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Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers

Hui-Zhong Wang · Zhen-Xing Wu · Jiang-Jie Lu · Nong-Nong Shi · Yan Zhao · Zhi-Tao Zhang · Jun-Jun Liu

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Abstract Spring orchid (*Cymbidium goeringii*) is a popular flowering plant species. There have been few molecular studies of the genetic diversity and conservation genetics on this species. An assessment of the level of genetic diversity in cultivated spring orchid would facilitate development of the future germplasm conservation for cultivar improvement. In the present study, DNA markers of intersimple sequence repeats (ISSR) were identified and the ISSR fingerprinting technique was used to evaluate genetic diversity in *C. goeringii* cultivars. Twenty-five ISSR primers were selected to produce a total of 224 ISSR loci for evaluation of the genetic diversity. A wide genetic variation was found in the 50 tested cultivars with Nei's gene diversity ($H = 0.2241$) and 93.75% of polymorphic loci. Fifty cultivars were unequivocally distinguished based on ISSR fingerprinting. Cultivar-specific ISSR markers were identified in seven of 50 tested cultivars. Unweighted pair-group mean analysis (UPGMA) and principal coordinates analysis (PCA) grouped them into two clusters: one composed the cultivars mainly from Japan, and the other contained three major subclusters mainly from China. Two Chinese subclusters were generally consistent with horticultural classification, and the third Chinese subcluster contained cultivars from various horticultural groups. Our results suggest that the ISSR technique provides a powerful tool for cultivar identification and establishment of genetic relationships of cultivars in *C. goeringii*.

Keywords *Cymbidium goeringii* · Genetic diversity · Inter simple sequence repeat · Phylogenetic analysis

H.-Z. Wang (✉) · Z.-X. Wu · J.-J. Lu · N.-N. Shi
Key Laboratory of Biochemistry and Molecular Biology, Hangzhou Normal University, Hangzhou 310018, China
e-mail: whz62@163.com

Present Address:
J.-J. Lu
Key Laboratory of Modern Forest Breeding Technology, Zhejiang Forestry College, Linan 311300, China

Y. Zhao
College of Food, Biological and Environmental Engineering, Zhejiang Gongshang University, Hangzhou 310035, China

Z.-T. Zhang
Zhejiang Orchid Society, Hangzhou 310018, China

J.-J. Liu (✉)
Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, Victoria, Canada V8Z 1M5
e-mail: juliu@nrcan.gc.ca

Introduction

As one of the most diverse and largest families of flowering plants, the *Orchidacease* family contains more than 25,000 species, accounting for about 10% of all flowering plants (Dressler 1993). Spring orchid (*Cymbidium goeringii*, Rchb. F.) is one of the most important and popular species in the orchid family, *Orchidaceae* (DuPuy and Cribb 1988). It has been cultivated for the variegated leaves, fragrant flowers and peloric flower structures for more than ten centuries in China (Hew 2001). For commercial production of cut flowers and pot plants with new floral characteristics and other beneficial traits, a large number of novel cultivars of *C. goeringii* have been produced through inter-specific hybridization and selection of somatic mutations during vegetative propagation in the past century (Wu 1993; Liu et al. 2006a), which complicates phylogenetic analyses and its interpretation in this species.

The cultivars of *C. goeringii* exhibit an incredible range of diversity in size, shape, and color in their flowers and leaves, and in plant length and resistance against diseases and pests. To assess the relationships amongst *C. goeringii* cultivars, the conventional method is based on morphological traits of leaf and flower (Jin and Yao 2006). Generally, *C. goeringae* cultivars are classified into a few horticultural types in China: Meiban (plum-petal-like sepals), Heban (lotus-petal-like sepals), Shuixianban (narcissus-petal-like petals), Dieban (butterfly-like sepals or petals), Suxin (white labellum with a single color for other structures of the flower), Sehua (whole flower with different brilliant colors), and Yeyi (leaves with various colors and shape patterns). Problems associated with variability, plant growth conditions, and individual biases have caused confusion in cultivar identification. In particular, horticultural classification of cultivars based on one or several morphological characters of leaves and flowers make it difficult to compare cultivars for the assessment of genetic resources in this flower species.

Understanding the genetic resources and diversity is very important for the breeding improvement of the genus *Cymbidium*. In recent years, genetic diversity and identification of *Cymbidium* cultivars have been measured by different molecular tools, including enzyme polymorphism marker (Obara-Okeyo et al. 1998), random amplified polymorphic DNA (RAPD) marker (Obara-Okeyo and Kako 1998; Wang et al. 2004; Choi et al. 2006), amplified fragment length polymorphism (AFLP) marker (Wang et al. 2004), and polymorphisms of internal transcribed spacers (ITS) of nuclear ribosomal DNA and plastid *matK* (Van den Berg et al. 2002).

