

Full Length Research Paper

Molecular characterization of *Cymbidium kanran* cultivars based on extended random amplified polymorphic DNA (ERAPD) markers

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Fifty-four *Cymbidium kanran* cultivars from China, Japan and Korea were examined and analyzed by using the successive screening of 3'-end extended random primer amplified polymorphic DNA (ERAPD) markers to determine their molecular diversity and relationships. In ERAPD analyses, the strand-specific DNA sequence of direct oligonucleotide extension sequencing primers was independently read from each of the RAPD fragments without recourse to cloning or strand separation. Eight primers, identified from 80 original RAPD primers, produced strong repetitive polymorphic bands that were used in 3'-end-extended random primer amplified DNA marker analysis. The products of primers ACTGAACGCCG + ACTGAACGCCG and ACTGAACGCC + ACTGAACGCC, linked to the same locus (2.5 - kb), were developed from the original ACTGAACGC RAPD primer; the products of this marker were more stable and specific than the original RAPD marker. Unweighted pair-group mean analysis (UPGMA) grouped them into two clusters based upon geographical traits. We demonstrated that the ERAPD technique is a powerful tool for cultivar identification and establishment of genetic relationships of cultivars in *Cymbidium kanran*.

Key words: *Cymbidium kanran*; genetic relationship; Extended random amplified polymorphic DNA (ERAPD).

INTRODUCTION

Orchids are members of the family *Orchidaceae*; one of the largest families of angiosperms and one of the most numerous epiphyte groups in many tropical and sub-tropical areas of the world (Benzing, 1989; Dressler, 2005; Benner et al., 1995). *C. kanran* Makino is one of the most important and popular species in the orchid family which represent a group of botanically significant and commercially important flowering plants because of their ornamental value (that is their variation in shape, form, size, and coloration surpasses the flowers of the other angiosperms) (DuPuy and Cribb 1988; Arditti, 1992; Judd et al., 1999).

Understanding the genetic resources and diversity is very important for the breeding improvement of the genus *ymbidium*. Presently, enzyme polymorphism marker

(Obara-Okeyo and Kako, 1998), random amplified polymorphic DNA (RAPD) marker (Wang et al., 2004; Choi et al., 2006), amplified fragment length polymorphism (AFLP) marker (Wang et al., 2004) and intersimple sequence repeats (ISSRs) (Wang et al. 2009) studies have been reported for *Cymbidium* cultivars. RAPD markers have been widely used for fingerprinting of horticultural cultivars. However, RAPD markers limit the sensitivity of PCR to reagent and template concentrations, pH, and other reaction parameters. An effort to reduce the problems associated with RAPD analysis led to the development of sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993). Mitchelson developed the direct oligonucleotide extension sequencing method (Mitchelson et al., 1999) without RAPD fragment cloning or DNA strand separation. This technique uses a set of decamer oligonucleotides that extend the nonamer sequence by a single base at the 3'-terminus (A, T, C, or G) as independent sequencing primers for each RAPD strand.

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Table 1. The information of the *Cymbidium kanran* cultivars.

Number	Name	Number	Name	Number	Name	Number	Name
1	Sanxingdie	15	Xueyu	29	Rixiangbailong	43	Senwu
2	Yincaihe	16	Fuya hong	30	Fuyong	44	Luying
3	Yinlunsan	17	Hanyusong	31	Fengxue	45	Xianhe
4	Jinlinghan	18	Hanjinjian	32	Shenlong	46	Luyiwen
5	Xiuhongmei	19	Yihua	33	Sizihua	47	Luo Zhao
6	Jinhe	20	Qifa	34	Hongwu	48	Queshe
7	Wucaichaoban	21	Yinling	35	Jileniaio	49	Xianlu
8	Fuguixindie	22	Shenzhifu	36	Huangchengzhiyue	50	Ziyipin
9	Wucailudie	23	Rixiangzhiyu	37	Shenqu	51	Huiyu
10	Santongdie	24	Baimiao	38	Shihuin	52	Zixia
11	Yahuangsu	25	Daxiong	39	Bailu	53	Caolu
12	Cuijinju	26	Nanguo	40	Baishazhiyu	54	Qiuyipin
13	Xuezhonghong	27	Meibanjin	41	Xiongnu		
14	Heizhizhu	28	Yushenjin	42	Xiongfei		

Note: 20 cultivars from number 1 to 20 originated from China, 20 cultivars from number 21 to 40 originated from Japan, 14 cultivars from number 41 to 54 originated from Korea.

