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Full Length Research Paper

Genetic diversity and population structure of 151 *Cymbidium sinense* cultivars

Jiangjie Lu[1,2], Xu Hu[1], Junjun Liu[3] and Huizhong Wang[1]*

[1]Key Laboratory for Systems Biology of Medicinal and Ornamental Plants, Hangzhou Normal University, Hangzhou 310018, China.
[2]Institute of Soybean, Nanjing Agricultural University, Nanjing 210095, China.
[3]Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, Victoria V8Z 1M5, Canada.

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Cymbidium sinense **cultivars exhibit an incredible range of diversity in the foliar morphology as well as the range of flower colors and shapes, which make them more popular among horticultural plants with great economic value. Understanding the genetic diversity and population structure in target populations will be of great importance for germplasm collection, breeding improvement and conversation of this species. In this study, Inter-simple sequence repeat (ISSR) markers were used to assess the genetic diversity and population structure of 151** ***C. sinense*** **cultivars collected from China and Japan.** ***C. sinense*** **cultivars exhibited moderate levels of genetic diversity (H = 0.24, I = 0.38, Ppl = 100%) and genetic differentiation (*Gst* = 0.17) with gene flow estimate *Nm* of 2.4 among six geographical groups. With neighbor joining (NJ) analysis, 151 cultivars were clustered into seven main groups, and approximately related to their geographical distribution. Population structure analysis revealed six subpopulations, generally consistent with NJ-clustering. The results in our study suggest that different provenance collection and** ***in situ*** **conversation are important for** ***C. sinense*** **conservation and genetic improvement.**

Key words: *Cymbidium sinense,* DNA polymorphism, genetic diversity, inter-simple sequence repeat, population structure.

INTRODUCTION

In the Orchidaceae family, many species are found with high morphological diversity. Many orchids are horticulture plants, including ancestral cultivars, adapted cultivars and natural hybrids. Orchid cultivation has become one of the favorites for the main leisure culture in China since Song Dynasty (960 to 1279 A. D.) and a Chinese book about orchid, the Pedigree of Orchid in Jin Zhang, was first published as early as 1233 A.D. (Liu et al., 2006).

Cymbidium sinense is a terrestrial orchid whose native habitat spreads from India through Thailand and into China as a lithophyte at an elevation up to 2000 m (David et al., 2007). *C. sinense* is also called Mo orchid in China ("Mo" means black, pitch-dark in Chinese), because its young leaves are light green and turn darker as they mature and the multi-flora are fragrant with dark color.

Cultivation of this species is relatively later than other orchids and the first record on *C. sinense* was about 200 years ago in China (Liu et al., 2006). *C. sinense* shows as erect plant-type with thick and beautiful flower stalks. Their blossoms are colored elegantly or gorgeously with highly ornamental value (Yan, 2001; Xu, 2002; Liu et al., 2006). These *C. sinense* cultivars exhibit an incredible range of diversity in the foliar morphology as well as the range of flower colors and shapes (Wu, 1993; Liu et al., 2006). Improvement of the cultivars with different horticulture traits in breeding needs us to understand the genetic diversity and population structure of this species. Related genetic diversity study would also help the conventional classification of *C. sinense* cultivars, which is mainly based on morphological traits of leaf and flower.

According to orchid horticultural classification (Jin and Yao, 2006), *C. sinense* cultivars are mainly grouped into

*Corresponding author. E-mail: whz62@163.com. Tel: 86-571-2886-5330. Fax: 86-571-2886-5198.

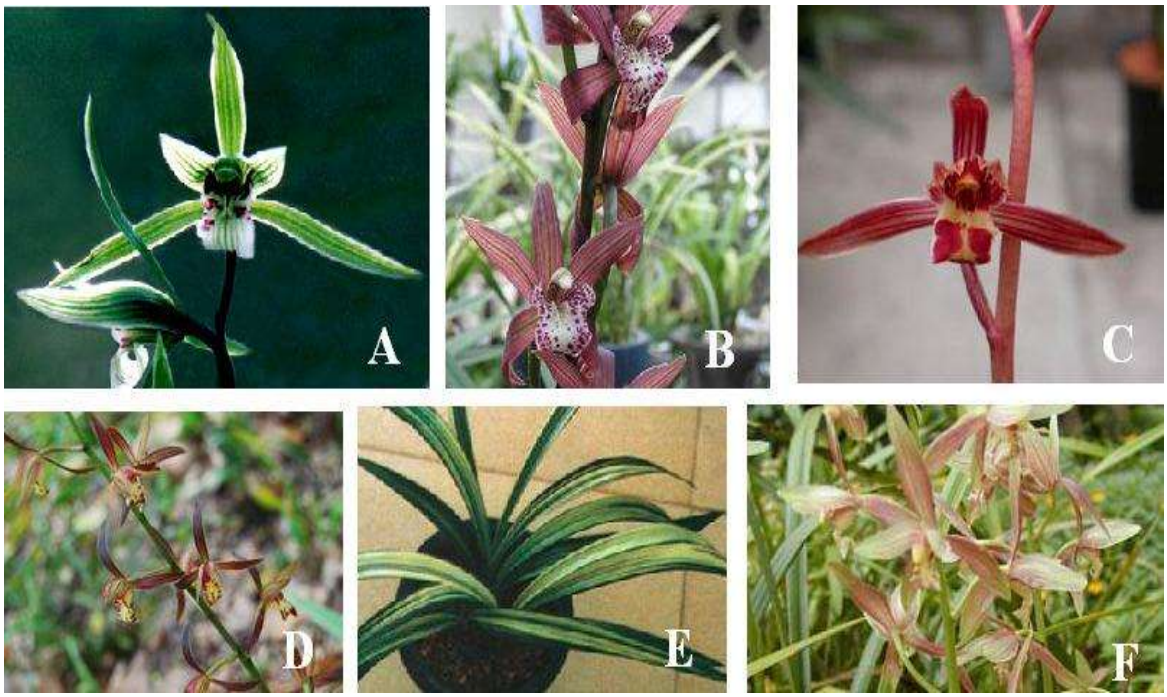

Figure 1. Morphological diversity of some *C. sinense* horticultural classes (Jin and Yao, 2006). A: Qihua class Heban type, B: Qihua class Meiban type, C: Qihua class Dieban type, D: Qihua class Shuixian type, E: Yeyi class, F: Sehua class Caihua type.