Intersimple sequence repeats (ISSRs) are highly polymorphic DNA markers. The consistency, reliability and codominancy make this type of molecular marker particularly suitable for evaluating genetic diversity and phylogeny among plant species and populations (Weber 1990; Zietkiewicz et al. 1994). ISSRs are polymorphic loci present in nuclear DNA and organellar DNA that consist of repeating units of one to six base pairs in length. They are typically neutral and are used as molecular markers which have wide-ranging applications (Jarne and Lagoda 1996).

Although SSR sequences are present in *Cymbidium* (Capesius 1976), this genotyping technology has rarely been used for the identification of *Orchidacease* species (Campbell et al. 2002). Phenotypic diversity of spring orchid has been documented (Jin and Yao 2006), while information is very limited on genetic diversity within and between horticultural types of this species. Knowledge on these aspects would guide flower breeders to select parental sources that potentially result in highly diverse populations for selection.

To develop a strategy for the conservation and sustainable utilization of this species, and to advance understanding of the genetic profile of this species, we investigated the genetic diversity of cultivars of *C. goeringae* using the ISSR technique. The objectives of this study were: (1) to search for ISSR markers for identification of *C. goeringii* cultivars; and (2) to detect the polymorphisms using these novel ISSR markers to assess genetic diversity in spring orchid.

Materials and methods

Plant materials

Fifty *Cymbidium goeringii* cultivars were selected to sample (Table 1). Thirty-seven of them originated in Zhejiang province of China, others from Jiangsu (three) and Shichuan (one) provinces of China, and Japan (nine). According to horticultural classification, they included 12 Suxin-type cultivars (S1 to S12), 11 Meiban-type cultivars (M1 to M11), 5 Heban-type cultivars (H1 to H5), 5 Shuixianban-type cultivars (X1 to X5), 5 Dieban-type cultivars (D1 to D5), 5 Sehua-type cultivars (C1 to C5), 6 Yeyi-type cultivars (Y1 to Y5), and 1 Qihua (miracle flower)-type cultivars.

Fresh leaf samples were collected from two or three individuals of each cultivar for genomic DNA extraction.

ISSR analysis

Genomic DNA was extracted from leave samples as described previously (Wang et al. 2004). The concentration of extracted genomic DNA was determined by UV spectrometer and necessary dilutions were done, followed by verification with 0.8% agarose gel electrophoresis.

ISSR primers (microsatellite set 9, University of British Columbia, Vancouver, Canada) were screened, and 25 of them were selected for ISSR amplification (Table 2). Polymerase-chain reaction (PCR) was performed using 10 μl of reaction mixture solution containing 1 μl of 10 × PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.1% gelatin), 1 μl of $MgCl_2$ (25 mM), 1 μl of primer (5 mM), 1 μl of dNTP (25 mM), 50 ng of genomic DNA, and 1 unit of Taq DNA polymerase (Dingguo, Beijing, China). Touch-down PCR was performed with conditions: 94°C for 5 min followed by 10 cycles of 94°C for 60 s, 53°C for 45 s (decreasing 0.5°C per cycle), and 72°C for 90 s, and 25 cycles of 94°C for 60 s, 48°C for 45 s, 72°C for 90 s, and a final extension at 72°C for 10 min.

For ISSR genotyping, PCR products were subjected to electrophoresis on 1.5% agarose gels, followed by staining with ethidium bromide. The electrophoretic patterns of the PCR products were recorded digitally using a Gel-Doc 2000 image analysis system (Bio-Rad, Philadelphia, PA, USA).

