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Full Length Research Paper

Genetic diversity of *Cymbidium kanran* detected by Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) markers

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Fifty-four *Cymbidium kanran* cultivars from China, Japan and South Korea were examined and analyzed by using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) markers to determine their molecular diversity and relationships. In chloroplast (cp) PCR-RFLP analyses, genetic differences were revealed in 4 of 6 primer sets (66.67%) and 19 of 72 primer-enzyme combinations (26.38%), 116 polymorphic bands were detected. For mitochondrion (mt) PCR-RFLP markers, genetic differences were revealed in 2 of 8 primer sets (25%) and 55 polymorphic bands (53.49%) were detected with 7 restriction primer-enzyme combinations. According to the PCR-RFLP marker profiling data, all the cultivars were classified into four groups. The group 1 has included three subclusters that two of them were generally consistent with geography classification. Subcluster 1 and Subcluster 2 were mainly composed of Chinese cultivars. Comparably, Subcluster 3 was composed of two cultivars which originated from Japan separately. Group 2 comprised Huangchengzhiyue and Xiongnu originated from Japan. Group 3 was included in seven Chinese cultivars and five Japanese cultivars. Group 4 was composed of eight Chinese cultivars and four Japanese cultivars. Therefore, we demonstrated that the PCR-RFLP technique could provide a powerful tool for cultivar identification and establishment of genetic relationships of cultivars in *cymbidium kanran.*

Key words: *Cymbidium kanran*, chloroplast (cp) polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), mitochondrion (mt) PCR-RFLP.

INTRODUCTION

Orchidaceae, one of the largest families of angiosperms, and one of the most numerous epiphyte groups in many tropical and subtropical areas of the world, are diverse in both their specialized pollination and ecological strategies. They provide a rich source of material for the investigation of evolutionary relationships and developmental biology (Dressler, 2005; Kuehnle, 2007). The orchids 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 (Kuehnle, 2007). A species' breeding system, its population size, and its colonizing ability play an important role in determining the distribution of genetic variation and genetic differentiation within and between populations (Hamrick and Godt, 1989; Sun, 1997). However, in recent decades, the over-collection of wild *Cymbidium* species has been a direct cause for the rarity and endangered nature of these species and populations, which led to a further loss of their genetic diversity and the alteration of their population genetic structure.

Studies in orchids have been hindered by several

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challenges, including their low transformation efficiency, and their long regeneration time and life cycle. To overcome such obstacles, random amplified polymorphic DNA (RAPD) (Choi et al., 2006; Goh et al., 2005; Ferreira et al., 2006), isozyme expression (Obara-Okeyo and Kako, 1997; Mi Yoon, 2008), and amplified fragment length polymorphism (AFLP) marker (Wang et al., 2004), inter-simple sequence repeats (ISSRs) (Hui-Zhong et al., 2009) studies have been reported for these species. However, there have been few reports of ISSR and PCR-RFLP in *C. Kanran* cultivars.

The plant cytoplasmic genome includes chloroplast (cp) DNA and mitochondrion (mt) DNA. Compared with the nuclear genome, the cytoplasmic genomes have simple genetics, little recombination, and a slow rate of structural evolution; thus it is a powerful tool for the study of land-plant evolutionary processes, both at the intraspecific level and in molecular systematic (Toshio et al., 2005; Roy et al., 2000; Yoshiya, 2001). Inter-Simple sequence repeats (ISSR) are one of the most powerful genetic markers in biology. They are a source of abundant, non-deleterious mutations that provide variation in the face of stabilizing selection (Qi-Lun and Fang, 2008). To improve the process of orchid breeding, we have identified the 54 *C. kanran* varieties and evaluated their molecular relationship and difference using the cpPCR-RFLP, and mtPCR-RFLP markers.

MATERIALS AND METHODS

Plant materials and DNA extraction

We used 54 *C. kanran* cultivars collected from China (40) and Japan (14) (Table 1) and extracted genomic DNA from leaves by a modified cetyltrimethylammonium bromide (CTAB) method (Knapp and Chandlee, 1996).

PCR- RFLP marker development

Approximately 1 mg each of extracted DNA was digested using six endonucleases that recognize four to six base pairs. The Primers are listed in Table 2. The 25 µl PCR mixtures contained 2.5 µl of buffer; 2 µl dNTPs; 0.4 µmol/L primers; 1.4 U *Taq* DNA polymerase and 300 ng of DNA. The cycle sequencing used the following procedure: step1, 94°C for 4 min; step 2, 55°C for 1 min, step 3, 72°C for 3 min, steps 1 to 3 were repeated for a total of 35 cycles; and finally, step 4, 72°C for 2 min. The PCR products were electrophoreses in a 1.8% agarose gels.