three classes in China (Figure 1). Qihua (flower with peculiar shape patterns), Sehua (whole flower with different brilliant colors), and Yeyi (leaves with various colors and shape patterns, Figure 1E). Based on sepal morphology, Qihua class is subdivided into Heban type (lotus-petal-like sepals, Figure 1A), Meiban type (plum-petal-like sepals, Figure 1B), Dieban type (butterfly-petal-like sepals or petals, Figure 1C), Shuixianban type (narcissus-petal-like sepals, Figure 1D). The Sehua class contains Suxin type (white labellum with a single color for other structures of the flower) and Caihua type (various brilliant colors for the whole flower, Figure 1F). The Yeyi class includes at least four types: Xianyi type (yellow or white lines/spots with inner green leaf), Aizhong type (dwarf leaf), Shuijing type (hyalo-white leaf tissue in apexes or margin, and even in the vein of leaf), and Duoyi type (the cultivars exhibiting combined morphological characteristics of multiple Yeyi types). Meanwhile, some cultivars share almost identical morphological characters with only a few differences, which usually results in confusion on the assessment of genetic resources at species level.

Characterization and assessment of plant genetic resources and diversity are essential for both germplasm collection and conversation. With the development of biotechnology, various molecular genotyping techniques have been applied in the studies of genetic diversity and (or) identification of *Cymbidium* plants. The first application of molecular polymorphism to measure *Cymbidium* genetic variation was enzyme polymorphism marker (Obara-Okeyo et al., 1998a). After that, various types of DNA markers, such as random amplified polymorphic DNA (RAPD) marker (Obara-Okeyo et al., 1998b; Wang et al., 2004; Choi et al., 2006), amplification fragment length polymorphism (AFLP) marker (Wang et al., 2004) have been explored to evaluate the genetic diversity and study the genetic relationship in this genus.

In this study, we explored the usefulness of the Inter-simple sequence repeat (ISSR) approach in study of genetic diversity and population structure of *C. sinense.* The ISSR technique, no need to define PCR primers for an individual species, easy to carry out, treated as dominant markers, provides genomic information for a range of applications including: population genetics, hybridizations and gene mapping (Wink, 2006), it has been applied widely in plants, notable in the conservation of rare species, used to estimate the genetic diversity at both plant species and population levels (Zietkiewicz et al., 1994; Treutlein et al., 2003; Gobert et al., 2006; Kothera et al., 2007). The ISSR technique was used for assessment of the genetic diversity and molecular identification of *Dendrobium* species (Wang et al., 2009a, 2009b). More recently SRR and EST-SSR markers were also reported to study diversity in the genus

*Cymbidium*resources in horticulture, it is of interest to characterize genetic diversity of this species. Here, we collected 151 *C. sinense* cultivars with various horticultural types originated in China and Japan. Their genetic variation (Huang et al., 2010; Moe et al., 2010).
For sustainable management of *C. sinense* genetic was

investigated at both species and geographical group levels by ISSR markers for the research objectives:

1) To reveal ISSR fingerprinting profiles for molecular identification of *C. sinense* cultivars.

2) To assess genetic diversity and population structure of *C. sinense* cultivars using those identified ISSR markers.

MATERIALS AND METHODS

Plant materials

One hundred and fifty-one cultivars of *C. sinense* with various horticultural types were collected from 5 provinces of China and Japan (Table 1) as six geographical populations: Taiwan population (No. 1 to No. 97), Guangxi population (No. 98 to No. 104), Guangdong population (No. 105 to No. 138), Fujian population (No. 139 to No. 141), Yunnan population (No. 142 to No. 145), and Japan population (No. 146 to No. 151). These cultivars covered all the Chinese traditional horticultural classification types (Table 1).

DNA extraction and ISSR analysis

Genomic DNA was extracted from young leaves by using the cetyltrimethyl-ammonium bromide (CTAB) method (Wang et al., 2004). Concentration and purity of total genomic DNA were determined by UV spectrometer and DNA electrophoresis with 1% agarose gel respectively.

Two ISSR primer sets, UBC # 9 (University of British Columbia UBC, Biotechnology Laboratory, Vancouver Canada) and # I (Wang et al., 2009) were synthesized (Sangon Co., Ltd., China). Four genomic DNA samples were used for primer screen. Eventually, 18 ISSR primers with production of clear and reproducible bands were selected for amplification of all genomic DNA samples (Table 2).

The ISSR amplification was performed as described previously (Wang et al., 2009). PCR products were separated by electrophoresis on 1.5% agarose gel. The electrophoretic patterns of the PCR fragments were visualized with ethidium bromide staining and recorded digitally with a Gel-Doc 2000 image analysis system (Bio-Rad, Philadelphia, PA, USA). The presence of amplified bands with different intensities and locations were detected and analyzed with the software Quantity One 4.62 (BioRad, Hercules, CA, USA) ISSR is a dominant marker, amplified fragments were considered as di-allelic and bands were scored as the two alleles of a locus (1 for presence and 0 for absence) to produce a set of binary data for genetic similarity comparison among cultivars.