Table 1 *Cymbidium goeringii* cultivar information and horticultural grouping

Horticultural type	Cultivar number	Cultivar name	Cultivar origin	Latitude/longitude (N/E)
Meiban	M1	Xishenmei	Fenghua, Zhejiang, China	29°40′/121°24′
	M2	Xiaochun	Hangzhou, Zhejiang, China	30°15′/120°10′
	M3	Xiaofeiyan	Shaoxing, Zhejiang, China	30°00′/120°35′
	M4	Caimeisu	Xiaoshan, Zhejiang, China	30°10′/120°15′
	M5	Changhuamei	Changhua, Zhejiang, China	30°11′/119°13′
	M6	Heshi	Suzhou, Jiangsu, China	31°18′/120°37′
	M7	Jiyuan	Hangzhou, Zhejiang, China	
	M8	Songmei	Shaoxing, Zhejiang, China	
	M9	Ruimei	Shaoxing, Zhejiang, China	
	M10	Fuxiongmei	Lanxi, Zhejiang, China	29°12′/119°28′
	M11	Danximei	Lanxi, Zhejiang, China	
Heban	H1	Huanqiuheding	Shaoxing, Zhejiang, China	
	H2	Xianhe	Fenghua, Zhejiang, China	
	H3	Cuigaihe	Shaoxing, Zhejiang, China	
	H4	Luyun	Hangzhou, Zhejiang, China	
	H5	Dafugui	Cixi, Zhejiang, China	30°11′/121°15′
Shuixianban	X1	Jialong	Kunshan, Jiangsu, China	31°23′/120°57′
	X2	Wangzi	Fenghua, Zhejiang, China	
	X3	Longzi	Yuyao, Zhejiang, China	30°04′/121°10′
	X4	Yipin	Fenghua, Zhejiang, China	
	X5	Yuxian	Fuyang, Zhejiang, China	30°03′/119°57′
Dieban	D1	Ruidie	Lanxi, Zhejiang, China	
	D2	Wucaihudie	Shaoxing, Zhejiang, China	
	D3	Daxiongmao	Fuyang, Zhejiang, China	
	D4	Wudie	Yuhang, Zhejiang, China	30°25′/120°18′
	D5	Yuhudie	Jinhua, Zhejiang, China	29°07′/119°39′
Qihua	Q	Jiaomeiren	Lanxi, Zhejiang, China	
Suxin	S1	Yuepeisu	Suzhou, Jiangsu, China	
	S2	Jiangnanxue	Hangzhou, Zhejiang, China	
	S3	Xiushuisu	Jiaxing, Zhejiang, China	30°46′/120°45′
	S4	Tiantongsu	Ningbo, Zhejiang, China	29°52′/121°31′
	S5	Zhanghesu	Shaoxing, Zhejiang, China	
	S6	Sudafugui	Fuyang, Zhejiang, China	
	S7	Jinsu	Shaoxing, Zhejiang, China	
	S8	Fuhesu	Shengxian, Zhejiang, China	29°36′/120°48′
	S9	Laowentuansu	Ningbo, Zhejiang, China	
	S10	Longchangsu	Longchang, Sichuan, China	29°21′/105°17′
	S11	Lanxihesu	Lanxi, Zhejiang, China	
	S12	Lanxisu	Lanxi, Zhejiang, China	
Sehua	C1	Rixin	Niigata, Japan	37°15′/138°45′
	C2	Fuzhiguang	Toyama, Japan	36°41′/137°13′
	C3	Hongyang	Ibaraki, Japan	36°10′/140°10′
	C4	Ningxiangzi	Shaoxing, Zhejiang, China	
	C5	Tianhongxiang	Saitama, Japan	36°25′/139°30′

Table 1 continued

Horticultural type	Cultivar number	Cultivar name	Cultivar origin	Latitude/longitude (N/E)
Yeyi	Y1	Anjimenghu	Fukushima, Japan	37°30′/140°15′
	Y2	Biancao (T1)	Fuyang, Zhejiang, China	
	Y3	Jinbo	Chiba, Japan	35°30′/140°07′
	Y4	Zhifujin	Saitama, Japan	
	Y5	Jiamaorijin	Niigata, Japan	
	Y6	Xueshan	Kumamoto, Japan	32°55′/130°55′

ISSR data analysis

For the genetic similarity analysis, ISSR DNA bands were scored as present (+) or absent (−) to produce a set of binary data (Liu et al. 2006b). Due to dominancy of ISSR markers, it was assumed that each DNA fragment position corresponds to an ISSR locus with two alleles revealed by band absence or presence (Powell et al. 1996). To analyze the genetic diversity between and among the cultivars of different horticultural types, the software POPGENE32, Version 1.32 (Yeh et al. 2000) was used to calculate mean Nei's (1973) gene diversity (*H*), mean Shannon's (Lewontin 1972) information index (*I*), and the percentage of polymorphic loci (Ppl).