Table 2. The primers for direct 3'-extension primer DNA sequencing.

Primer	Nucleotide sequence (5'-3')
BO311	AGGGAACGAG
BO312	TTCCGAACCC
BO317	GGGAACGTGT
BO319	ACTGAACGCC
BO327	GGCGGTTGTC
BO324	CCTTGACGCA
BO323	GTTGCGATCC
BO321	CCACACTACC

Knowledge of the level of variation among these cultivars would be of great value to breeders because many cultivars can be used in crosses to produce new cultivars. Thus, we investigated the molecular character of *C. kanran* cultivars using the ERAPD technique.

MATERIALS AND METHODS

Plant materials and DNA extraction

We used 54 *C. kanran* cultivars collected from China (20 cultivars), Japan (20 cultivars) and Korea (14 cultivars) (Table 1). Genomic DNA was extracted from leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Knapp and Chandless, 1996). Fresh leaf tissue (100 mg) was placed in a mortar and ground to a powder in liquid nitrogen. A volume of 600 μ l of cold extraction buffer (3% CTAB, 1.42 M NaCl, 20 mM ethylene-diaminetetraacetic acid (EDTA), 100 mM Tris-Cl pH 8.0, 2% polyvinylpyrrolidone, and 5 mM ascorbic acid) was added and the tissue was further ground for 2 min. Ground samples were left at 65°C for 15 min and extracted once with chloroform-isoamyl alcohol (24:1, v/v) to obtain a clear supernatant. Supernatant containing plant genomic DNA

was transferred to a fresh tube after centrifugation at 12,000 rpm for 5 min. One-fifth volume of 5% CTAB solution in 0.7 M NaCl was added to the aqueous phase, the samples were treated at 65°C for 15 min and then extracted once more with chloroform-isoamyl alcohol. DNA was precipitated from the super-natant by the addition of two volumes of cold absolute ethanol, incubation at -80°C for 15 min and centrifugation at 12,000 rpm for 20 min at 4°C. DNA was dried under a vacuum after rinsing the pellet containing the DNA in cold 70% ethanol. The dried DNA was re-suspended in 100 μ l of double distilled water.

RAPD amplification

Eighty random decamer primers were tested in all samples. PCR was performed in 20 μ l volumes containing 2 μ l 10 \times PCR buffer (10 \times buffer: 500 mM KCl, 100 mM Tris-HCl pH 8, and 15 mM MgCl₂), 1 μ l dNTPs (10 mM of each nucleotide), 0.2 μ l Taq DNA polymerase (5U), 2.0 ng of template DNA, 1 μ l of primers, and 13.8 μ l of ddH₂O. Amplifications were performed using an MJ. Research PT-100 thermal cycler with the following temperature profile: step 1, denaturation at 94°C for 3 min; step 2, thermal ramp to 94°C for 40 s; step 3, thermal ramp to 37°C for 1 min, steps 1-3 were repeated for a total of 40 cycles, and finally, step 4, thermal ramp to 4°C and hold. We analyzed 15 μ l of the PCR products on 1.2% agarose gel and stained with ethidium bromide. From the produced clear and repeatable fragments, eight primers (Table 2) were identified from the 80 initial random primers and were further analyzed.