Data analysis

For genetic diversity analyses, POPGENE32 Version 1.32 (Yeh, 2000) was used to measure mean Nei's (1973) gene diversity (*H*), mean Shannon's (Lewontin, 1972) information index (*I*), the percentage of polymorphic loci (Ppl), observed number of alleles (Na), effective number of alleles (*Ne*) (Kimura and Crown, 1964), the total genetic diversity all over the populations (Ht), the average genetic diversity within populations (*Hs*), and differentiation (*Gst*) and gene flow estimate (*Nm*) among *C. sinense* cultivar of different geographical populations.

The UPGMA distance method was used to analysis the band data usually. However, this method does not fully take into account the evolutionary pattern and does not search for the optimal tree. Therefore, we used neighbor-joining (NJ) cluster analyses with Power Marker version 3.25 (Liu and Muse, 2005) and the tree was displayed using MEGA 4 (Tamura et al., 2007).

To infer the approximate number of genetic clusters present in the data set (*K*) and to assign individuals to these cluster, we used STRUCTURE version 2.2 (Pritchard et al., 2000; Falush et al., 2007). The optimum number of population (*K*) was selected after four independent runs of a burn-in of 500,000 iterations followed by 500,000 iteration for each value of *K* (testing from *K* = 2 to *K* = 10). A model-based clustering with distinctive allele frequencies placed individuals into *K* clusters, where *K* is chosen in advance but can be varied for independent runs of the algorithm. The most likely number of cluster (*K*) was selected by comparing the logarithmized probabilities of data [Pr(*X*|*K*] and α value for each value of *K* according to Pritchard et al. (2000).

RESULTS

High ISSR polymorphism in *C. sinense*

Eighteen ISSR primers of UBC # 9 and # I primer sets, which produce clear and reproducible bands with 4 DNA samples of *C. sinense* cultivars, were selected for the genomic DNA amplification of all 151 *C. sinense* cultivars. As showed in Table 2, 15 primers were for di-nucleotide repeats while 3 were for tri-nucleotide repeats. Of 15 di-nucleotide repeat primers, 10 were (AG or GA) repeat primers and 5 were (AC or CA) repeat primers. All of these primers were informative and they revealed polymorphic loci in *C. sinense.*

A total of 14, 478 DNA fragments with sizes ranging from 100 to 2500 bp were scored from 151 cultivars with 18 ISSR primers. An example ISSR profile is shown in Figure 2. DNA fragments with the same size from the same primer were considered as the same ISSR locus. Finally, 251 ISSR loci were revealed across 151 cultivars, with an average of 13.94 ISSR loci per primer, ranging from 9 (UBC840) to 19 loci (UBC825). Of all 18 primers used in the present study, each primer revealed 100% polymorphism of ISSR loci at the species level.

Moderate genetic differentiation among *C. sinense* geographical populations

One hundred and fifty-one cultivars originated from 6 geographical regions were found with 100% ISSR polymorphic loci at species level (Table 2). Genetic diversity was measured in six geographical populations (Table 3). Within them, the Guangdong population has the highest genetic diversity (*H* = 0.2489, *I* = 0.3894, and Ppl = 92.43%). In contrast, the Fujian population showed the lowest genetic diversity (*H* = 0.1420, *I* = 0.2084, and Ppl = 35.86%). The diversity was moderate (*H* = 0.2380, *I* = 0.3780, Ppl = 100%), for all 251 ISSR loci at species level. Coefficient of genetic differentiation (Gst) was 0.1715, with estimate of the gene flow *Nm* of 2.4149

Table 1. *Cymbidium sinence* cultivars materials used in this study.

Cultivar code	Cultivar name	Origin	Traditional classification	Simulation population
1	Shi Ba Jiao	Taiwan	Sehua	V
2	Wen Shan Xian Die	Taiwan	Qihua (Dieban)	II
3	Wen Shan Qi Die	Taiwan	Qihua (Dieban)	IV
4	Jin Ying	Taiwan	Qihua (Shuixian)	IV
5	Jin Niao Huang Zhua	Taiwan	Sehua	V
6	Liu Xian Nv	Taiwan	Qihua (Shuixian)	IV
7	Feng Huang Zhua	Taiwan	Sehua	V
8	Bi Lv Yi	Taiwan	Sehua	IV
9	Fu Ji Cui	Taiwan	Yeyi	IV
10	Yu Fei Guan	Taiwan	Yeyi	IV
11	Xin Pu Wang Yue	Taiwan	Qihua (Heban)	VI
12	Huang Yu	Taiwan	Sehua	II
13	Yu Shi Zi	Taiwan	Sehua	IV
14	Shi Men Zhua	Taiwan	Yeyi	V
15	Da Shi Men	Taiwan	Yeyi	IV
16	Bao Dao Qi Hua	Taiwan	Sehua	V
17	Wen Shan Long	Taiwan	Yeyi	IV
18	Hong Yu	Taiwan	Sehua	V
19	Wan Dai Fu Zhua	Taiwan	Yeyi	IV
20	Da Mo Shi Ren Gong Yi	Taiwan	Qihua (Heban)	V
21	Long Feng Guan	Taiwan	Yeyi	III
22	Tian Luo Tou	Taiwan	Qihua (Heban)	V
23	Bi Lv	Taiwan	Sehua	IV
24	Wen Shan Jia Long	Taiwan	Sehua (Suxin)	V
25	Yin Long	Taiwan	Yeyi	II
26	Huang Guan Jin Die	Taiwan	Qihua (Dieban)	V
27	Dai Dai Fu	Taiwan	Yeyi	IV
28	Guo Xiang Mu Dan	Taiwan	Qihua (Meiban)	V
29	Cai Long	Taiwan	Qihua (Meiban)	I
30	Xin Niang	Taiwan	Sehua	V
31	Bai Weng	Taiwan	Qihua (Dieban)	II
32	Da Mo	Taiwan	Yeyi	II
33	Hua Guang Die	Taiwan	Qihua (Dieban)	II
34	Wu Cai Long	Taiwan	Qihua (Meiban)	IV
35	Tai Yang	Taiwan	Yeyi	III
36	Shui Jing Long Liu	Taiwan	Sehua (Suxin)	II
37	Fu Lu Shou	Taiwan	Qihua (Shuixian)	I
38	Xue Bai Zhua	Taiwan	Yeyi	II
39	Jin Niao	Taiwan	Yeyi	IV
40	Tao Ji Zhua	Taiwan	Yeyi	IV
41	Tian Long	Taiwan	Qihua (Heban)	VI
42	Yu Qi Lin	Taiwan	Qihua (Meiban)	IV
43	Lv Yun	Taiwan	Qihua (Heban)	VI
44	Ri Xiang	Taiwan	Yeyi	IV
45	Feng Huang	Taiwan	Sehua	IV
46	Niao Jin Zhi Bao	Taiwan	Sehua	IV
47	Da Mo Guan	Taiwan	Yeyi	II
48	Jin Ru Yi	Taiwan	Qihua (Meiban)	V
49	Gui Fu Ren	Taiwan	Sehua	III
50	Fu Cui	Taiwan	Qihua (Heban)	II

