifera x *V. davidii*	80	Bayan Shirei x Jinan-2 No. 1	81	Muscat Ottonel x Jinan-2 No.2		
V. vinifera x *V. wilsonae*			82	Blue French x Yangxian-1 No.4		
V. riparia Michx	83	*V. riparia* No. 2				

Note: ♂ = male; / = female, not annotated; / ♂ = perfect flower type; No. 1-9 are perfect flower cultivars.

ume with 2.5 µl 10 x reaction buffer, 1.5 mM $MgCl_2$, 150 µm each dNTPs and 1 unit Taq DNA polymerase (Sino-American Company of Biotechnology, SACB) and 4 pM primer covered with a drop of mineral oil. Amplification was performed in a Perkin Elmer-480 thermal cycler programmed for 45 cycles (94 °C for 1 min; 36 °C for 1 min; 72 °C for 2 min) followed by an extension at 72 °C for 10 min. Amplification products were resolved by electrophoresis on 1.5 % agarose gels in 1 x TAE buffer at 5 $V \cdot cm^{-1}$ for 2 h. Gels were stained with ethidium bromide and visualized under UV light. In all cases a PCR marker (SACB) and λ DNA/*Hind*III were used as size marker.

Data analysis: Intense and reproducible bands on the gel were visually scored: 1 (for presence) or 0 (for absence), and reported in a binary matrix.

Polymorphic percentage (%) =
(Number of polymorphic bands/Total bands) x 100.

Results and Discussion

185 out of the 280 primers produced amplified products, especially kits G, H, J, P, Q, U, V and W revealed a high level of polymorphism. 20 oligonucleotides (Tab. 2) were selected to analyze the 83 samples; they yielded profiles with intense and well-separated bands, 191 polymorphic bands of total 278 bands, intense and easy to score, were chosen as markers. The size of the amplified fragments ranged from 100 to 3,000 bp, most bands having 300 to 2,200 bp. 87 bands (31.3 %) were monomorphic among all samples. The proportion of polymorphic bands was 68.7 %. Amplification of each primer is presented in Tab. 2.

Fig. 1 illustrates the results of 83 samples obtained with primer OPJ07. The high number of polymorphic bands (*i.e.* present in at least one sample and/or absent in at least one sample) of wild, native grapes was very different from that of *V. vinifera*, *V. riparia*, Kyoho and SO 4. RAPD markers displayed high polymorphism in wild, native grapes. The lowest number of total bands was 6 which were only produced by primer OPV10, including one polymorphic band. The highest number of total bands was 27 produced by primer OPQ04, including 20 polymorphic bands. OPJ07 was the second with 16 polymorphic bands out of a total of 18 scored bands. The average of amplified bands per primer/template was 5.7 (Tab. 2).

According to each template combination banding patterns, OPJ07 could distinguish all accessions excluding Pingli-2 and Pingli-7, Jiangxi-2 and Liuba-11 of *V. romanetii* Rom. (Figs 1 and 2). OPQ04 could not differentiate between Pingli-2 and Pingli-7 of *V. romanetii* as well as Zhuoshan-2 (74003) and Zhuoshan76097 of *V. amurensis*, the remaining samples could be identified by OPQ04. OPJ01 could not dif-

Table 2

Twenty polymorphic primers

Primer	Sequences (_5'-3')	Total amplified bands	Polymorphic bands	Average amplified bands per template	Polymorphic percentage
OPH19	CTGACCAGCC	14	12	7.0	85.7
OPV10	GGACCTGCTG	6	1	6.0	16.7
OPV18	TGGTGGCGTT	17	15	7.0	88.2
OPP02	TCGGCACGCA	17	14	6.5	82.3
OPV02	AGTCACTCCC	13	8	4.5	61.5
OPA15	TTCCGAACCC	16	12	5.0	75.0
OPV07	GAAGCCAGCC	13	8	5.0	61.5
OPG14	GGATGAGACC	14	11	6.5	78.6
OPW02	ACCCCGCCAA	11	10	5.5	90.9
OPU20	ACAGCCCCCA	8	3	5.5	37.5
OPO05	CCCAGTCACT	12	2	8.0	16.7
OPU16	CTGCGCTGGA	13	9	6.5	69.2
OPO10	TCAGAGCGCC	15	10	5.0	66.7
OPU13	GGCTGGTTCC	9	6	7.0	66.7
OPQ04	AGTGCGCTGA	27	20	7.5	74.1
OPJ07	CCTCTCGACA	18	16	4.0	66.7
OPO06	CCACGGGAAG	17	12	6.0	70.6
OPJ01	CCCGGCATAA	14	11	6.0	50.0
OPH08	GAAACACCCC	12	9	5.0	75.0
OPW08	GACTGCCTCT	12	9	6.0	75.0
Total		278	191	5.7	68.7