A similarity matrix of ISSR banding patterns was generated using Dice's coefficient of similarity measured as in Nei and Li (1979) for all pair-wise comparisons among the 50 cultivars as follows: $Sab = 2Nab/(Na + Nb)$, where *Nab* is the number of bands shared by individuals a and b, and *Na* and *Nb* are the number of bands present in individuals *a* or *b*, respectively. The resultant similarity matrix was employed to construct a dendrogram by cluster analysis using the unweighted pair group method of the arithmetic averages (UPGMA) in the software package NTSYS-pc, version 2.01 (Applied Biostatistics Inc., NY, USA). Principal coordinate analysis (PCA) was performed further to demonstrate multiple dimensional distributions of the *C. goeringii* cultivars in a scatter-plot (NTSYS-pc, version 2.01). To verify the matrix correspondence between ISSR data and geographical distance, the Mantel test was implemented using a software package for population genetic analyses (TFPGA, Miller 1997).

Results

ISSR profile and analysis

To screen primers useful for *C. goeringii* cultivar genotyping, the primers of the UBC microsatellite set 9 were tested with a subset of genomic DNA samples. Twenty-five primers produced clear and repeatable amplicon profiles, and they were chosen for fingerprinting of all 50 cultivars (Table 2). Using these primers, a total of 4,405 scorable and repeatable DNA fragments were generated from all 50 cultivars. The size of fragments ranged from 150 to 2,000 bp. Fragments of the same size were considered as the same locus. In total, 224 ISSR loci were detected with repeatability across 50 cultivars. An average ISSR locus frequency was 8.96 loci per primer, ranging from 4 to 15 loci per primer. Fifteen of 25 primers revealed ISSR loci with 100% polymorphism at species level, while other primers detected polymorphic loci from 60% (primer I74) to 90.9% (primer UBC825), leading to an average of 8.4 polymorphic loci per primer (Table 2). As summarized in Table 2, primers for di-nucleotide repeats were generally more informative and polymorphic as compared with those primers with tri- or tetra-nucleotide repeats.

Cultivar-specific ISSR markers

Because of the many polymorphic loci, all 50 *C. goeringae* cultivars showed unique ISSR profiles. Among 25 ISSR primers, eight amplified DNA fragments that were unique to seven *C. goeringii* cultivars. Cultivar-specific markers included ISSR DNA marker I2-180 bp for Suxin-type cultivar S8, UBC866-500 bp for Suxin-type cultivar S10, UBC868-1100 bp for Suxin-type cultivar S12, both I4-500 bp and UBC818-600 bp for Meiban-type cultivar M10, UBC840-180 bp for Shuixianban-type cultivar X1, UBC864-750 bp for Heban-type cultivar H1, and I65-780 bp for Dieban-type cultivar D1. These ISSR primers amplifying cultivar-specific DNA fragments would provide molecular tools for cultivar identification.

Genetic diversity in horticultural groups

The 50 cultivars were divided into eight horticultural types based on morphological features (Table 1). A high level of genetic variation was observed using ISSR markers, with 93.75% polymorphic loci at the species level. Genetic diversity was measured in seven horticultural groups (Table 3). The Suxin group had the highest diversity ($H = 0.1877$, $I = 0.2852$, and Ppl = 60.71%), while the

Table 2 Polymorphism of ISSR markers in *Cymbidium goeringii* cultivars

Primer name	Primer sequence[a]	Total bands	Ratio of polymorphic ISSR loci[b]	Fragment size range (Kb)
UBC807	(AG)8T	187	12/12 (100%)	0.15–1.0
UBC811	(GA)8C	159	10/10 (100%)	0.15–0.8
UBC812	(GA)8A	173	10/10 (100%)	0.15–0.8
UBC814	(CT)8A	229	11/11 (100%)	0.2–2.0
UBC818	(CA)8G	75	6/7 (85.71%)	0.2–0.75
UBC825	(AC)8T	184	10/11 (90.90%)	0.2–1.0
UBC827	(AC)8G	149	7/9 (77.78%)	0.2–0.8
UBC829	(TG)8C	194	9/9 (100%)	0.15–1.0
UBC834	(AG)YT	216	13/13 (100%)	0.2–2.0
UBC835	(AG)8YC	221	15/15 (100%)	0.2–1.2
UBC840	(GA)8YT	314	14/14 (100%)	0.15–1.1
UBC855	(AC)8YT	54	6/6 (100%)	0.2–0.5
UBC862	(AGC)6	172	7/8 (87.50%)	0.3–1.2
UBC864	(ATG)6	115	8/8 (100%)	0.4–1.3
UBC866	(CTC)6	119	9/10 (90%)	0.4–1.8
UBC868	(GAA)6	126	8/8 (100%)	0.3–1.3
UBC870	(TGC)6	121	6/6 (100%)	0.5–1.3
I2	(AC)8AT	192	8/8 (100%)	0.15–0.5
I4	(AC)8AG	48	5/6 (83.33%)	0.3–0.7
I25	(AC)8CA	205	5/7 (71.42%)	0.15–1.0
I34	(AG)8AA	171	6/8 (75%)	0.3–0.8
I39	(ACG)6	126	3/4 (75%)	0.5–1.3
I44	(AC)8GA	163	8/8 (100%)	0.4–1.0
I65	(AG)8CC	193	9/9 (100%)	0.3–1.5
I74	(ACTG)4	100	3/5 (60%)	0.3–1.2
Total		4,405	210/224 (93.75%)	0.15–2.0