Direct 3'-extension primer DNA sequencing

Extra bases of A, T, C, or G were added to the primers for use in 3'-extension primer DNA sequence, which generated four kinds of primers; examples are primer-A, primer-T, primer-C, and primer-G. At the same time, by assembling these primers, we obtained ten primers, (primer-A + primer-A), (primer-T + primer-T), (primer-C + primer-C), (primer-G + primer-G), (primer-A + primer-T), (primer-A + primer-C), (primer-A + primer-G), (primer-T + primer-C), (primer-T + primer-G) and (primer-C + primer-G).

Amplifications were performed differently for RAPD. Typically,

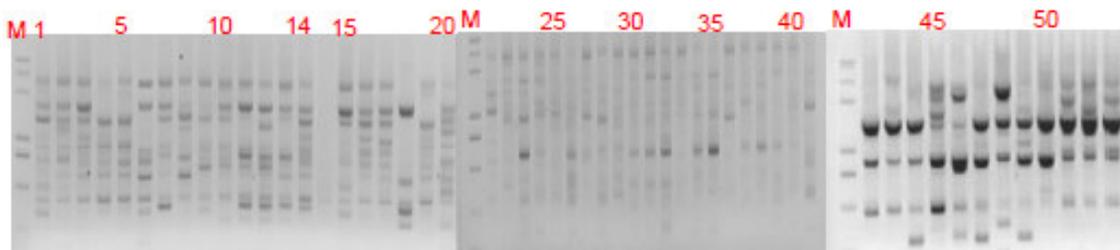


Figure 1. DNA banding profiles of amplified products from the one-base-extended primers (ACTGAACGCC + ACTGAACGCG). Note: Cultivar numbers are the same as in Table 1.

primers were annealed to the denatured RAPD template at 45°C and were cycle sequenced using the following procedure: step 1, 96°C for 30 s; step 2, 40°C for 15 s; step 3, 60°C for 4 min, steps 1-3 were repeated for a total of 35 cycles and finally step 4, 4°C and hold.

Further 3'-extension primer DNA sequencing

RAPD fragments, which were not amenable to direct 3'-extension decamer primers because both of the terminal sequences were the same, was sequenced by the use of an additional set of four decamer extension primers in which the sequencing primer was 3'-extended by two nucleotide bases from the sequence of the original nonamer amplification primer. These products (primer-C + primer-G) having the same fragment with the direct 3'-extension primer DNA sequences, were extended (primer-CA, primer-CT, primer-CG, primer-CC, primer-GA, primer-GC, primer-GG, and primer-GT), which generated 16 double primers (primer-CA + primer-GA, primer-CA + primer-GC, primer-CA + primer-GG, primer-CA + primer-GT, primer-CT + primer-GA, primer-CT + primer-GC, primer-CT + primer-GG, primer-CT + primer-GT, primer-CC + primer-GC, primer-CC + primer-GG, primer-CC + primer-GT, primer-CT + primer-GT, primer-CG + primer-GA, primer-CG + primer-GC, primer-CG + primer-GG, and primer-CG + primer-GT).

Amplifications were performed with different conditions for RAPD. Typically, primers were annealed to the denatured RAPD template at 40°C and were cycle sequenced using the following procedure: step 1, 96°C for 30 s; step 2, 50°C for 15 s; step 3, 60°C for 4 min; steps 1-3 were repeated for a total of 35 cycles and finally, step 4, 4°C and hold.

Data analysis

The presence and absence of amplified bands were detected and analyzed with the Quantity One 4.1 (BioRad, Hercules, CA, and USA) software. Bands were scored for their presence (1) or absence (0) and genetic distances were calculated using Nei's coefficient of genetic distance (Nei and Li, 1979). The dendrogram of these cymbidiums cultivars were constructed based on the similarity matrix data by applying unweighted pair group method with arithmetic averages (UPGMA) cluster analysis using the NTSYS program (Exeter Software, Setauker, and NY).