Table 1. Contd.

51	Rui Bao	Taiwan	Sehua (Suxin)	V
52	Chang Le Shou	Taiwan	Yeyi	IV
53	Hua guang Die Zhua	Taiwan	Sehua (Suxin)	V
54	Da Tun Qi Lin Lv Mao	Taiwan	Yeyi	II
55	Rui Hua	Taiwan	Qihua (Meiban)	V
56	Wen Han Qi Die Lv Zhua	Taiwan	Sehua	II
57	Da Tun Qi Lin Lv Zhua	Taiwan	Sehua	V
58	Hao Guang Si She	Taiwan	Yeyi	II
59	Da Mo Liu He	Taiwan	Yeyi	II
60	Da Tun Qi Lin	Taiwan	Sehua	VI
61	Yu Guan Yin	Taiwan	Qihua (Dieban)	II
62	Wan Dai Fu Zhua	Taiwan	Yeyi	II
63	Da Mo Zhong Tou Yi	Taiwan	Yeyi	II
64	San Xiong Hong Die	Taiwan	Qihua (Dieban)	III
65	Hong Ju	Taiwan	Sehua	II
66	Yan Ji	Taiwan	Qihu (Heban)	VI
67	Bai Yu	Taiwan	Qihua (Shuixian)	IV
68	Hua Wang Jin	Taiwan	Yeyi	IV
69	Long Feng Zhua	Taiwan	Yeyi	IV
70	Nian Nian Fu	Taiwan	Yeyi	I
71	Yu Jin Jiao	Taiwan	Yeyi	II
72	Yu Fei	Taiwan	Qihua (Heban)	V
73	Yang Ming Jin	Taiwan	Yeyi	II
74	Lv Bao	Taiwan	Yeyi	V
75	Wang Dai Fu	Taiwan	Yeyi	II
76	Lan Yang Qi Die	Taiwan	Qihua (Dieban)	V
77	Ji Fu Long Mei	Taiwan	Qihua (Dieban)	IV
78	Jin Bao Bei	Taiwan	Sehua (Suxin)	II
79	Shuang Mei Ren	Taiwan	Yeyi	II
80	Tian Tang Niao	Taiwan	Qihua (Meiban)	V
81	Long Feng	Taiwan	Yeyi	IV
82	Feng Guan Shui Jin	Taiwan	Yeyi	I
83	Hong Bao Shi	Taiwan	Qihua (Meiban)	V
84	Liu Feng	Taiwan	Qihua (Meiban)	II
85	Xin Gao Shan	Taiwan	Yeyi	I
86	Huan Dao Zhua	Taiwan	Yeyi	II
87	Shuang Long Guan	Taiwan	Yeyi	I
88	Bao Shan Zhua	Taiwan	Yeyi	II
89	Fu Gui	Taiwan	Qihua (Meiban)	V
90	Tai Zhong Wang Yue	Taiwan	Yeyi	II
91	Ai Zhi Bao	Taiwan	Yeyi	V
92	Tao Ji	Taiwan	Sehua	IV
93	La Ba Ji	Taiwan	Sehua	I
94	Yu Zhou Wang	Taiwan	Yeyi	I
95	Shi Ba Jiao Mei Zhua	Taiwan	Yeyi	I
96	Xu Huang	Taiwan	Yeyi	II
97	Xu Huang Zhua	Taiwan	Yeyi	VI
98	Xue Yu	Guangxi	Yeyi	III
99	Wu Cai Fei Long	Guangxi	Qihua (Meiban)	III
100	Feng Lai Chao	Guangxi	Yeyi	III
101	Jin Ying	Guangxi	Qihua (Shuixian)	III
102	Gong Zhou Long	Guangxi	Sehua (Suxin)	III

Table 1. Contd.