Data and cluster analysis

The presence of amplified bands were detected and analyzed with the Quantity One (BioRad, Hercules, CA, USA) software .Bands were scored for their presence (1) or absence (0) for numerical analysis. Genetic distances were calculated using Nei's coefficient of genetic distance (Nei and Li, 1979).The dendrogram of these *C. kanran* 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, NY).

RESULTS

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) maker development

In cpDNA PCR-RFLP analysis, four out of six primers could amplify more than one clear band that could be digested by the restriction endonucleases EcoRI, HindIII, BamHI, EcoR321, BsuR1, and EcoR881, Nine out of seventy-two primer-enzyme combinations amplified 518 bands, and 116 bands revealed genetic differences. The product of trnL-trnR digested by EcoRI obtained relatively higher levels of polymorphisms (24 bands) than the others. Few primers could produce polymorphic bands in different *C. kanran* varieties.

In mtDNA PCR-RFLP analysis, two out of six primers could amplify one clear band, and they were digested by the restriction Endonucleases EcoRI, HindIII, BamHI, EcoR321, BsuR1, and EcoR881. Only one primer-enzyme combination (Cox1) amplified 109 bands and 55 (53.49%) polymorphic bands were revealed in 54 *C. kanran* cultivars**.**

Cluster analysis

The PCR-RFLP classified all cultivars into four groups (Figure 1); the group 1 has included three subclusters that two of them were generally consistent with geography classification. Subcluster 1 and Subcluster 2 were composed of Chinese cultivars. Comparably, Subcluster 3 was composed of four cultivars which originated from Japan separately. Group 2 comprised Huangchengzhiyue and Xiongnu originated from Japan. Group 3 was mainly composed of Chinese *C. kanran* cultivars which was included in seven Chinese cultivars and five Japanese cultivars. Group 4 was composed of eight Chinese cultivars, Four Japanese cultivars a. The results from this study demonstrated that most cultivars have genetic polymorphisms that correspond with the ecological diversity in *C. Kanran*.

DISCUSSION

In view of the large and increasing numbers of *C. kanran* cultivars and the importance of wild exotic germplasm for breeding programs, it is necessary to characterize and understood their genetic structure and phylogenetic relationship in this horticulture species. DNA methodologies have become a clear and powerful impact on understanding of the origin, evolution and genome relationships among the plant species. As a very useful discriminative power of DNA markers tool, PCR-RFLP technologies have been extensively used to study the intra-specific genetic diversity as well as to establish the inter-specific relationships in several animal and higher plants groups. For example, *mulberry* (Kar et al., 2008);

Table 1. Description of *C. kanran* species.

Horticultural type	Cultivar number	Cultivar name	Cultivar origin
Meiban	1	'Hongmei'	Sichuan, China
	2	'Xilemei'	Fujian, China
	3	'Cuiyuqi'	Guizhou, China
	4	'Lubao'	Fujian, China
	5	'Zhonghuahongmei'	Guizhou, China
	6	'Hanxiangmei'	Fujian, China
	7	'Zixiuhongmei'	Yunan, China
	8	'Longjihongmei'	Guangxi , China
	9	'Xiaoqingmei'	Guangxi , China
	10	'Sanxingmei'	Guangxi , China
	11	'Xinpinhongmei'	Sichuan , China
	12	'Meibanjin'	Japan
Shuixianban	13	'Yinlunshan'	Guangxi , China
	14	'Jinlinghan'	Fujian, China
Dieban	15	'Baguihuangdie'	Guangxi , China
	16	'Sanxindie'	Guangxi , China
	17	'Banbiandie'	Guangxi , China
	18	'Wucailudie'	Guangxi , China
	19	'Fuguidiexin'	Guangxi , China
	20	'Yanshanqidie'	Guangxi , China
	21	'Xiangyanghongdie'	Guangxi , China
	22	'Santongdie'	Guangxi , China
	23	'Wufushuangxi'	Guangxi , China
	24	'Sixidie'	Fujian, China
Heban	25	'Honghe'	Guizhou, China
	26	'Xiashanhe'	Guizhou, China
Suxin	27	'Lubaiqisu'	Fujian, China
	28	'Baguisu'	Guizhou, China
	29	'Dongfangsu'	Fujian, China
	30	'Hanhaosu'	Guangdong China
	31	'Fengxue'	Japan
	32	'Baimiao'	Japan
	33	'Daxiong'	Japan
	34	'Rixiangbailong'	Japan
	35	'Yinling'	Japan
	36	'Yushenjin'	Japan
	37	'Sizhihua'	Japan
Shuangyi	38	'Huangyuezhicheng'	Japan
	39	'Rixiangzhiyu'	Japan
Xianyi	40	'Shuangxi'	Guangdong, China
	41	'Huban'	Jiangxi, China
Sehua	42	'Xuezhonghong'	Fujian, China
	43	'Shenlong'	Japan
	44	'Shenqu'	Japan
	45	'Jileniao'	Japan
	46	'Fuzhishen'	Japan