ferentiate between Fujian-4 and Lueyang-4 of *V. davidii* Foex, Jiangxi-2 ? and Pingli-2 ? of *V.romanetii* and clones of *V. pseudoreticulata* W. T. Wang. OPH19 could not identify clones of *V. adstricta* Hance and *V. bashanica*. OPP02 could not distinguish between Bai-35-1 ?, Guangxi-2 ? and Shangnan-1 ? of *V. pseudoreticulata*, Pingli-2 and Pingli-7 of *V. romanetii*. OPA15 could not differentiate between most clones of *V. quinquangularis* and Liu-9 and Liu-6 of *V. piasekii* Maxim. OPU16 could not identify Nanzheng-1 and 83-4-1 and Shang-24 of *V. quinquangularis,* Fujiang-4 and Jinan-1 and Lueyang-4 of *V. davidii*, Zhuoshan-2 (74003) and Zhuoshan76097 and Tonghua-3 of *V. amurensis*.

Based on combined banding patterns, all 73 wild, native grapes were identified by using only one of OPQ04 and OPJ07 together with one of OPJ01, OPH19, OPP02, OPA15 and OPU16. RAPD fingerprints of each wild grape sample were different from the remaining clones. They were converted into polymorphic band data arranged in the form of a 73 x 191 matrix (data not shown); which is part of the Chinese Wild Grape Germplasm Databank. RAPD analysis could identify clones belonging to the same species with similar morphological traits. Bands suitable to distinguish different accessions are summarized in Tab.3, with used primer molecular length of the amplified band. The frequency of these polymorphic bands was less than 10 %.

Polymorphism among the wild grapes of China is very large, since a high number of polymorphic bands have been detected using a few random 10-mer primers. The amount of polymorphism we encountered (191 markers obtained with 20 primers) is much higher than the polymorphism previ-

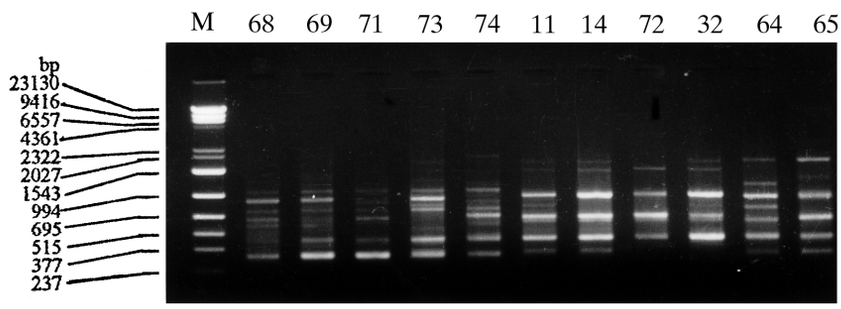

Fig. 1: RAPD fingerprints amplified by primer OPJ07 in some grape germplasms. Number of the clones see Tab. 1.