RESULTS

Direct 3'-extension primer analysis

The direct 3'-extension primer DNA sequencing technique

utilizes the likelihood that different nucleotides occur immediately internally to the primer-specified terminal. One of the 4 possible direct 3'-extension primer DNA sequencing primers of each set (A, G, C, or T at the 3'-terminus) will independently prime direct sequencing of one strand of a RAPD fragment, while the other direct 3'-extension primer DNA sequencing primer from the set will prime sequencing of the complementary RAPD fragment strand in an independent reaction.

Among the prescreened primers with a GC content of 60 and 70%, 8 primers (Table 2), identified from an initial 80 random primers, yielded highly amplified polymorphic DNA bands by the direct 3'-extension.

Eight primers produced a total of 2,959 bands and 2,527 (85.40%) were polymorphic bands, according to the method used for band scoring. The bands were characterized based on size and ranged from approximately 0.2 to 2.5 kb. The number of amplified bands varied for every primer (Figure 1). An average ERAPD bands was 6.85 per primer, and ranged from 4 to 10 bands per primer. Compared with RAPD markers, the identified new and high quality DNA fragments (0.5, 1.5, and 1.8 kb) from the RAPD fragment read by the direct 3'-extension primer DNA sequencing, were amplified because the primers were changed by the one base extension (Figure 1). The amplified products of the direct 3'-extended 11-mer (ACTGAACGCC+ACTGAACGCG) oligonucleotide extension sequencing from 10-mer (ACTGAACGCC) RAPD amplification primers identified the same strand (2.5 kb) and increased the strand's intensity, possibly because of the addition of the C nucleotide. At the same time, other new bands identified in the 54 *C. Kanran* cultivars.

Cluster analysis

The dendrogram that resulted from the UPGMA cluster analysis shows that the UPGMA separated the orchids into two major clusters (Figure 2). The second cluster (II) included 4 cultivars, Xueyu and Qifa (China), Hanyusong (Japan) and Luozhao (Korea). According to horticultural and geography classification, the first cluster (I) was comprised of 50 cultivars which formed subgroups I-1, I-2, I-3,