Table 1. Contd.

Heban	47	'Xiaocuixian'	Fujian, China
	48	'Hongguifei'	Fujian, China
	49	'Yangguifei'	Fujian, China
	50	'Xiaohanye'	Fujian, China
	51	'Doushepingjian'	Fujian, China
	52	'Shuixianhe'	Fujian, China
	53	'Xinpin1'	Sichuan, China
	54	'Xinpin2'	Guizhou, China

Table 2. The primer sequences and source for cpDNA and mtDNA RFLP markers.

Primer	Sequence	Type	Primer	Sequence	Type
trnT-trnL	5'-CATTACAAATGCGATGCTCT-3' 5'-TCTACCGATTTCGCCATATC-3'	cpDNA	trnH-trnK	5'-ACGGGAATTGAACCCGCGCA-3' 5'-CCG ACTAGTTCCGGGTTCGA-3'	cpDNA
trnL-trnR	5'-CGAAATCGGTAGACGCTACG-3' 5'-ATTTGAACTGGTGACACGAG-3'	cpDNA	nad1B-nad1C	5'-GCATTACGATCTGCAGCTCA-3' 5'-GGAGCTCGATTAGTTTCTGC-3'	mtDNA
trnD-trnT	5'- ACCAATTGAACTACAATCCC-3' 5'- CTACCACTGAGTTAAAAGGG-3'	cpDNA	Cox1	5'-CTAACCACAAGGATATTGGGAC-3' 5'-AGTTCTCCAAAAGTATGAAAGGC-3'	mtDNA

Saffron (Angela et al., 2009); *Spartina maritime* (Yannic et al., 2004).

In this study, PCR-RFLP markers were be used for the first time to measure genetic variation and determinate of genetic relationships in *C. kanran* cultivars, which could be produced complete, very reliable, reproducible and highly polymorphic fingerprints among 54 *C. kanran* cultivars.

According to different genetic and chemical characteristics of orchid, the use of PCR-RFLP fingerprints needed to modify optimal pattern in *C. kanran* cultivars. Four cpDNA and two mtDNA PCR-RFLP primers were selected and provided robust, complete and polymorphic fingerprints which could be clearly distinguished all the tested *C. kanran* cultivars.

In fifty-four *C. kanran* cultivars, we detected 290 cpDNA PCR-RFLP fragments using four primers and 109 mtDNA PCR-RFLP fragments using two primers, indicating that there was abundance information in the genome of *C. kanran*.

Polymorphisms were useful for genotype identification, genotype relationship and genetic variability. A high level of DNA polymorphism (94%) was detected across special *C. kanran* cultivars using PCR-RFLP genotyping. Lower polymorphism (78%) using RAPD marker was found in 36 Cymbidium cultivars (Obara-Okeyo and Kako 1997). High polymorphism for ISSR markers was also reported , such as C. goeringii cultivars(Hui-zhong, 2009), which was proved that the genetic variation may be more directly related to the number of polymorphisms detected by the marker system and indicated that PCR-RFLP and ISSR markers were equally effective for assessing molecular diversity and identifying cultivars in *C. kanran.*

The high similarity between Yihua (China) and Meibanjin (Japan), Nanguo (Japan) and Qiuyipin (Japan), Cuijinju (China) and Qifa (China); Santongdie (China) and Hongwu (Japan) were detected in PCR-RFLP analysis. The results showed that these genotypes of cultivars are closely related.