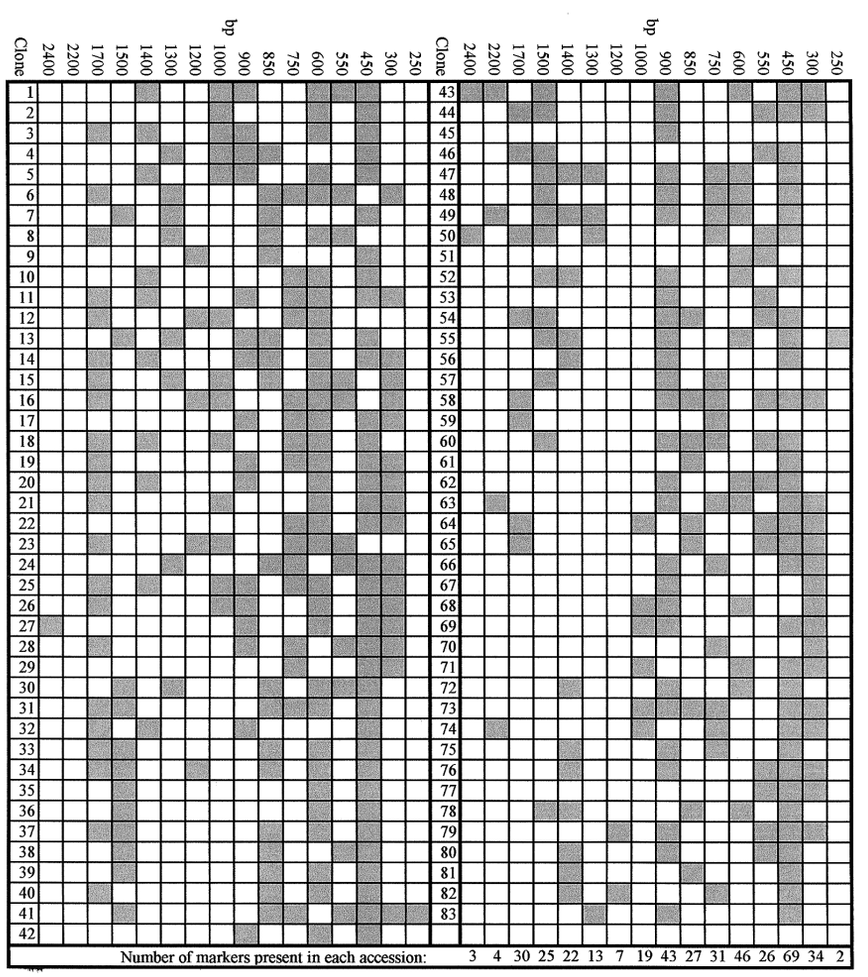

Fig. 2: Polymorphic bands of the 83 clones amplified by primer OPJ07. A dark box represents the presence, a white box the absence of the marker. Number of the clones see Tab. 1.

T a b l e 3

Bands suitable to distinguish different accessions

No.	Specific bands*					No.	Specific bands*				
1	H19-400	P02-600	G14-800	O10-1400	J01-1200	42	P02-350	U16-250	O06-2000	O06-1400	W08-1200
2	V18-1300	V18-400	G14-500	U13-700		43	P02-350	A15-850	O06-2000	J07-2200	
	O10-300	W08-600					W08-1200	J07-2400			
3	P02-600	G14-800	U13-700	Q04-750	W08-600	44	V18-400	P02-350	G14-1400	O06-2000	W08-1200
4	G14-800	W02-500	O10-300			45	V18-400	V18-200	J01-700	W08-600	
5	G14-800	O10-300	O10-1400			46	V18-400	A15-2000	A15-500	U16-250	
6	H19-1500	H19-400	G14-800	W02-500	O10-300	47	P02-1100	A15-850	Q04-1100	J01-700	
7	O10-300	J01-1200				48	P02-1800	15-500	W08-500		
8	H19-1500	H19-400	J01-1200			49	P02-1100	A15-850	A15-500	U16-250,	
9	P02-600	V07-1600	W02-500	J07-1200			H08-1500	J07-2200			
10	A15-2000	W02-500	Q04-250			50	P02-1800	P02-1100	J07-2400	H08-1500	
11	V18-350	P02-600	A15-800	Q04-250	J07-1200	51	P02-1800	A15-850	O06-1400	W08-500	
12	G14-800	J01-600				52	A15-850	Q04-750	J01-1200	O06-1800	
13	G14-1400	O10-1100	O10-350				W08-1200	W08-500			
14	V18-400	Q04-250	J01-900			53	H19-1100	W08-500			
15	A15-800	W02-500	O10-1400	O10-1100	J01-900	54	V18-1500	W02-1300			
16	V18-400	W02-500	J01-900	J07-1200		55	H19-1100	P02-1100	Q04-750	J07-250	
17	P02-600	A15-2000	A15-800	O10-1000	O10-350	56	V18-1300	V18-1000	O06-1400		
18	H19-1500	P02-1100	A15-800	O10-1000	O10-350	57	P02-1100	Q04-750	O06-1800		
19	A15-2000	A15-800	O06-600			58	V18-600	P02-600	G14-500	H08-1500	
20	V18-400	O10-1000	O10-350	U13-900		59	U16-250	H08-1500	W08-600		
21	V18-350	P02-800	A15-2000	A15-800		60	V18-1000	Q04-550			
	W02-1300	O10-350				61	U16-250	Q04-1100	Q04-750		
22	A15-2000	O10-350	U13-900			62	A15-2000	A15-850	Q04-550		
23	V18-1300	P02-1100	U13-900	J07-1200		63	V18-1300	U16-250	O06-1400	J07-2200	
24	V18-1300	O10-1000	U13-900			64	U16-250	U13-900	Q04-600		
25	V18-350	Q04-550				65	V18-1300	U13-900	Q04-750	O06-1400	
26	P02-800	O10-1000				66	V18-1300	Q04-600			
27	V18-400	V18-350	P02-800	U13-900	J07-2400	67	P02-1800	A15-2000	Q04-1100		
28	Q04-250	J01-700				68	Q04-600	Q04-250			
29	P02-800	Q04-600				69	V18-600	U16-250	Q04-250		
30	V18-200	A15-500	G14-400			70	Q04-250	O06-700			
31	P02-1100	A15-500	G14-500	G14-400		71	A15-1200	G14-500	O06-700	H08-1200	
32	P02-1200	G14-800	H08-1400			72	P02-1800	V02-1200	A15-1200	U16-250	
33	G14-800	J01-2300					O06-700	H08-1200			
34	P02-400	Q04-250	O06-2000	J07-1200		73	Q04-1700	O06-700			
35	V18-200	P02-800	P02-400	G14-500		74	H19-300	P02-1700	U20-1300	H08-1200	J07-2200
	G14-400	O06-1800				75	V02-1200	O10-1100	Q04-1100	J01-1200	H08-1200
36	H19-300	G14-500	G14-400	O06-1800		76	V18-1300	V18-1100	V02-1200	O10-1100	
37	H19-1500	V18-1100	G14-500	G14-400			J01-1200	H08-1200			
	Q04-750	O06-1800				77	O10-350	Q04-1100			
38	H19-1500	P02-800	G14-500	G14-400		78	A15-850	J01-1200			
	O06-1800	O06-1400				79	Q04-1100	H08-1200	J07-1200		
39	H19-1500	P02-1800	V07-1600	Q04-250	006-1400	80	P02-1800	V02-1200	A15-850	O10-1100	Q04-1100
40	V07-1600	G14-800	J01-700			81	P02-1800	V02-1200	H08-1200		
41	V18-200	P02-400	P02-350	G14-500		82	V18-600	V02-1200	A15-850	O10-1100	J07-1200
	O06-2000	W08-1200	J07-250			83	V18-400	O10-750			