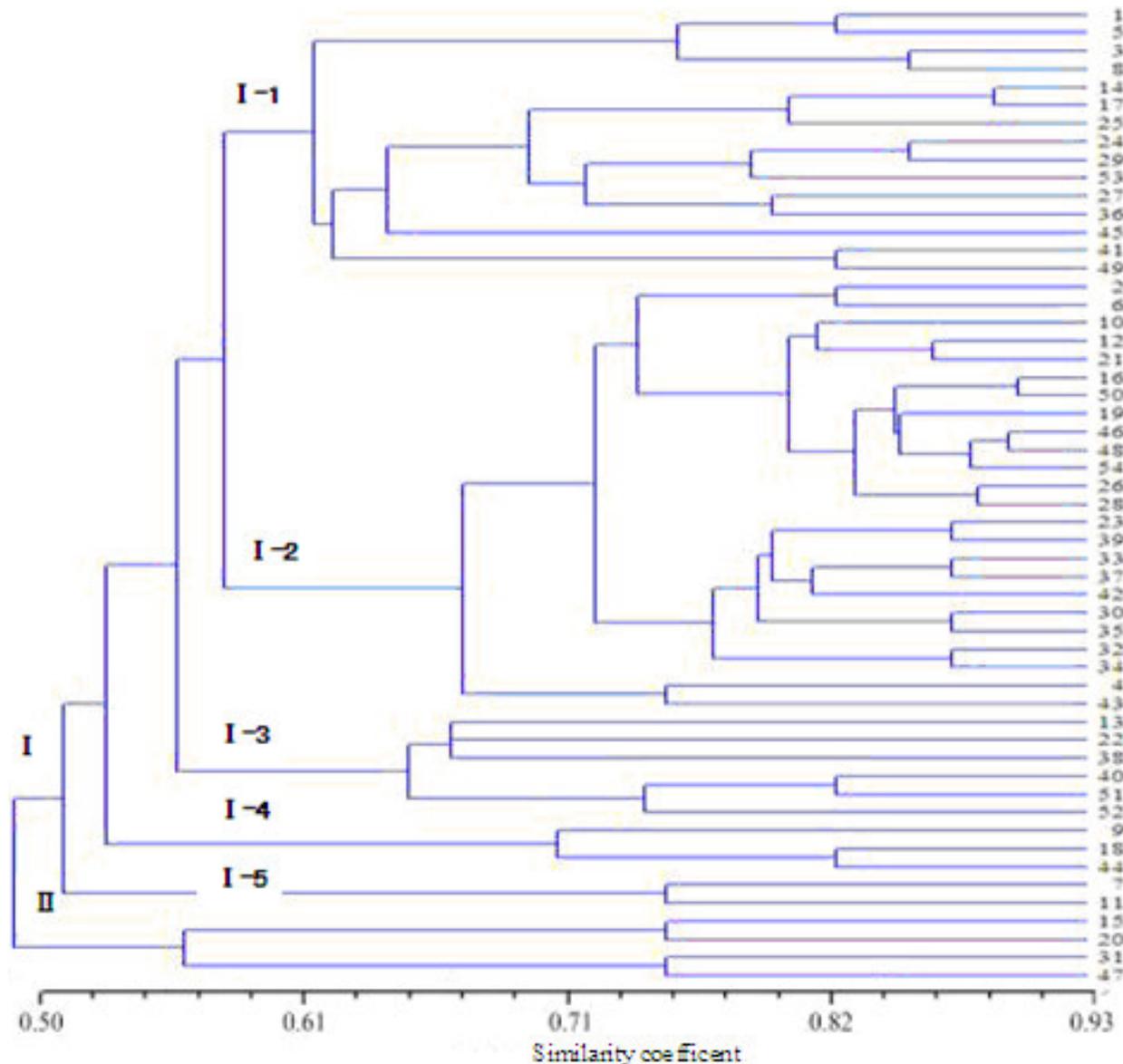


Figure 2. Dendrogram of 54 *C. kanran* cultivars using ERAPD markers as per unweighted pair group method with arithmetic average (UPGMA) clustering. The scale shown at the bottom is the measure of genetic similarity Dice's coefficients as calculated according to Nei and Li (1979).

I-4, I-5 in five subclusters. According to orchid horticulture classification, subcluster I-4 and I-5 comprises 2 of 20 Chinese cultivars, respectively. Also, according to orchid geography classification, they were produced in small subcluster in I-1, I-2, I-3, respectively. Similarity coefficient ranged from 0.50 to 0.91 among 54 *C. kanran* cultivars, the lowest genetic similarity (45%) originated from China, Korea, Japan Xuezhonghong, Xianhe and Shenzhifu cultivars, respectively. The highest genetic similarity (91%) was between Fuya hong (China) and ziyipin (Korea). The results from this study demonstrated that they have genetic polymorphisms that correspond with the phenotypic and ecological diversity in cymbidium Kanran.

Further 3'-extension primer sequencing

Oligonucleotide extension sequencing primers obtained after further sequencing also shared the common sequence motif with the 11-mer, the ten nucleotide bases from the 5'-terminal and varied for the last two 3'-terminal nucleotides. Further 3'-extension primer sequencing was performed and the results indicated that there were high different strands by the addition of two bases to the direct 3'-extension primers. For example, the primers ACTGAA CGCCCG and ACTGAACGCCCG only amplified a single 2.5 - kb DNA sequence from the RAPD fragments (Figure 3). These primers were developed from the primers