103	Chao Yang He	Guangxi	Qihua (Heban)	I
104	Yi Die	Guangxi	Qihua (Dieban)	IV
105	Bai Yu Su Jin	Guangdong	Yeyi	I
106	Tian Fu	Guangdong	Qihua (Shuixian)	II
107	Ji Guan Shui Jing	Guangdong	Yeyi	V
108	Yuan Dong He	Guangdong	Qihua (Heban)	VI
109	Jin Feng Die	Guangdong	Qihua (Dieban)	I
110	Gui He	Guangdong	Qihua (Heban)	III
111	Shan She Qi Hua	Guangdong	Qihua (Meiban)	VI
112	Cai Xin Jin Si Ma Wei	Guangdong	Yeyi	I
113	Qing Yu	Guangdong	Sehua	I
114	Jin Hua Shan	Guangdong	Yeyi	III
115	Zhu Hai Yu Nv	Guangdong	Qihua (Dieban)	III
116	Nan Guo Shui Xian	Guangdong	Qihua (Shuixian)	VI
117	Dong Fang Ming Zhu	Guangdong	Qihua (Shuixian)	VI
118	Shi Zi Tou	Guangdong	Yeyi	V
119	Huang Zhong Wang	Guangdong	Sehua	I
120	Yi Pin He	Guangdong	Sehua	VI
121	Yin Feng Bai Hua	Guangdong	Sehua	VI
122	Tang Shan Hu	Guangdong	Yeyi	VI
123	Jin Yao Shi	Guangdong	Yeyi	IV
124	Shan Dian	Guangdong	Yeyi	V
125	Nan Hai Mei	Guangdong	Qihua (Meiban)	III
126	Shen Zhou Qi	Guangdong	Qihua (Shuixian)	III
127	Yin Feng	Guangdong	Yeyi	II
128	Fu Se Hua	Guangdong	Sehua (Suxin)	III
129	Hu Po Jin Long	Guangdong	Yeyi	III
130	Hu Bi Jin Long	Guangdong	Yeyi	III
131	Huang Jin Guan	Guangdong	Yeyi	II
132	Huang Fei	Guangdong	Sehua	III
133	Bai Tian E	Guangdong	Yeyi	III
134	Jin Bi	Guangdong	Sehua	I
135	Xin Pin Die	Guangdong	Qihua (Dieban)	II
136	Yin Hua	Guangdong	Yeyi	III
137	Tian Fu Shou	Guangdong	Sehua	III
138	Shui Jing Long	Guangdong	Yeyi	III
139	Tian Sheng	Fujian	Qihua (Shuixian)	III
140	He Zhi Hua	Fujian	Sehua	III
141	Ying Zui Shui Jing	Fujian	Yeyi	III
142	Qing Long Jian	Yunnan	Qihua (Shuixian)	VI
143	Huang Chun Shui Jing	Yunnan	Yeyi	III
144	Feng Die	Yunnan	Qihua (Dieban)	VI
145	Bai Mei	Yunnan	Qihua (Meiban)	VI
146	Ai Guo	Japan	Yeyi	V
147	Da Xun Zhua	Japan	Sehua (Suxin)	II
148	Sheng Ji Guang	Japan	Yeyi	VI
149	Jin Yu Man Tang	Japan	Qihua (Dieban)	VI
150	Huang Jin Yang Lao	Japan	Qihua (Shuixian)	IV
151	Ri Yu	Japan	Sehua	I

among six populations. This means that the main genetic diversity (82.8%) is within the population and only

Table 2. Polymorphism of ISSR markers in *Cymbidium sinense* cultivars.

Primer name	Primer sequence [a]	Total bands	Ratio of polymorphic ISSR loci [b] (100%)	Fragment size range (bp)
UBC807	$(AG)_8T$	753	14/14	150-1500
UBC808	(AG)8C	1131	16/16	100-1600
UBC809	(AG)8G	1040	16/16	200-1400
UBC811	(GA)8C	819	17/17	150-1750
UBC825	$(AC)_8T$	789	19/19	180-2000
UBC826	$(AC)_8C$	549	14/14	200-1400
UBC827	$(AC)_8G$	775	16/16	250-2100
UBC834	(AG)8Y*T	907	14/14	250-1100
UBC835	(AG)8Y*C	676	10/10	200-1300
UBC836	(AG)8Y*A	882	18/18	180-1700
UBC840	(GA)8Y*T	415	9/9	150-2000
UBC864	$(ATG)_6$	647	11/11	160-1350
UBC866	(CTC)6	905	9/9	100-1750
I2	A $(CA)_8T$	643	11/11	220-1900
I4	A$(CA)_8G$	807	14/14	350-2000
I34	(AG)8AA	1018	12/12	160-1550
I39	$(ACG)_6$	823	15/15	150-1100
I65	(AG)8CC	899	16/16	250-1300
Total		14 478	251/251	100-2100

[a] Y=C + T, [b] Number of polymorphic fragment in total number of fragments amplified by each primer