[a] $Y = C + T$

[b] Number of polymorphic fragment in total number of fragments amplified by each primer

Table 3 Genetic diversity within morphological groups of *Cymbidium goeringii* cultivars based on ISSR data

Horticultural group[a]	Cultivar number	Na*	Ne	*H*	*I*	Npl	Ppl (%)
Suxin	12	1.60 ± 0.48	1.31 ± 0.36	0.18 ± 0.19	0.28 ± 0.27	136	60.71
Meiban	11	1.55 ± 0.49	1.28 ± 0.35	0.17 ± 0.19	0.26 ± 0.27	125	55.80
Shuixianban	5	1.49 ± 0.50	1.27 ± 0.35	0.16 ± 0.19	0.25 ± 0.27	108	48.21
Heban	5	1.38 ± 0.48	1.24 ± 0.36	0.14 ± 0.19	0.21 ± 0.28	87	38.84
Dieban	5	1.37 ± 0.48	1.22 ± 0.34	0.13 ± 0.18	0.19 ± 0.27	84	37.50
Sehua	5	1.37 ± 0.48	1.23 ± 0.35	0.13 ± 0.18	0.19 ± 0.27	81	57.04
Yeyi	6	1.34 ± 0.47	1.20 ± 0.32	0.11 ± 0.17	0.17 ± 0.25	70	31.25
Group level	5–12	1.44 ± 0.10	1.25 ± 0.0	0.14 ± 0.02	0.22 ± 0.04	98.71	47.05
Species level	49	1.93 ± 0.24	1.36 ± 0.34	0.22 ± 0.17	0.34 ± 0.24	210	93.75

[a] Qihui group is not included here because it contained only one cultivar (Q) for investigation in the present study

* Na = Observed number of alleles; Ne = Effective number of alleles (Kimura and Crow 1964); H = Nei's (1973) gene diversity; I = Shannon's Information index (Lewontin 1972); Npl = The number of polymorphic loci; Ppl = The percentage of polymorphic loci

Yeyi group displayed the lowest diversity ($H = 0.1144$, $I = 0.1729$, and Ppl = 31.25%). At the species level, the coefficient of gene differentiation (Gst) for all 224 ISSR loci was 0.2217, and the estimate of the total genetic diversity (Ht) was 0.1895. The within group genetic diversity (Hs) was 0.1475, indicating that the total genetic diversity in this

species (about 78%) was primarily from genetic divergence inside horticultural groups.

Cluster analysis

A total of 224 ISSR loci with 4,405 DNA fragments were selected for cluster analysis based on Dice's coefficient of DNA banding pattern similarity. Among the 50 cultivars studied, the lowest pairwise genetic similarity (42%) was between Shuixianban-type cultivar X1 and Yeyi-type cultivar Y2, and the highest genetic similarity (93%) was between two Suxin-type cultivars S5 and S6.

A dendrogram was constructed to infer phylogenetic relationships among *C. goeringii* cultivars using UPGMA analysis. Two major clusters are observed in the dendrogram (Fig. 1) which roughly correspond to geographical origins. The first cluster comprised all cultivars originated in China, plus one Sehua-type cultivar (C1) from Japan. The other major cluster contains all six Yeyi-type cultivars (Y1 to Y6) and three Sehua-type cultivars (C2, C3, and C5), all of which originated from Japan except Yeyi-type cultivar Y2. Both cultivars C1 and Y2 were found to be genetically diverse from others that shared the same geographical origin (China or Japan); further investigation is needed to elucidate in detail their genetic differentiation.