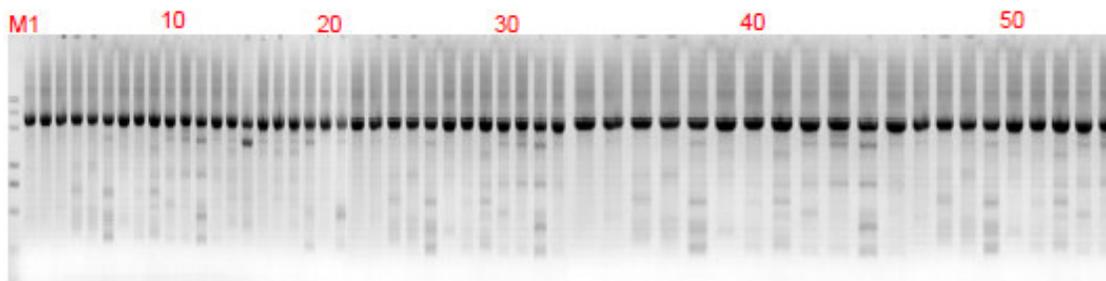


Figure 3. DNA banding profile of the amplified products from the two-base extended primers of ACTGAACGCCCG + ACTGAACGCGG. Note: Cultivar numbers are the same as in Table 1.

ACTGAACGCC and ACTGAACGCC, which were both derived from the RAPD primer ACTGAACGCC. Distinct patterns of RAPD fragments were amplified despite the 3'-extended primers sharing the same common sequence with each other and only differing at the 3'-terminus. However, the majority of the distinct RAPD fragments were not amplified with the 2-base 3'-extended primer, but were detected with the 1-base 3'-extended primer.

DISCUSSION

Oligonucleotide extension sequencing could also allow a large number of informative RAPD fragments to be converted readily into mapped sequence-tagged sites (STS) or SCAR loci. Although, the direct oligonucleotide extension sequencing technique and SCAR markers share many similar steps, that is the re-amplification of an excised RAPD fragment to ensure purity, fragment sizing, and re-purification by gel electrophoresis, however, the former technique is simple and quick to perform, while the latter requires the isolation of a particular RAPD fragment, cloning, clone analysis, and sequencing of the selected plasmids.

This is the first report on the use of ERAPD markers in the measurement of genetic variation and determination of genetic relationships in *C. kanran* cultivars. ERAPD genotyping was also used in other plant species (Mitchelson et al., 1999). Genetic diversity analysis and assignment of cultivars to heterotic groups would provide valuable information for plant breeding programs. 2959 bands in 54 *C. kanran* were detected using 8 primers, about 7 bands per primer. Most importantly, specific bands (2.5kb) were obtained, indicating that there was considerably high level of genomic polymorphism and abundant information throughout the genome of *C. kanran*.

C. kanran has been cultivated for many centuries in Japan, Korea and China which is divided into numerous horticultural types and varieties that have disparate geographic origins, distributions and considerable ecological and morphological variations (Liu, 2006). The long-term cultivated orchid showed a large number of diverse populations within species. Correspondingly, there is a high probability that nuclear DNA mutations occurred

during the horticultural selection process, resulting in diversification and genetic polymorphism in growth habit, vigor and leaf and flower characteristics. Most mutants of individual orchid plant were selected and propagated by vegetative means such as colour and leaf characteristics.

Choi et al. (2006) reported that the level of polymorphic bands from RAPD analysis of cymbidiums was high (95%). A high level of DNA polymorphism (93.75%) using ISSR marker was detected (Wang et al., 2009). The current investigation indicates that the ERAPD technique is also effective for assessing genetic diversity and identifying cultivars in *C. kanran* cultivars. It was found that most *C. kanran* cultivars were cluster according to their geography character by ERAPD analysis. Comparably, their genetic compositions revealed their sole geographical origin in subcluster (Figure 2).

Therefore, direct 3'-extension primer ERAPD markers could provide useful information on the origin, biology and genotyping system in orchid breeding program, at the same time, further 3'-extension primer ERAPD markers could also provide specific gene for all cymbidium *Kanran*. In conclusion, a genotyping system using the ERAPD technique was established in *C. kanran*. This genotyping system can be used efficiently for specific genes that confer commercially important traits.

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