Conclusion

Molecular markers are scattered throughout the genome and their association with various agronomic traits is influenced by the cultivator under selection pressure induced by domestication. In conclusion, we have established a genotyping system using the PCR-RFLP technique in *C. Kanran*. This genotyping system can be used efficiently for specific genes that confer commercially important traits, and exploration of diversity among these *C. kanran* cultivars would be of great significance for breeding programmers.

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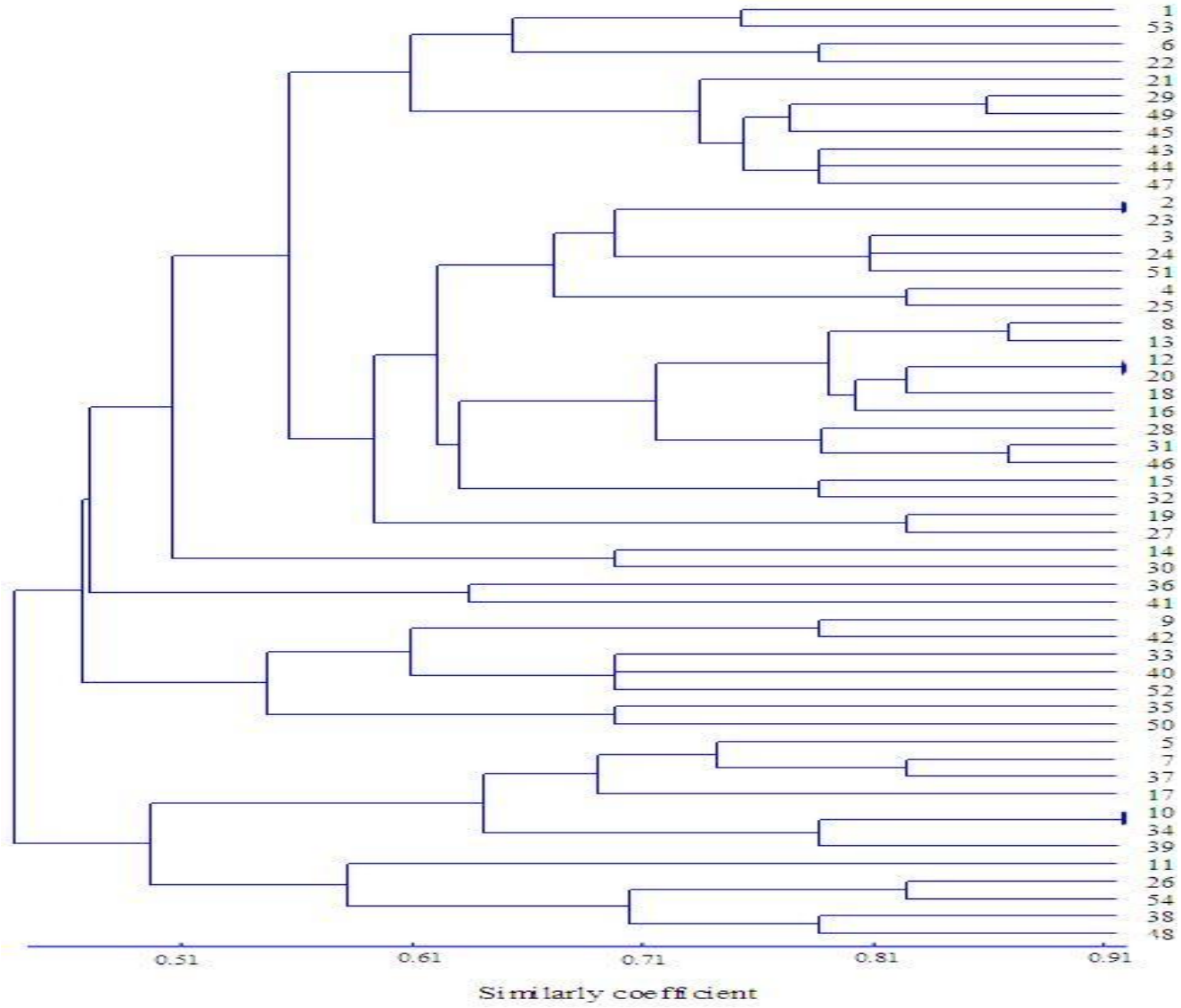

Figure 1. Dendrogram of 54 *C. kanran* cultivars using PCR-RFLP markers as per unweighted pair group method with arithmetic average (UPGMA) clustering. Cultivar numbers are the same as in Table 1.

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