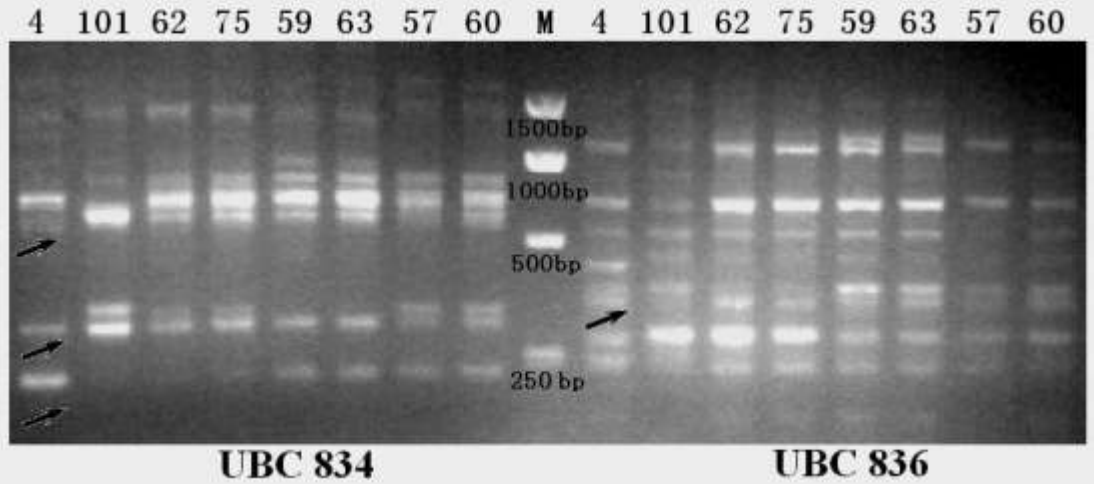

Figure 2. Example of the ISSR profiles in the *C. sinense* cultivars. Electrophoresis patterns from two ISSR primers (UBC 834 and UBC 836) are shown for identification of cultivars No. 4 and No. 101 with the same horticultural name “Jin Ying”. Three pairs of cultivars: No. 57 and No. 60, No. 59 and No. 63 or No. 62 and No. 75, were very similar with each other respectively. Arrows indicate the PCR bands amplified differentially between cultivars No. 4 and No. 101. The same ISRR band patterns are observed in three sets of close cultivars.

moderate genetic differentiation (17.2%) occurs among these populations. *C. sinense* was revealed with the total genetic diversity all over the populations (*Ht* = 0.2336) and the average genetic diversity within populations (*Hs* = 0.1935).

NJ clustering analysis in *C. sinense*

The genetic similarity of 151 cultivars in this study was calculated by means of Dice’s coefficient of total 14,478 DNA fragments from 251 ISSR loci. The lowest pairwise

Table 3. Genetic diversity within morphological populations of *Cymbidium sinense* cultivars based on ISSR data.

Horticultural population	Cultivar number	Na	Ne	*H*	*I*	Npl	Ppl (%)
Taiwan	97	1.97±0.17	1.36±0.32	0.22±0.17	0.36±0.23	244	97.21
Guangxi	7	1.64±0.48	1.32±0.34	0.20±0.18	0.30±0.26	161	64.14
Guangdong	34	1.92±0.27	1.40±0.33	0.25±0.16	0.39±0.22	232	92.43
Fujian	3	1.36±0.48	1.25±0.37	0.14±0.20	0.21±0.29	90	35.86
Yunnan	4	1.44±0.50	1.27±0.36	0.16±0.19	0.24±0.28	111	44.22
Japan	6	1.58±0.49	1.32±0.37	0.19±0.19	0.29±0.28	146	58.17
Species level	151	2.00±0.00	1.38±0.31	0.24±0.16	0.38±0.21	251	100.00
Group level	4-97		164	65.34			

* Na = Observed number of alleles; Ne = Effective number of alleles (Kimura and Crown1964); *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.

genetic similarity (39.3%) was between cultivars No. 29 and No. 135, and highest (86%) between cultivar No. 59 and No. 66 among the 151 cultivars. The dendrogram tree (Figure 3A), constructed by NJ analysis with the Nei's similarity among ISSR loci, showed that 151 *C. sinense* cultivars could be divided into two major clusters upon seven groups. Group 2, 4, 5 and 7 contained 80 of 97 cultivars originated from Taiwan in one cluster. Group 1, 3 and 6 contained 30 of 34 cultivars originated from Guangdong in the other cluster. The cultivars originated from Guangxi, Fujian, Yunnan and Japan were not clustered in an independent group, but scattering in others (Figure 3A).

Bayesian structure analysis in *C. sinense*

The model-based simulation of population structure using ISSRs showed that likelihood was maximized and minimized when the number of populations was set at six, suggesting that these cultivars can be grouped into six subpopulation, inferred from the model, showed as POP 1, POP 2, POP 3, POP 4, POP 5 and POP 6, respectively (Figure 4, Tables 1 and 4). The genetic structure of *C. sinense* is obviously complex.

DISCUSSION

This study is aimed to measure molecular biodiversity and investigate genetic structure of 151 *C. sinense* cultivars using ISSR markers. With advantages of low-cost and high efficiency, the PCR-based ISSR genome fingerprinting technique has gradually become a common molecular tool for genetic variation assessment among plant populations originated in different geographical sites since its development in1994 (Zietkiewicz et al., 1994; Fang and Roose, 1997; Raina, 2001; Pradeep et al., 2002). In view of the large and increasing numbers of both cultivars and wild exotic germplasm available for collection and *in situ* conversation, it is necessary for us to evaluate genetic diversity and population structure among various populations from different geographical origins for sustainable management of the orchid species *C. sinense*. Our investigation findings on genetic diversity, population structure and gene-flow among 151 cultivars would provide valuable information for horticultural practice in *C. sinense*. The present study indicates that ISSR technique is useful in *C. sinense*, as 18 primers revealed 251 ISSR loci with an average of 13.94 loci per primer across 151 cultivars (Table 2). We found 35.86 to 97.21% ISSR polymorphisms inside geographical populations (Table 3), suggesting a considerably high level of genomic polymorphism within these populations.

Due to its cultivation history for many centuries in China, *C. sinense* has been divided into numerous horticultural classes and types. Largely for their horticultural value, some *C. sinense* cultivars were transplanted from place to place in the history, which result in the confusion and difficulty to trace their evolutionary origins. In our study, NJ clustering and structure analysis both showed some information of this, as cultivars originated from Taiwan (in Groups 2, 4, 5 and 7) and Guangdong (in Groups 1, 3 and 6) separated each other. Because of sampling difficulty, only a few cultivars were collected from Guangxi, Fujian, Yunnan and Japan, their NJ-clustering may not reveal the real relationship of these four geographical populations with other two (Figure 3A). Compared the structure population simulation with the NJ clustering, both showed similar results in general (Figures 3B, 4 and Table 4), 12 of 18 POP1 cultivars were found in Group 1; 25 of 31 POP 2 cultivars were found in Group 2; 16 of 25 POP 3 cultivars were in found Group 3; 12 of 18 POP 6 cultivars were found in Group 6; 19 of 29 cultivars in POP 5 came from Groups 5 and 7; while the POP 4 contained 6 of 8 Group 4 cultivars and others scattering in all the seven NJ groups.