In three Chinese subclusters, two of them were generally consistent with horticultural classification. Subcluster Ia comprises 11 of 12 Suxin-type cultivars (S1 to S9, S11, and S12) and one Meiban-type cultivar (M2). The Suxin-type cultivar S10 from Sichuan, a southwest province of China, was revealed to be genetically diverse from other Suxin-type cultivars originated from the Zhejiang and Jiangsu areas. Subcluster Ib includes 10 of 11 Meiban-type cultivars (M1, M3–M11), two Shuixianban-type cultivars (X1 and X2), and one Qihua (miracle flower)-type cultivar (Q). Subcluster Ic is most complex, and it has four Dieban-type cultivars (D1 to D4), four Heban-type cultivars (D1 to D4), three Shuixianban-type cultivars (X3–X5), and one Sehua-type cultivar (C1). In Cluster I, cultivars S10, H5, D5, and C4 were positioned outside subclusters Ia, Ib, and Ic.

To visualize the genetic relationships among *C. goeringii* cultivars in greater detail, we performed principal coordinate analysis (PCA) based on genetic similarity for a two-dimensional display of the relationships among cultivars. Similar to the UPGMA clustering pattern, PCA located most of the 50 cultivars into four distinct groups. This analysis also shows that several Dieban- and Suxin-type cultivars appear intermediate between groups, such as Suxin-type cultivar S10 and Dieban-type cultivar D5.

The 50 *C. goeringii* cultivars in the present study originated from three Chinese provinces and Japan (Table 1). The Mantel test was conducted to investigate a possible correlation between genetic relationships and geographic distances. We found that the correlation was relatively high

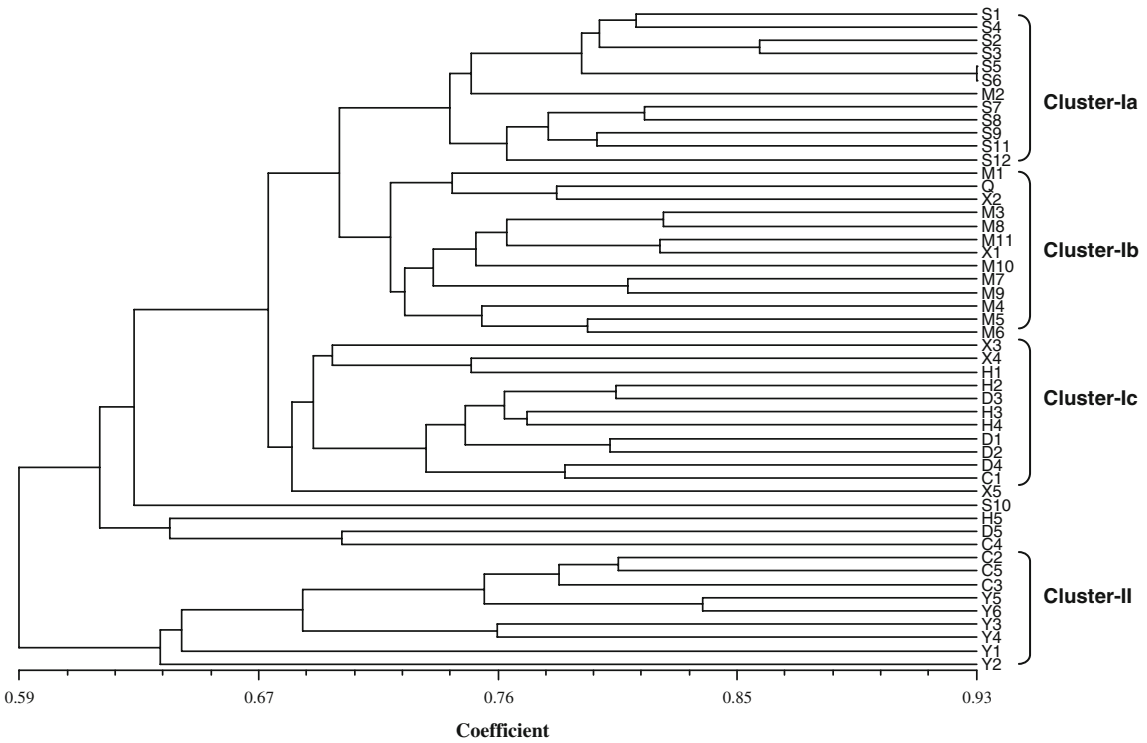

Fig. 1 Dendrogram of 50 *C. goeringii* cultivars using ISSR markers as per unweighted pair group method with arithmetic average (UPGMA) clustering. The scale shown at the bottom is the measure of genetic similarity Dice's coefficients as calculated according to Nei and Li (1979)

with highly statistical significance ($r = 0.5099$, $P < 0.01$) between the matrix of ISSR markers and distances between cultivars' geographical origins.