"No." is the same as clone No. in Tab. 1.

* Marker notation: last 3 positions of the primer name in Tab. 2 refers to the kit (first letter) and the primer (number) purchased from Operon Technologies followed by the size (base pairs) of amplified DNA fragment.

ously reported for other *Vitis* species (Büscher 1993; Xu 1995; Grando 1996; This 1997). One reason is that RAPD analysis is primer- and sample-dependent, another reason might be that wild, native grapes have different geographical origins and have various flower types. Many species originate from different ecological environments, like *V. amurensis* originating from the cold north-east of China, or *V. quinquangularis* growing in the warm and humid tropics and subtropics.

Intraspecific clones have similar phenotypes but differ in their disease resistance genes, for example, clones of *V. amurensis, V. quinquangularis, V. pseudoreticulata, V. romanetii* and *V. piasekii* differ largely in their resistance to *Plasmopara viticola* (Wang Guoyin 1986), *Agrobacterium tumfaciens* (Chai 1997) or *Elsinoë ampelina* (de Bary) Shear (Wang 1998). Intraspecific variation of RAPD profiles was considerably large although some markers were also present for most of the clones of one species. Within clones of *V. quinquangularis*, three markers (O10-350, O10-1000, U13-900) were present on 5 clones respectively, but all accessions' profiles differed from each other. Of the 5 specific bands which appeared in 83-4-67(17) and Wei-3(18), three were the same (A15-800,O10-1000 and O10-350) and two were different bands (A15-2000 and P02-600 presented in 83-4-67; P02-1100 and H19-1500 presented in Wei-3). Only two primers, OPA15 and OPP02 or OPH19, were needed to differentiate between 83-4-67(17) and Wei-3(18) with similar morphological traits. Nine primers (OPP02, OPQ04, OPA15, OPU13, OPO06, OPW02, OPO10, OPV18 and OPJ01) displayed useful polymorphic banding patterns (Tab. 3) to discriminate 13 clones of *V. quinquangularis*. Only one out of two primers (OPP02 and OPQ04) and one out of the other 7 primers were needed for any single individual to determine its identity. Accessions of other wild species also displayed high variation in RAPD profiles. RAPD marker technology potentially provides many polymorphic markers that can be used to survey the plant genome quickly. With this class of markers, the existence of intraspecific variation in wild, native grapes can be examined. We were able to distinguish between accessions. Further studies should be conducted on clone and sequence specific RAPD markers in order to discriminate clones by SCAR (sequence characterized amplified region) markers exactly.

Identifying wild, native grapes in China by RAPD is becoming a practical necessity. The relatively narrow range of morphological traits and the limited number of polymorphic isoenzyme systems are not adequate to discriminate all the clones of any given species. RAPD analysis could discriminate all wild clones of any given native species, especially similar intraspecific clones with different disease resistance genes. With prudent selection of primers and strictly controlled reaction conditions, it offers a reliable method for the identification of wild grapes and has advantages over many morphologic and chemotaxonomic methods used for plant identification, which are susceptible to environmental variation. Genetic diversity among Chinese wild grapes has been investigated based on RAPD analysis before long (Luo *et al.* 2001). Our results show that RAPD markers are useful for identifying Chinese wild grapes and analyzing their relationships. In the future these markers may be used to tag disease resistance genes, which would be important to use the high resources of wild *Vitis* native to China.

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