Some confusion of horticultural classification also came from documentation with different cultivars given the same name or the same cultivar named differently by flower breeders and growers. Taken two cultivars in our current study for example the cultivars No. 4 (originated

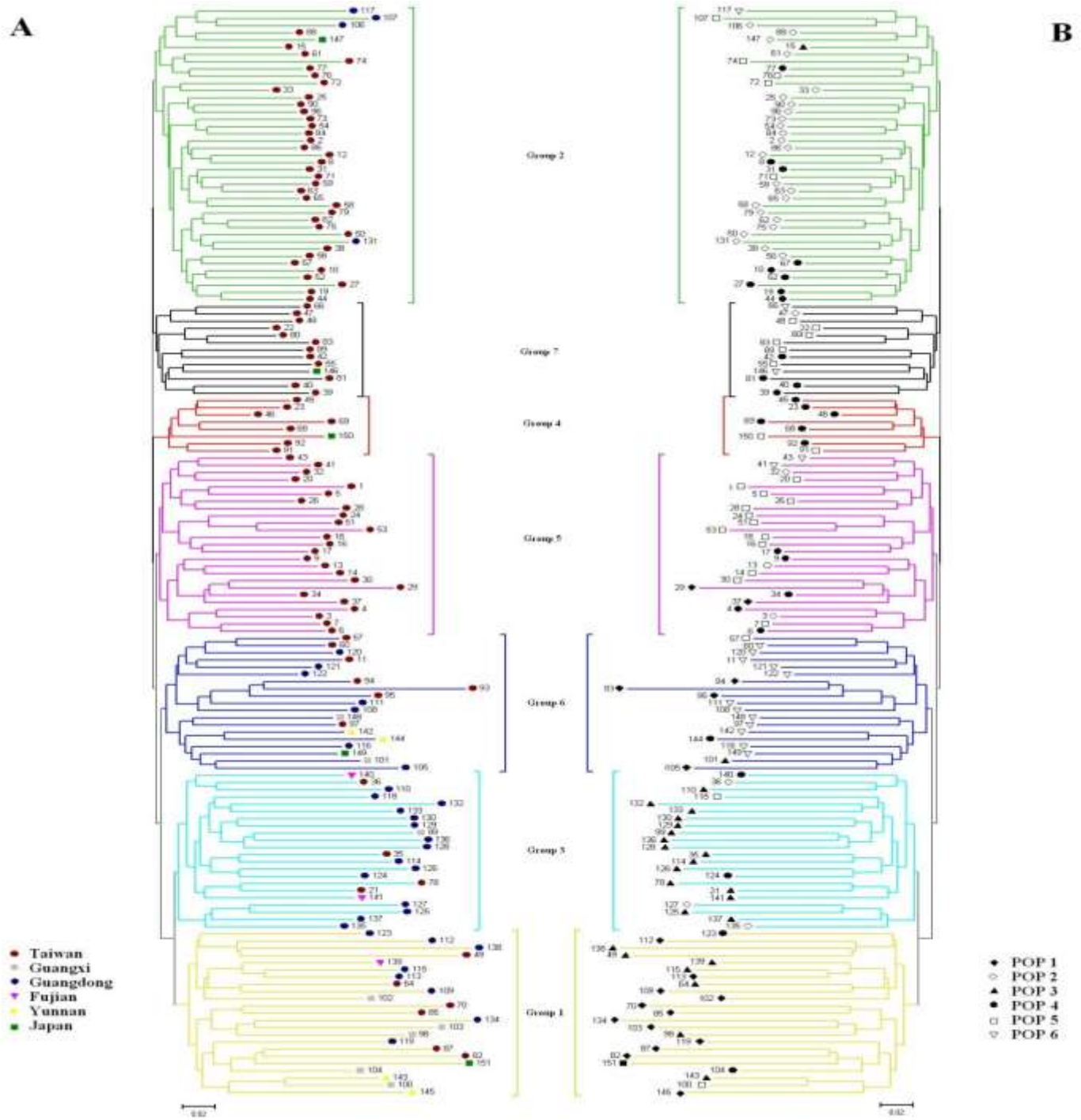

Figure 3. The comparison of simulation populations with geographical populations in a dendogram produced using NJ cluster analysis. 151 *C. sinense* cultivars were clustered into seven groups (Groups 1 to 7) by NJ analysis based on Nei's similarity of ISSR markers. Each individual cultivar was labeled according to their six geographical origins (A), or labeled according to their distribution among six simulation populations (B). Cultivars numbers were shown as in Table 1.

in Taiwan) and No. 101 (originated in Guangxi) have the same name "Jin Ying" (Table 1), but they were distinguished from each other on ISSR fingerprinting profiles (Figure 2). This suggests that these two cultivars might share certain common flower features despite their different genetic backgrounds resulting from geographical isolation.