Discussion

Because it is sensitive to low levels of genetic variation, ISSR technology provides a very useful molecular tool for studying population genetics on a wide range of plant species, as well as investigation of genetic discrimination of populations from the same species (Zietkiewicz et al. 1994; Jarne and Lagoda 1996; Fang and Roose 1997; Raina et al. 2001). As a PCR-based genome fingerprinting technique, ISSR analysis is low-cost and effective. In view of the large and increasing numbers of spring orchard cultivars and the importance of wild exotic germplasm for breeding programs, it is necessary to evaluate genetic diversity and phylogenetic relationship in this important horticulture species.

We report the first use of ISSR markers in measuring genetic variation and determination of genetic relationships in *C. goeringii* cultivars. ISSR genotyping is also used to reveal high levels of genetic polymorphism in other plant species, including citrus cultivars (Fang and Roose 1997), *Plantago major* (Wolff and Morgan-Richards 1998), and mulberry (Kar et al. 2008). Genetic diversity analysis and assignment of cultivars to heterotic groups would provide valuable information for plant breeding programs.

We detected 224 ISSR loci in *C. goeringii* using 25 primers, about nine loci per primer, indicating that ISSRs are abundant throughout the genome of *C. goeringii* (Table 2). Furthermore, for all detected ISSR loci, 31.25–60.71% polymorphisms were revealed inside horticultural groups and 93.75% polymorphism was scored at species level (Table 3), showing a considerably high level of genomic polymorphism in *C. goeringii*.

Cymbidium goeringii has been cultivated for many centuries in China and divided into numerous horticultural types and varieties that have disparate geographic origins and distributions and considerable ecological and morphological variations (Jin and Yao 2006). The long-term selection of spring orchid implies 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, vigour, and of leaf and flower characteristics. Most spring orchid cultivars were selected from mutants of individual plant and propagated predominately by vegetative means. For example, the evolution of the Yeyi-type cultivars, which mainly originated from Japan, demonstrates significant variation ($H = 0.11$) in response to selection pressures just on the leaf color patterns and characteristics.

A high level of DNA polymorphism (93.75%) was detected across 50 *C. goeringii* cultivars using ISSR genotyping. Lower polymorphism (78%) using RAPD marker was found in 36 *Cymbidium* cultivars (Obara-Okeyo and Kako 1998). Higher polymorphism for ISSR markers than RAPD markers was also reported in other species, such as citrus (Fang and Roose 1997), lentil (Sonnante and Pignone 2001), chickpea (Chowdhury et al. 2002), and barley (Fernández et al. 2002). Our current investigation indicates that the ISSR technique is also effective for assessing genetic diversity and identifying cultivars in spring orchid.

The correlation between ISSR genetic distances and distances between geographical origins of cultivars was relatively high ($r = 0.5099$). We found that most spring orchid cultivars were grouped into a Chinese cluster (cluster I) or a Japanese cluster (cluster II) according to their geographical origins by ISSR analysis (Fig. 2). The Yeyi- and Sehua-type cultivars, most of which originated from Japan, are grouped into one cluster (cluster II), suggesting that their genetic compositions revealed by ISSR markers may reflect their sole geographical origin.

The cultivars originated in China were further divided into three major subclusters. The Chinese cultivars representing horticultural Suxin- or Meiban-types were grouped together, suggesting a potential monophyletic origin for each of these two horticultural types. Among those cultivars of horticultural Suxin- or Meiban-types, a general correspondence between observed morphological and ISSR molecular diversity suggested a genetic basis for their horticultural classification. However, most cultivars from Heban- and Dieban-types were grouped in the third subcluster (cluster Ic), in contrast to their differentiation from morphological data. The ISSR molecular diversity of cultivars of both Heban- and Dieban-types is not reflected in their morphological classification. The Shuixianban-type cultivars (X1 to X5) were scattered widely across different clusters, suggesting their wide genetic backgrounds.

Diversity of ISSR markers is assumed to be neutral, and it may not reflect diversity of morphological traits. Horticultural classification in spring orchid cultivars used simple traits such as flower shape and leaf color. These morphological characters vary widely and are useful for horticultural purposes, but may have little taxonomic value. For example, alteration of one or a few MADS genes could result in marked morphological changes in flower structures (Liu and Podila 1997; Liu et al. 2003). On the other hand, the relationship observed from molecular markers probably provides useful information on the origin and biology of cultivars, but it may not reflect observed horticultural traits. Further investigation with other genotyping

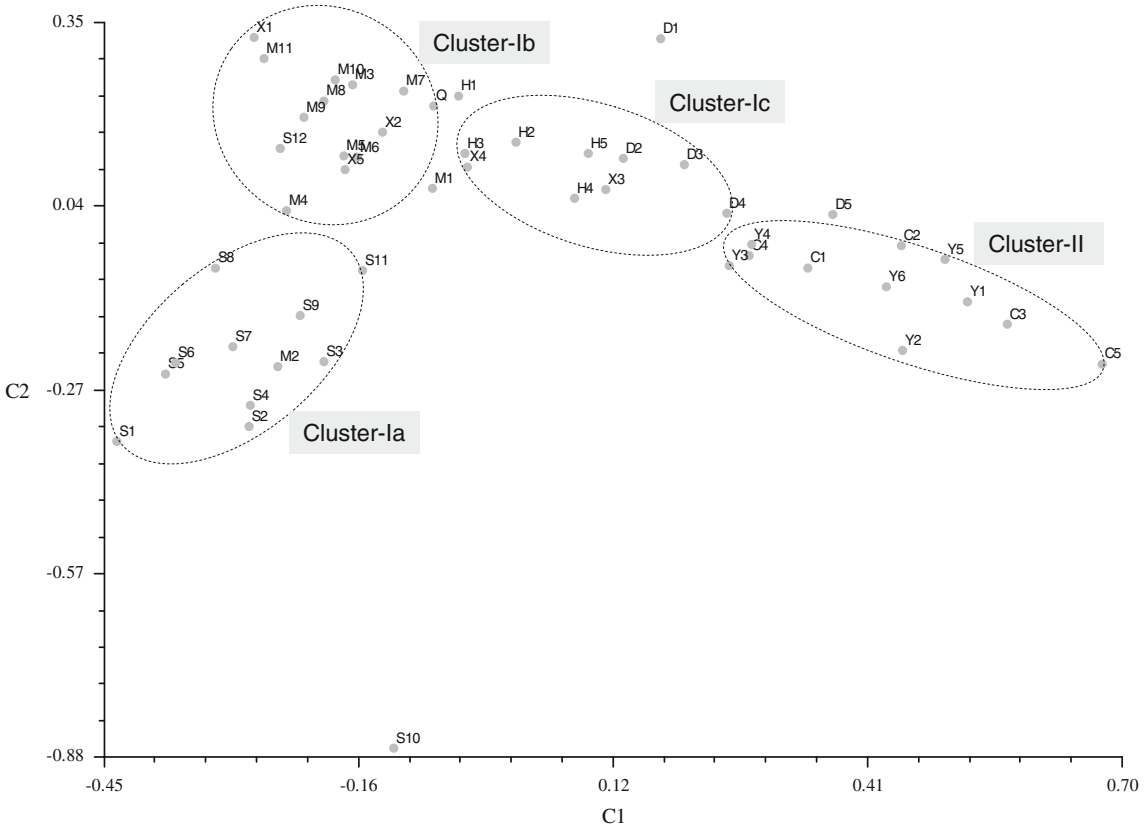

Fig. 2 Two-dimensional picture of principal coordinate analysis using ISSR genetic similarity matrix of 50 cultivars of *C. goeringii*. The plot was generated from a Dice's similarity matrix using NTSYS-pc software

technologies is necessary to determine the phylogenic origin of these spring orchid cultivars.

The *C. goeringii* ISSR genotyping patterns can differentiate 50 cultivars with 25 primers; even the two closest Suxin-type cultivars S5 and S6 had just 93% similarity. In strawberry, a single ISSR primer was employed for the differentiation of 30 cultivars (Arnau et al. 2003). ISSR fingerprinting was also able to distinguish 27 cultivars out of 30 *Leucadendron* cultivars (Pharmawati et al. 2005). Because it is simple, fast, cost-effective, highly discriminant and highly reliable, the cultivar-specific ISSR markers could be applied as diagnostic tools for cultivar identification in spring orchid. The elucidation of the relationships among the 50 cultivars and identification of cultivar-specific ISSR markers are important milestones for the breeding and management of spring orchid germoplasm.

In conclusion, we have established a genotyping system using the ISSR technique in *C. goeringii*. This genotyping system can be used efficiently for tagging genes that confer commercially important traits such as flower colors and structural alterations. We have documented high genetic diversity within horticultural groups relative to total species genetic diversity. This pattern suggests that cultivars from the same or different horticultural groups are equally important for flower breeders in the search for valuable traits.

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