The long-term horticultural selection of *C. sinense* has

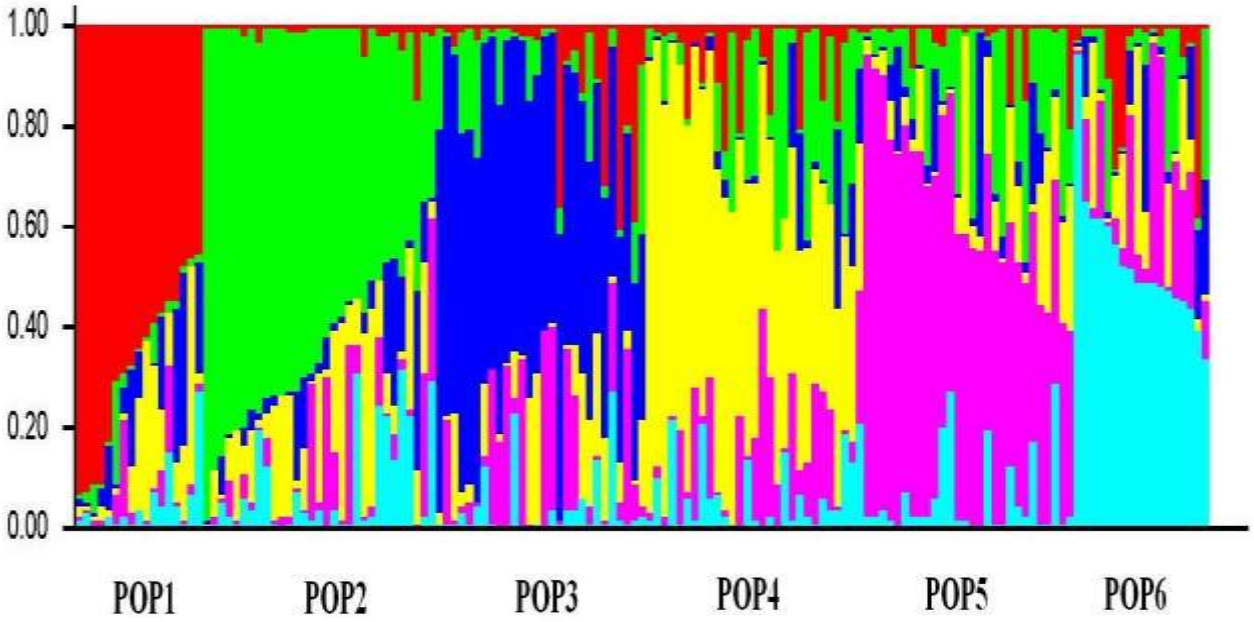

Figure 4. Population structure of 151 *C. sinense* cultivars based on 251 ISSR loci (K = 6). Each cultivar is represented by a thin vertical line, which can be partitioned into six colored segments that represent the estimated membership probabilities (Q) of the individual to the six clusters, and then all accessions were sorted by Q.

Table 4. The number of *C. sinense* cultivars in simulation structure populations (Pop 1-6) and NJ clusters (Groups 1-7).

Population	Cluster							
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Total
Pop 1	**12**	0	0	0	2	4	0	18
Pop 2	0	**25**	3	0	2	0	1	31
Pop 3	7	1	**16**	0	0	1	0	25
Pop 4	2	9	2	**6**	6	1	4	30
Pop 5	1	5	1	2	**13**	1	**6**	29
Pop 6	1	1	0	0	2	**12**	2	18
Total	23	41	22	8	25	19	13	**151**

resulted in large numbers of cultivars within this orchid species. Nuclear mutations that occurred during the selection process probably lead to the polymorphisms of a set of functional genes, which may be responsible for the phenotypic variations of horticultural traits that enable us to select vigorous orchid plants with desired leaves and flower characteristics. In our collection, some cultivars were selected and bred from bud mutants of individual plant, so they shared close relationship with each other based ISSR profiling (Figure 2A). For example, our ISSR study found 86% genetic similarity between cultivars No. 59 (Da Mo Liu He) and No. 63 (Da Mo Zhong Tou Yi). Cultivars No. 59 was bred from a floral mutant of cultivar No. 63. Both No. 59 and No. 63 belonged to Yeyi class based on leaf characteristics while No. 59 could also be classified as a Heban-type cultivar when floral features are considered. Sehua-class cultivar No. 57 (Da Tun Qi Lin Lv Zhua), featured with special green petals, was cultured from a Yeyi-class cultivar No. 60 (Da Tun Qi Lin), both sharing 85.09% ISSR genetic similarity. Similarly, a mutant of the cultivar No. 75 (Wan Dai Fu) resulted in a novel cultivar No. 62 (Wan Dai Fu Zhua), both cultivars had 85.57% genetic similarity between each other. Some *C. sinense* cultivars may have closer phylogenetic relationship if they have originated directly or indirectly from mutants of the same ancestor cultivar though their morphological characters vary widely. Horticultural classification of *C. sinense* generally emphasizes only one or two characters, such as shape and color patterns of plant leaf and flower for economic

purposes, and its taxonomic value is very limited. Those cultivars of the same horticultural class with similar horticultural characters were scattered throughout the NJ cluster (Figure 2A), suggesting that the ISSR marker-based genetic diversity is far beyond that underlying variations of morphological traits selected in horticultural practice. The ISSR-based phylogenetic relationship probably provides useful information on the origin and biology of *C. sinense* cultivars, but it may not reflect genetic variation of a small set of genes for the horticultural traits with cultivation interests. Future investigation with other genotyping technologies would provide further insight into evolutionary origin of the *C. sinense* cultivars and natural germplasm.

We applied the ISSR genotyping system for genetic characterization of *C. sinense* cultivars. Our results of ISSR molecular biodiversity and genetic structure among 151 cultivars are important for further discovery of genes responsible for commercially important traits such as flower colors and fragrance, and structure alterations in future research on *C. sinense*. An indirect gene flow estimate of Nm was 2.4 among 6 geographical regions, within Nm range from 1 to 4, indicating that gene flow among populations inhabiting different regions was sufficient to deter population differentiation if they were at equilibrium between migration and random genetic drift (Wright, 1931). High variation levels (*Gst* = 0.1715) within geographical groups suggest that *C. sinense* cultivars in each region are equally important for germplasm collection and genetic conservation.

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